STRUCTURAL CHARACTERIZATION OF HUMAN PARAINFLUENZA VIRUS 3 AND CLONING OF VIRAL SPECIFIC GENES

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ABSTRACT

Two approaches were taken to examine the role of viral proteins in virus-host cell interactions. The first approach was to examine the molecular mechanism of vesicular stomatitis virus (VSV) induced cell fusion. The second approach involved the analysis of human parainfluenza virus 3 (HPIV3) virion components, intracellular viral components, the primary structure of the viral genes and the amino acid sequence of certain viral proteins.

Characteristically, cultured cells rapidly die when infected with VSV. Thus, the typical cytopathic effect of a VSV infection is cell rounding and degeneration. However, when viral protein synthesis was inhibited early in infection, both Indiana and New Jersey serotypes of VSV induced cell fusion. Pulse-chase experiments were done to determine the rates of synthesis and transport of each viral protein. The viral protein most rapidly transported from the cell was the M protein. Therefore, inhibition of protein synthesis would have the greatest effect on the intracellular pool of M protein. A temperature-sensitive mutant, defective in the M protein, was the only mutant of five complementation groups which spontaneously induced cell fusion at the non-permissive temperature. In conclusion, VSV-infected cells fuse when the intracellular pool of functional M protein is reduced and G protein is present on the cell surface.

The second part of this research dealt with the analysis of human parainfluenza virus 3 (HPIV3). Initially, the virion components were examined. The genome of HPIV3 was shown to be a linear RNA molecule with a molecular weight of 4.6×10^6. The virion contains seven major viral proteins. Three proteins with molecular weights of 195,000, 87,000 and 67,000 are associated with the nucleocapsid and have been designated L, P

and NP respectively. A protein of molecular weight 35,000, designated M protein, was associated with the envelope components of the virion and aggregated in low salt buffer. Three glycoproteins which labeled with [^{14}C]-glucosamine were designated HN, F_0 and F_1, and had molecular weights of 69,000, 60,000 and 46,000, respectively. The HN protein was associated with the virus envelope, had intramolecular disulfide bonds, and did not form disulfide-linked multimers. F_1 was derived from a complex of two disulfide-linked polypeptides ($F_{1,2}$). $F_{1,2}$ had an electrophoretic mobility similar to that of F_0 under non-reducing conditions.

Five monocistronic viral mRNA species were identified in infected cells and corresponded to five viral structural proteins (P,HN,NP,F and M). In addition, two structurally bicistronic mRNA species were identified: a NP/P mRNA and a M/F mRNA. M protein was translated in vitro from the M/F bicistronic mRNA.

The nucleotide sequence of HPIV3 hemagglutinin-neuraminidase (HN) gene was determined using cDNA clones derived from both HPIV3 genomic and messenger RNA. The HN mRNA contains 1882 nucleotides not including the poly (A) tail. The HPIV3 mRNA has one large open reading frame that codes for a polypeptide of 572 amino acids with a deduced molecular weight of 64,178. Potential polymerase recognition signals for the HN and L genes are located in the flanking regions. The HN protein of HPIV3 shares some common features with the previously sequenced HN proteins of Sendai virus and simian virus 5. These features include: a N-terminal membrane anchor, four regions of highly conserved amino acid sequence and conserved positions for the cysteine residues. The relationship of the HN sequences is closest between Sendai virus and HPIV3. The hydropathy profile of the HPIV3 HN protein indicates that the HN protein may be a transmembrane protein.

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DEDICATION

This thesis is dedicated to Monica.

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GLOSSARY OF ABBREVIATIONS

BPIV3	Bovine parainfluenza virus 3
BHK-21	Baby Hamster Kidney cells (clone 21)
cDNA	Complementary deoxyribonucleic acid
CDV	Canine distemper virus
CPE	Cytopathic effect
C-terminal	Carboxy-terminal
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphospate
ddATP	Dideoxyadenosine triphosphate
ddCTP	Dideoxycytidine triphosphate
ddGTP	Dideoxyguanosine triphosphate
ddTTP	Dideoxythymidine triphospate
DI particles	Defective interfering particles
DMEM	Dulbecco's modified minimal essential medium
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
HPIV1	Human parainfluenza virus 1
HPIV2	Human parainfluenza virus 2
HPIV3	Human parainfluenza virus 3
HPIV4	Human parainfluenza virus 4
IgA	Immunoglobulin A
Kb	Kilobase
Kbp	Kilobase pairs

LLC-MK2	Monkey kidney cells
MOI	Multiplicity of infection
M.W.	Molecular weight
MEM	Minimal essential medium
mRNA	Messenger ribonucleic acid
NDV	Newcastle disease Virus
N-terminal	Amino-terminal
P.F.U.	Plaque forming units
[R(B77)]	Avian Sarcoma Virus transformed Rat cells
RF DNA	Replicative form of M13 deoxyribonucleic acid
RNP	Ribonucleoprotein
SDS	Sodium Dodecyl Sulfate
SSC buffer	0.15 M Sodium chloride, 0.015 M Sodium citrate
SSPE	Subacute sclerosing panencephalitis
SV5	Simian virus 5
TCA	Trichloroacetic acid
TE buffer	0.02 M Tris-HCl [pH 7.4], 0.001 M EDTA
ts	Temperature sensitive
VSV	Vesicular stomatitis virus

CHAPTER 1 INTRODUCTION

1.1 HISTORICAL AND LITERATURE REVIEW OF VESICULAR STOMATITIS VIRUS

Vesicular stomatitis virus (VSV) causes disease in cattle, pigs and horses (Brown and Crick, 1979; Emerson, 1985). In cattle, manifestations of this disease include an inability to eat, frothing, excessive salivation and ulceration of the mouth (Brown and Crick, 1979). The disease caused by VSV is regarded as a disease of the Western Hemisphere since there are few occurrences reported outside of this area (Brown and Crick, 1979). Although VSV is not a human pathogen, the economic impact of the disease in cattle and swine is considerable.

VSV is classified as a member of the family Rhabdoviridae (Matthews, 1982). The Rhabdoviridae family consists of 3 genera. VSV is the prototype of the Vesiculovirus genus. The genus Lyssavirus is comprised of rabies-like viruses and the third genus is comprised of plant rhabdoviruses. VSV can be further classified into two serotypes, designated as Indiana or New Jersey. Unless it is specified the VSV referred to in this thesis belongs to the Indiana serotype.

1.1.1 The virion structure of Vesicular stomatitis virus

The virions of VSV have a bullet shaped outline (Stone et al., 1961, Howatson and Whitmore, 1962) with a particle length of 160-180 nm (Murphy and Harrison, 1979). VSV is composed of 74% protein, 20% lipid, 3% carbohydrate, and 3% RNA (Wagner, 1975). Each virion contains a single strand of RNA which can not serve as a messenger but which is complementary to viral messenger RNA's (mRNA) (Prevec and Whitmore, 1963; Huang and Wagner, 1966; Huang et al., 1970; Mudd and Summers, 1970). A RNA-dependent RNA polymerase, needed for the synthesis of viral mRNA's, must accompany the genome of VSV into the cell (Emerson and Wagner, 1972).

From the point of view of both structure and function, VSV can be divided into two units: the ribonucleoprotein complex (RNP) and the envelope. There are two stages in the formation of the RNP complex. First, the genomic RNA of VSV is surrounded with the nucleocapsid (N) protein to form a helical nucleocapsid (Blumberg et al., 1981). Then the helical nucleocapsid associates with the L and the NS proteins (Kang and Prevec, 1969; Wagner et al., 1970; Cartwright et al., 1970; Bishop and Roy, 1972). The L and the NS proteins of VSV make up the active RNA-dependent RNA polymerase (Emerson and Wagner, 1972). The functions of the envelope of VSV are to protect the RNP from degradation and to aid in the entry of the genomic RNA into the host cell (Bishop et al., 1975). The envelope consists of a lipid bilayer studded with glycoproteins on the external surface and lined internally by the membrane (M) protein (Cartwright et al., 1969; Schloemer and Wagner, 1975; Landsberger and Compans, 1976; Dubovi and Wagner, 1977). In the virion the M protein is sandwiched between the lipid bilayer and the RNP complex. Presumably, the M protein serves as a link between the envelope and the RNP (Dubovi and Wagner, 1977).

1.1.2 Vesicular stomatitis virus replication

A. Viral Attachment and Penetration

The glycoprotein (G protein) of VSV mediates attachment of the virion to the host cell receptors (Kelly et al., 1972; Wiktor et al., 1972). The nature of the receptor on the host cell is unknown. However, VSV infects a wide range of cell types so the receptor must be a common component of the eukaryotic cell plasma membrane. Phosphotidylserine is a candidate for the receptor for several reasons. It is a common component of a number of cell membranes. Moreover, it inhibits both binding of VSV to membranes and plaque formation of VSV. Additionally, phosphotidylserine-containing liposomes will bind to VSV (Schlegel et al., 1982; 1983).

The mechanisms of penetration and uncoating of VSV are unclear. The prevailing thought is that after attachment to clathrin-coated regions on the cell, the virus is ingested into the cell by endocytosis of coated vesicles (Matlin et al., 1981; Schlegel et al., 1982). Once the vesicle is internalized, the pH within the vesicle drops, triggering the membrane fusion activity of VSV. The G protein of VSV is responsible for this pH-dependent membrane fusion activity (Hughes et al., 1979a; Handa et al., 1982; Mifune et al., 1982; Florkiewicz and Rose, 1984; Riedel et al., 1984). Once the pH drops, the G protein induces fusion of the viral and vesicle membrane which results in entry of the VSV nucleocapsid into the cytoplasm (Mifune et al., 1982).

B. RNA Transcription

As soon as the infecting virus uncoats, transcription is initiated by the viral RNA-dependent RNA polymerase. Transcription begins at the 3' end of the genome with the synthesis of a small RNA product (48 base leader sequence) (Colonno and Banerjee, 1976). The small RNA product does not contain a 5'-terminal 7-methylguanosine nor a 3'-terminal polyadenylate sequence. In contrast, the viral mRNA's produced have both these features (Abraham et al., 1975; Moyer et al., 1975). Transcriptional mapping studies have shown that transcription begins at the 3' end of the genome and proceeds in a sequential manner toward the 5' end of the genome (Ball and White, 1976). The amount of mRNA produced decreases as the distance to the gene from the 3' end of the genome increases. Thus, the order of mRNA production from most abundant to least abundant is N, NS, M, G, and L (Ball and White, 1976).

C. Genome Replication

The replication of the genome of VSV is coupled to translation of the

N, NS and L proteins (Wertz and Levine, 1973; Wertz, 1980). Replication of VSV RNA occurs in two stages. First, a full length RNA strand complementary to the genomic RNA is synthesized and the N protein associates with the RNA to form a nucleocapsid (Blumberg et al., 1981). In the second stage of replication, the complementary RNA is used as a template to generate a full length genomic RNA. Immediately after synthesis, the genomic RNA is surrounded with N protein. The mechanism involved in switching viral transcription to viral replication has not been determined. Possibly, control of genome replication is determined by an interaction between the N protein and leader RNA (Blumberg et al., 1981).

D. Protein Synthesis

Translation of the five viral mRNA's occurs immediately after transcription. VSV mRNA's are efficiently translated throughout the infectious cycle. The small leader RNA, produced during transcription, is not translated. Regulation of viral protein synthesis occurs at the transcriptional level rather than at the translational level. Thus, N protein is the most abundant protein and the L protein is the least abundant protein synthesized (Hsu et al., 1979).

Synthesis of the G protein is carried out on membrane bound polyribosomes (Knipe et al., 1977a). The G protein remains associated with the membrane throughout synthesis, glycosylation, processing and transport to the plasma membrane of the cell (Knipe et al., 1977a; Rothman and Fine, 1980). Once the G protein reaches the plasma membrane it is inserted so that the carboxyl terminus extends into the cytoplasm but the bulk of the protein is located on the external surface of the membrane (Emerson, 1985). The other four VSV proteins are translated on cytoplasmic polyribosomes (Morrison and Lodish, 1975).

E. Virion Assembly

Assembly of the virion occurs only when membrane-associated G protein, soluble M protein and the RNP complex come together at a discrete site on the membrane (Knipe et al., 1977a). The exact mechanism of assembly has not been worked out. However, it is likely that the M protein promotes clustering of the G protein, at numerous membrane sites, followed by binding of the RNP and budding of the virion (Reidler et al., 1981; Wilson and Lenard, 1981).

1.1.3 Temperature sensitive mutants of vesicular stomatitis virus

Temperature sensitive (ts) mutants of VSV can be divided into five groups on the basis of complementation studies. These mutations are conditional lethal mutations. Therefore, at the non-permissive temperature very little progeny virus is produced. Each of the complementation groups can be phenotypically linked to a specific protein of VSV (Indiana) as follows: I-L protein (Hunt et al., 1976), II-NS protein, III-M protein, IV-N protein, and V-G protein (Pringle, 1977).

1.1.4 Cytopathic effects induced by vesicular stomatitis virus

Virus-induced changes in cells, known as cytopathic effects (CPE) have been used since the early 1950's as a diagnostic tool for the identification of viruses (Malherbe, 1985). The cytopathic effects characteristic of viral infections include: swelling or shrinkage of cells, cell fusion (syncytial formation or polykaryocytosis), lysis or necrosis of the cells, inclusion formation (intranuclear or cytoplasmic), distortion or displacement of the nucleus, nucleolar alterations, vacuolation of the cytoplasm, or various combinations of these changes (Craighead, 1978; McIntosh, 1985; Malherbe, 1985). The cytopathic effects associated with common viral infectious agents are outlined in Table 1 (Craighead, 1978).

TABLE ONE: CYTOPATHIC CHANGES ASSOCIATED WITH COMMON VIRAL INFECTIOUS AGENTS [1]

Cytopathic effect	Infectious Agents
Necrosis	Enteroviruses Herpesviruses Arboviruses Rhabdoviruses
Inclusions intranuclear	Herpesviruses Adenoviruses Papovaviruses
intracytoplasmic	Rabies virus Poxviruses numerous other viruses
Multinucleated Giant cells (Polykaryocytosis)	Paramyxoviruses Herpesviruses Myxoviruses
Cytomegaly	Herpesviruses Adenoviruses Papovaviruses
Cytoplasmic modifications and vacuolation	"Slow" viruses Hepatitis viruses SV40

[1] Table modified from Craighead, (1978).

VSV is a cytocidal virus which quickly kills the infected host cell in tissue culture. Thus, the predominant cytopathic effect of VSV, a rhabdovirus, is necrosis (Table 1). Cell death resulting from VSV infection can follow one of two courses of cytopathology. A rapid cellular response occurs if the cells are infected with VSV at a multiplicity of infection of greater than 200 plaque forming units/cell. This course of cytopathology does not require active viral synthetic functions (Baxt and Bablanian, 1976a). The second course of cytopathology is slower and does require replication of the virus (Baxt and Bablanian, 1976a). Unless otherwise specified, this thesis deals with the second slower course of VSV cytopathology. The observed result of a VSV infection of cultured cells is cell swelling and rounding, leading to a rapid destruction of the cells due to disruption of the membranes (Baxt and Bablanian, 1976a; Marcus and Sekellick, 1974; Murphy and Harrison, 1979). This characteristic cytopathic change in the infected cell has long been used as a morphological indicator of VSV infection.

In addition to morphological changes in infected cells, VSV infection also causes an efficient inhibition of the cellular RNA, DNA and protein synthesis (McGowan and Wagner, 1981). Viral transcription, but not replication, is required for inhibition of macromolecular synthesis and for cell killing (Marcus and Sekellick, 1974; Marcus et al., 1977). Transcription of only a small portion of the genome (probably the leader sequence) is required for the inhibition of RNA synthesis (Weck et al., 1979). The kinetics of inhibition of cellular RNA and DNA synthesis are identical (McGowan and Wagner, 1981). Thus, it is likely that the same VSV function shuts off both cellular DNA and RNA synthesis. Viral infection can proceed without cellular RNA and DNA synthesis but must utilize the cellular protein synthesis machinery. Clearly, there are different

requirements for the inhibition of protein synthesis than for either DNA or RNA synthesis. Transcription of at least the N gene, and possibly the NS gene, is required for inhibition of cellular protein synthesis (Marvaldi et al., 1978). Furthermore, it is possible that cellular protein synthesis is not inhibited until the N and NS mRNA's are translated (Marvaldi et al., 1978).

1.1.5 Vesicular stomatitis virus induced cell fusion

Virus-induced cell fusion (polykaryocytosis) is the formation of a multinucleated cell through the fusion of two separate cells, as a result of viral infection (Poste, 1970; 1972). A number of enveloped RNA and DNA viruses induce cell fusion (Poste, 1970; 1972; Takehara, 1975; Nishiyama et al., 1976). There are two types of virus-induced cell fusion, designated fusion from within and fusion from without. Fusion from without can be produced by either infectious or inactivated virions and does not have a requirement for either viral protein synthesis or replication (Poste, 1972). Characteristically, fusion from without requires a high multiplicity of infection and occurs in a short period of time (Poste, 1972). In contrast, fusion from within occurs at low multiplicity of infection and takes longer. In addition, both infectious virus and viral protein synthesis are required for fusion from within (Poste, 1972). Production of progeny virus is not a requirement for fusion from within because conditional lethal mutants grown under non-permissive conditions can still mediate cell fusion (Poste, 1972). Little is known about the molecular mechanisms involved in virus-induced cell fusion. However, viral glycoproteins are involved in both fusion from without (Scheid and Choppin, 1974; 1976) and fusion from within (Chany-Fournier et al., 1977; Manservigi et al., 1977; Hughes et al., 1979a).

VSV and some of its temperature sensitive mutants do not always show the typical cytolytic response but rather induce cell fusion in specific host cells (Takehara, 1975; Nishiyama et al., 1976; Chany-Fournier et al., 1977; Hughes et al., 1979a; Handa et al., 1982). For example, VSV (Indiana) induces marked cell fusion in two clones of BHK-21 cells but shows the typical cytolytic effect in LLC-MK2 cells, L cells and HeLa cells (Takehara, 1975). Furthermore, VSV (New Jersey) induces a high level of polykaryocytosis in mouse L cells and a much lower level of cell fusion in BHK-21 cells (Nishiyama et al., 1976). Temperature sensitive mutants of VSV (Indiana), ts O52(II) and ts G31(III) induce cell fusion in rat XC cells (Chany-Fournier et al., 1977; Handa et al., 1982). The mutant ts G31(III) also induces polykaryocyte formation in mouse Ehrlich ascitic tumor cells and in murine neuroblastoma cell lines N-18 and N-2A (Hughes et al., 1979a; Handa et al., 1982). Clearly, a specific combination of host cell and virus is required for VSV-induced cell fusion. Additionally, VSV-induced cell fusion requires a low multiplicity of infection and viral protein synthesis (Takehara, 1975; Nishiyama et al., 1976; Chany-Fournier et al., 1977; Hughes et al., 1979a; Handa et al., 1982). Thus, VSV infections of specific host cells results in cell fusion from within. Environmental factors may influence VSV-induced cell fusion. For instance, if the pH of the medium is lowered to below 6.0, VSV infection can result in cell fusion (White et al., 1981).

The molecular mechanism of VSV-induced cell fusion has not been determined. However, at least one viral protein is involved. A cDNA clone encoding the VSV glycoprotein gene has been expressed in a stable cell line (Florkiewicz and Rose, 1984; Riedel et al., 1984). In the presence of low pH (5.5), the cells expressing the G protein at the cell surface fused (Florkiewicz and Rose, 1984). Cell fusion could be prevented by incubation

of the cells with a monoclonal antibody directed against the G protein (Florkiewicz and Rose, 1984). Clearly, the G protein of VSV promotes cell fusion in the absence of the other viral proteins (Florkiewicz and Rose, 1984; Riedel et al., 1984). However, other factors must also be involved in VSV-induced cell fusion. Indeed, G protein is expressed at the cell surface during a cytolytic infection yet cell fusion does not occur. Furthermore, at physiological pH, G protein expressed on the cell surface does not result in cell fusion (Florkiewicz and Rose, 1984). Further work is needed to identify other factor(s) involved in VSV-induced cell fusion.

1.2 HISTORICAL AND LITERATURE REVIEW OF HUMAN PARAINFLUENZA VIRUS 3

1.2.1 Taxonomy of the Parainfluenza Viruses

The parainfluenza viruses belong to the family Paramyxoviridae, genus Paramyxovirus (Kingsbury et al., 1978). The family Paramyxoviridae includes two other genera, Morbillivirus and Pneumovirus. Table 2 outlines some of the human and animal viruses in each genus. The four parainfluenza viruses are separated on the basis of serological differences. In this study comparisons will be made between human parainfluenza virus 3 and the other members of the family Paramyxoviridae.

1.2.2 Clinical significance of human parainfluenza virus 3 infections

Human parainfluenza virus 3 (HPIV3) was first isolated from children with acute respiratory disease in 1956 (Chanock, 1956; Chanock et al., 1958). HPIV3 has been shown to cause pneumonia, bronchiolitis and croup (Chanock et al., 1958; 1959; Parrott et al., 1962; Glezen and Denny, 1973). In fact, HPIV3 ranks second only to respiratory syncytial virus (RSV) as a cause of lower respiratory tract disease in infants (Parrott et al., 1962; Glezen and Denny, 1973). HPIV3 infections characteristically occur early in

TABLE TWO: PARAMYXOVIRIDAE

Genus	Characteristic Feature	Human Viruses	Related Animal Viruses
Paramyxovirus	Virions contain membrane fusion, hemagglutinin and neuraminidase activities	PIV1	Sendai virus (murine)
		PIV2	Simian virus 5 (canine and simian)
		PIV3	Shipping fever virus (bovine)
		PIV4	-
		-	Newcastle disease virus (avian)
Morbillivirus	Virions lack neuraminidase activity	measles	Canine distemper virus
Pneumovirus	Virions lack hemagglutinin and neuraminidase activity	respiratory syncytial virus	-

life. Serological evidence indicates that at least 60% of children are infected by five months of age and that 80% or more are infected by 2 years of age (Parrott et al., 1962). When every individual of a population is followed from birth, infection rates of 75% to 100% are found for children in the first two years of life (Glezen et al., 1981; Hope-Simpson, 1981). Re-infection with HPIV3 occurs commonly in children and adults (Welliver et al., 1982; Glezen et al., 1984). The combination of high infection rates early in life, coupled with high frequency of re-infection suggests that HPIV3 spreads efficiently from person to person. These characteristics make HPIV3 infections particularly troublesome in semi-closed populations such as nurseries or pediatric hospital wards (Mufson et al., 1973). For example, in a number of outbreaks of HPIV3 infections, in nursery schools, virtually every susceptible individual was infected (Chanock et al., 1963).

1.2.3 The virion structure of Paramyxoviruses

Despite the clinical significance of HPIV3 infections, the virus has not been examined in any detail at the molecular level. However, extensive work has been carried out on other paramyxoviruses such as Sendai virus (a murine parainfluenza virus 1), simian virus 5 (a canine parainfluenza virus 2), and Newcastle disease virus (an avian parainfluenza virus). These three viruses have been found to be structurally and functionally similar. Thus, for illustrative purposes Sendai virus is generally used as an example of the paramyxoviruses.

The virions of the paramyxoviruses are roughly spherical, but vary in both size and shape (Matsumoto, 1982). They range in diameter from 150 to 300 nm (Matsumoto, 1982). The composition of the simian virus 5 (SV5) virion as a percentage of dry weight was determined to be 73% protein, 20% lipid, 6% carbohydrate and 1% RNA (Klenk and Choppin, 1969). Newcastle disease virus has a similar composition. Sendai virus contains one large

single stranded RNA genome with a molecular weight of approximately $5x10^6$ (Barry and Bukrinunskaya, 1968; Blair and Robinson, 1968; Kingsbury et al., 1970; Kolakofsky et al., 1974b). The genome of Sendai virus cannot serve as a messenger RNA and is complementary to Sendai virus messenger RNA (Chanock and McIntosh, 1985). Like VSV, Sendai virus must carry a RNA-dependent RNA polymerase within the virion for virus replication (Chinchar and Portner, 1981).

Virions of Sendai virus are composed of two functionally separate entities, the ribonucleoprotein complex (RNP) and the envelope. The ribonucleoprotein complex consists of the viral genomic RNA surrounded by three proteins, the L, P, and NP proteins (Mountcastle et al., 1970; Stone et al., 1972). The L (large) protein and the P (phosphoprotein) are present in minor amounts on the RNP (Lamb et al., 1976). The NP (nucleocapsid) protein is the most abundant component of the RNP complex (Lamb et al., 1976). The L, NP, and P proteins have all been shown to be involved in RNA polymerase activity (Mountcastle et al., 1970; Stone et al., 1972; Chinchar and Portner, 1981; Hamaguchi et al., 1983). The envelope of the paramyxoviruses consists of a lipid bilayer studded externally with two types of glycoproteins and lined internally with the M (membrane) protein (Lamb and Mahy, 1975). The M protein, located between the envelope and the RNP, plays a central role in viral assembly and budding (Choppin and Compans, 1970; Shimizu and Ishida, 1975; Yoshida et al., 1979). The two glycoproteins (HN and F proteins) of Sendai virus, located on the external surface of the virus membrane, are believed to be transmembrane proteins (Lyles, 1979).

The HN (hemagglutinin/neuraminidase) protein is present on the surface of the virion as a dimer joined by disulfide bonds in the hydrophilic

region and by hydrophobic bonds at the base (Scheid et al., 1978; Choppin and Scheid, 1980; Markwell and Fox, 1980). The HN proteins of Sendai virus, NDV and SV5 are believed to be oriented with the N-terminus inserted into the membrane (Schuy et al., 1984; Blumberg et al., 1985; Heibert et al., 1985a; Shioda et al., 1986). The F (fusion) glycoprotein is involved in virus-induced hemolysis, membrane fusion and initiation of infection (Homma and Ohuchi, 1973; Scheid and Choppin, 1974; 1976). The active fusion protein ($F_{1,2}$) of Sendai virus is formed by proteolytic cleavage of an inactive precursor (F_0) to form two disulfide linked proteins (F_1 and F_2) (Scheid and Choppin, 1974; 1976).

1.2.4 Paramyxovirus replication

The HN glycoprotein is responsible for adsorption to the neuraminic acid residues of the host cell receptors (Scheid and Choppin, 1974). Presumably, the RNP complex enters the cell after fusion of the viral and cellular membrane. This fusion of membranes is independent of pH (Nagai et al., 1983). Furthermore, Sendai virus replication is not inhibited if endocytosis of the virus into lysosomal vesicles is blocked (Nagai et al., 1983). Once the RNP has gained access to the cytoplasm the viral genome is transcribed without losing its protein coat (Stone et al., 1971). Its genetic information is coordinately expressed as a series of mRNA's transcribed from the viral genome. The entire genome of Sendai virus has been sequenced and the gene order has been found to be 3'-NP-(P+C)-M-F-HN-L-5' (Shioda et al., 1986). Five of the Sendai virus mRNA species specifying the L, HN, F, NP and M proteins are monocistronic and a sixth mRNA is polycistronic specifying the P, C, and C' proteins (Giorgi et al., 1983, Shioda et al., 1983). The C and C' proteins are Sendai virus specific non-structural proteins (Lamb and Choppin, 1978; Dethlefsen and Kolakofsky, 1983). An equivalent protein to the Sendai virus C protein is also

produced in SV5; this protein is designated V (Paterson et al., 1984a). Recent evidence suggests that the C protein is translated from a second open reading frame in the P mRNA (Giorgi et al., 1983; Shioda et al., 1983).

After transcription the viral mRNA's are translated. The two glycoproteins of NDV are synthesized on the rough endoplasmic reticulum (Nagai et al., 1976). They are then transported, via smooth intracellular membranes, to the plasma membrane (Nagai et al., 1976). It is not clear where synthesis of the other proteins occurs. Presumably, synthesis occurs in association with cytoplasmic polyribosomes. After translation, the M protein immediately incorporates into the plasma membrane (Nagai et al., 1976). As a result, intracellular pools of the M protein remain low (Nagai et al., 1976). After synthesis, the NP and P proteins of Sendai virus rapidly associate with the viral RNA (Portner and Kingsbury, 1976). However, in contrast to the M protein, intracellular pools of the NP and P protein accumulate during infection (Portner and Kingsbury, 1976).

Replication and transcription may be regulated by the NP protein of Sendai virus (Carlsen et al., 1985). Viral protein synthesis is required for RNA replication since addition of cycloheximide to an infected cell inhibits replication (Robinson, 1971; Carlsen et al., 1985). In contrast, transcription does not require protein synthesis (Carlsen et al., 1985). The RNA replicase of the Paramyxoviruses has not been identified. Furthermore, the RNA-dependent RNA polymerase cannot simultaneously serve as a transcriptase and a replicase (Choppin and Compans, 1975). Genomic RNA replicates using a full length complementary RNA as a template (Carlsen et al., 1985).

The first step in paramyxovirus assembly is the insertion of the viral

glycoproteins into the membrane of the host (Klenk et al., 1970). The M protein is then selectively associated with the plasma membrane regions containing the viral glycoproteins (Nagai et al., 1976). Finally, the RNP complex associates with the region of the membrane containing the M protein. Subsequently, the virion is formed by the process of budding (Matsumoto, 1982). Sendai virus nucleocapsids packaged into the virion contain either genomic sense or anti-genomic sense RNA (Kolakofsky and Bruschi, 1975). However, virions containing genomic sense negative strand RNA predominate.

1.2.5 The role of the glycoproteins of the paramyxoviruses in the immune response of the host

Antibodies to the viral surface (glyco)proteins are important for the development of immunity to infection (Choppin and Scheid, 1980). For the paramyxoviruses, two glycoproteins form spikes on the external surface of the viral lipid bilayer. The larger glycoprotein (HN) is responsible for hemagglutinin and neuraminidase activities and for adsorption of the virus to the host cell (Scheid et al., 1972). The other glycoprotein (F) is involved in hemolysis and in virus induced membrane fusion (Homma and Ohuchi, 1973; Scheid and Choppin, 1974; 1976). Presumably, the HN and F proteins are the critical antigens recognized by the immune system of the host. Indeed, if antiserum, directed against either the HN or F protein, is mixed with the virus prior to inoculation of the cells, then the infectivity of the virus is neutralized (Merz et al., 1980; 1981). However, if a few cells are infected and then the antiserum added, the anti-HN antibody fails to inhibit the spread of the virus (Merz et al., 1980; 1981). On the other hand, if anti-F antiserum is added after a few cells are infected, spread of the infection is completely inhibited. The interpretation of these experiments is that although any released virus is

neutralized by the anti-HN antiserum, spread of the infection by cell fusion between adjacent cells is not stopped. In contrast, anti-F antibody completely inhibits the spread of the virus by both neutralizing the released virus and preventing spread of infection by fusion of adjacent cells (Merz et al., 1980; 1981). These studies suggest that anti-HN antibodies alone may not be sufficient to protect against a parainfluenza virus infection. Furthermore, protection against parainfluenza virus infections may be critically dependent on the development of anti-F antibody.

1.2.6 The immune response of the host to infection by human parainfluenza virus 3

Immunity to HPIV3 infection, shortness of the duration of illness and less severe symptoms correlates to the presence of serum neutralizing antibodies (Chanock et al., 1963). The correlation of immunity to neutralizing antibody is partial because one third of infants and children with a high level of serum antibody still become infected (Chanock et al., 1963). During the first four months of life the risk of infection by HPIV3 is inversely related to the level of neutralizing antibody present in the cord sera at birth (Glezen, cited in Chanock and McIntosh, 1985). This correlation is not absolute because some infants with a moderately high level of maternally-derived antibody become infected with HPIV3. The importance of the secretory immune system in protection against HPIV3 has not been addressed. However, for HPIV1 and HPIV2 a strong correlation exists between resistance to infection or illness and higher levels of secretory IgA neutralizing antibodies in the respiratory tract mucosa (Smith et al., 1966; Tremonti et al., 1968).

Infection with HPIV3 leads to the development of antibodies against

the HN and F glycoproteins (Kasel et al., 1984). However, in serum specimens taken either after primary infection or from cord serum immediately after birth, the anti-HN antibody titre was consistently higher than the anti-F antibody titre (Kasel et al., 1984). It is only after repeated exposure to the virus that infants show a rise in the level of anti-F antibody. Indeed, low anti-F titres may account for the incomplete immunity which develops after HPIV3 infection. The poor immune response to the F protein may be due to low amounts of F protein on the surface of the virus. Thus, repeated exposure to the virus is needed to stimulate an immune response to the F protein. However, the relationship between protection and the level of F antibody is not yet clear. An alternative explanation for the high frequency of reinfection among infants and young children could be a poor local immune response. In adults the response to infection is an increase in local secretory IgA antibody which neutralizes the infectivity of the virus (Smith et al., 1966; Tremonti et al., 1968). However, in young infants undergoing primary infection, the local IgA antibody response may be weak or ineffective in neutralizing the virus (Yanagihara and McIntosh, 1980). This factor coupled with a poor serum immune response to the F antigen may lead to poor resistance to infection in infants even after the initial exposure to HPIV3.

Formalin-inactivated viral vaccines against the parainfluenza viruses have not been successful. Indeed, higher infection rates occur in the vaccinated populations than in the unvaccinated populations (Chin et al., 1969; Fulginiti et al., 1969; Vella et al., 1969). However, these vaccines are antigenic as indicated by the development of hemagglutination-inhibiting antibodies and complement-fixing antibodies (Chin et al., 1969; Fulginiti et al., 1969; Vella et al., 1969). Two characteristics of the vaccines may account for their failure to protect against infection. One

characteristic is that the vaccines fail to elicit a local secretory IgA response (Chanock and McIntosh, 1985). Another characteristic of formalin-inactivated vaccines is that the antigenic structure of the F protein is altered (Orvell and Norrby, 1977; Chanock and McIntosh, 1985). An alteration in the antigenic structure of the F protein could lead to an incomplete immune response and to immunopathological processes (Orvell and Norrby, 1977).

HPIV3 infections pose a major clinical problem because they cause serious lower respiratory tract infections in infants and young children, are readily transmissible and are likely to recur. Yet, no effective immunoprophylaxis is available. Clearly, a strategy for the prevention of HPIV3 infections by immunological or antiviral means is needed.

1.3 The Role of Viral Glycoproteins in Interactions Between the Virus and Host Cell

Viral glycoproteins play a multifaceted and critical role in the initiation of viral infection and the response of a host to that infection. For every enveloped virus in which adsorption and membrane fusion has been investigated, the viral glycoprotein(s) mediate these functions (Choppin and Scheid, 1980; Wiley, 1985). Membrane fusion may occur at the cell surface such as that mediated by the fusion protein of Sendai virus (Homma and Ohuchi, 1973; Scheid and Choppin, 1974; 1976). Alternatively, viruses like vesicular stomatitis virus, influenza virus, and Semliki forest virus, which undergo receptor-mediated endocytosis, have a glycoprotein which fuses the virus membrane and the vesicle membrane intracellularly.(Marsh and Helenius, 1980; Matlin et al., 1981; 1982). The fusion activity of these viruses is activated by the acidic pH of the vesicle (White et al.,

1983). The ability of viruses to undergo multiple cycles of replication, to spread in the host and to cause disease are dependent on repeated rounds of adsorption and penetration (Choppin and Scheid, 1980). Therefore, the involvement of viral glycoproteins in adsorption and penetration suggests that these proteins play a role in the pathogenesis of viral infections. Additionally, viral glycoproteins can directly cause cytopathic effects (Holmes and Choppin, 1966; Homma and Ohuchi, 1973; Scheid and Choppin, 1974; McSharry and Choppin, 1978; Dietzschold et al., 1983). However, not only are viral glycoproteins virulence factors but they are also the most obvious and accessible antigens for the immune response of the host to recognize (Sissons and Oldstone, 1985). The importance of viral glycoproteins as antigens stems from their presentation on the surfaces of infected cells and the virion (Sissons and Oldstone, 1985). The external presentation allows viral glycoproteins to stimulate numerous immunological responses, especially the induction of virus neutralizing antibodies (Choppin and Scheid, 1980; Crumpacker 1980; Wiley, 1985).

In spite of the critical role that viral glycoproteins play in infection, only recently is information emerging on the specific involvement of glycoproteins in virus-host cell interactions. The focus of my study was on the involvement of viral glycoproteins in virus-host cell interactions. Two model systems were examined to gain insight into virus-host cell interactions. One aspect of this thesis examines the molecular mechanisms of vesicular stomatitis virus (VSV) induced cell fusion. The other aspect of this thesis deals with the analysis of human parainfluenza virus 3 (HPIV3): virion components, intracellular viral-specific components and the primary structures of the viral genes and proteins. Identification of the viral glycoproteins, elucidation of their biosynthesis, analysis of

their primary structure and their relationship to other viral components are important first steps toward understanding their role in virus-host cell interactions.

1.4 STATEMENT OF OBJECTIVES

A 1. To characterize the requirements for VSV-induced cell fusion with specific emphasis on the modulating factors of membrane fusion.

B 1. To characterize the structural components of the virions of HPIV3 (viral genomic RNA(s) and proteins).

2. To characterize the viral specific intracellular components of virus infected cells (viral specific RNA's and proteins).

3. To elucidate the nucleotide sequence of the gene coding for the hemagglutinin-neuraminidase protein of HPIV3.

CHAPTER 2: MATERIALS AND METHODS

2.1 The Study of Cell Fusion Induced by Vesicular Stomatitis Virus

2.1.1 Cells and viruses

The VSV Indiana (Heat Resistant variant) used in this study, which will be referred to as VSV (IND-HR), was provided by Dr. L. Prevec, McMaster University, Hamilton. VSV Indiana (New Mexico), was provided by C.H. Calisher, The Center for Disease Control, Fort Collins. This isolate originated from an Aedes species at Rancho De Aliquin, New Mexico in 1966. VSV Indiana (Glasgow) was obtained from Dr. C. Pringle, Glasgow, Scotland. VSV New Jersey (Concan) was isolated in Concan, Texas in 1949 from material harvested from the dental pad of an infected cow. It was obtained from Dr. R. Simpson, Rutgers University, who received it from Dr. R.R. Wagner, University of Virginia. Further descriptions of these virus stains can be found in Clewley et al. (1977).

Baby hamster kidney cells (BHK-21) derived from clone 21, were obtained from the American Type Culture Collection. Cultures of rat cells transformed with the B77 strain of avian sarcoma virus [R(B77)] were originally obtained from Dr. Howard Temin, McArdle Laboratory, University of Wisconsin, Madison. All cell lines were grown in Dulbecco's modified minimal essential medium (DMEM) (Grand Island Biological Co.) supplemented with 5% heat treated fetal bovine serum (Grand Island Biological Co.). The formulations of DMEM and the other tissue culture media used in these studies are outlined in Appendix 1.

2.1.2 Growth of cell cultures in amino acid deficient media

The concentrations of amino acids in the media were varied by adding a concentrated amino acid mixture to a medium lacking amino acids. The medium lacking amino acids contained Earle's balanced salt solution (50ml), sodium

bicarbonate (2.54 g), minimal essential medium vitamins (20ml), 0.2 M L-glutamine (5ml), heat-treated fetal bovine serum (25ml) and distilled H_2O (355ml). The normal concentration of amino acids was obtained with the addition of 20ml of a minimal essential medium amino acids mixture. Earle's balanced salt solution, minimal essential medium vitamins, fetal bovine serum and minimal essential medium amino acids mixture were all obtained from Grand Island Biological Co. The formulations of these products are outlined in Appendix 1. To prepare amino acids deficient media containing 1/5, 1/10, 1/20 or 1/30 of the normal amino acids concentration, 4, 2, 1, or 0.67 ml respectively of the minimal essential amino acids mixture was added to the medium lacking amino acids. The amount of distilled H_2O was adjusted to bring the volume up to 500ml.

Twelve hours before infection, medium in the cultures was removed and replaced with medium containing the appropriate concentration of amino acids. The cells were infected with VSV at a multiplicity of infection (MOI) of 1. After the 1 hour adsorption period, fresh medium containing the appropriate concentrations of amino acids was added to the cell cultures. Microscopic examinations of the cultures were made every four hours.

2.1.3 Inhibition of host cell specific protein synthesis

Duplicate sets of cultures were prepared in media containing either the normal concentrations of amino acids or 1/20 of the normal concentration of amino acids. The compositions of these media are detailed in Section 2.1.2. To one set of cultures, actinomycin D (Merck, Sharp, and Dome) was added to yield a final concentration of 1 ug/ml. Twelve hours after the addition of actinomycin D the cultures were infected with VSV at a MOI of 1. After the 1 hour adsorption period, fresh medium containing the appropriate concentrations of amino acids and actinomycin D was added back to the cultures. The other set of cultures did not receive actinomycin D

but was treated in an identical manner. Microscopic examinations of the cultures were carried out at 18 hours after infection.

2.1.4 Inhibition of total protein synthesis

Puromycin and cycloheximide were purchased from Aldrich Chemical Co. DMEM containing one of the inhibitors at a concentration of 0, 1, 10, 50, or 100 ug/ ml was added to the cell monolayers 15 hours prior to infection. Immediately before infection, the cells were washed once with phosphate buffered saline to remove any residual inhibitor remaining in the medium. The cells were infected with VSV at a MOI of 1. Fresh DMEM free of inhibitors was added to the cultures after the adsorption period of 1 hour. Photographs were taken at 12 and 24 hours after infection.

To determine the levels of protein synthesis in each of the cultures the following procedure was used. Cell cultures were grown in the presence of either puromycin (at a concentration of either 1 or 100 ug/ml) or cycloheximide (at a concentration of 1, 10, 50, or 100 ug/ml) for 15 hours prior to infection. Control cultures containing neither inhibitor were grown in parallel. The cells were infected with VSV at an MOI of 1 with an adsorption period of 1 hour. Fresh medium containing 5 uCi/ml of [^{3}H]-amino acids mixture (Amersham Corp., see product TRK 440 for details on specific activities of the individual amino acids) was added to the cultures after the adsorption period. Twelve hours after the addition of the radioactive amino acids, the cells were scraped from the plates and washed with 2 ml of phosphate-buffered saline. The cells were pelleted by centrifugation at 3000 x g for 20 minutes. The supernatant was carefully drained and 1 ml of 10% SDS was added to the pellet. A 10 ul aliquot of the lysed pellet was mixed with 40 ul of bovine serum albumin (Sigma)(10mg/ml) and 2 ml of 10% trichloroacetic acid (TCA). The sample was precipitated on ice for 10

minutes and centrifuged at 3000 x g for 10 minutes. The pellet was resuspended in 5% TCA and precipitated on ice for 10 minutes. The sample was centrifuged at 3000 x g for 10 minutes and drained. The pellet was solubilized with 2 ul of 2 N NaOH and diluted to 40 ul with H_2O. The sample was counted in 10 ml of Aquasol (New England Nuclear) using a liquid scintillation counter (Beckman).

2.1.5 Preparation of pulse-labeled viral proteins for kinetic study

Cells were grown for 18 hours in DMEM, containing 5% heat-treated fetal bovine serum and 2 ug/ml of actinomycin D and then infected with VSV at a MOI of 1. After the 1 hour adsorption, methionine-free Eagle's modified minimal essential medium (Flow Laboratories) was added to the cultures which were then incubated for 1 hour. [^{35}S]-methionine (1000 Ci/mmol, Amersham) was added to the cultures to yield a final concentration of 3.5 uCi/ml. The cells were incubated in the presence of the radioactive label for 3 hours. The medium containing the radioactive label was removed, and the cells washed with fresh medium. Medium containing an excess of unlabeled methionine was added to the cultures to begin the chase period. Hourly during the chase period, samples were taken. The extracellular virus was harvested by centrifugation of the culture medium at 113,000 x g, for 90 minutes at 4^0 C. The virus pellet was resuspended in 30 ul of 0.00625 M Tris-HCl [pH 6.8]. To obtain intracellular proteins the cell monolayer was scraped from the plate, washed in 2 ml of phosphate-buffered saline and pelleted by centrifugation at 3000 x g for 10 minutes. The pellet was resuspended in 1ml of electrophoresis sample buffer (0.00625 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate (SDS), 4% dithiothreitol, 0.02% bromophenol blue, and 20% glycerol). The intracellular and extracellular viral specific proteins were analyzed in SDS-polyacrylamide gels as outlined in section 2.1.6.

2.1.6 Analysis of viral proteins by SDS-polyacrylamide gel electrophoresis.

In general protein samples were prepared by combining the sample with ten times its volume of electrophoresis sample buffer (0.00625 M Tris-HCl [pH 6.8], 4% SDS, 4% dithiothreitol [DTT], 0.02% bromophenol blue, and 20% glycerol). The samples were heated to 100°C for 1 minute just prior to loading and electrophoresis. SDS-polyacrylamide gel electrophoresis of the viral proteins was carried out in a 15% acrylamide-0.09% bisacrylamide separating gel with a 3% acrylamide-0.09% bisacrylamide stacking gel. The discontinuous buffer system has been previously described (Laemmli, 1970). Electrophoresis was carried out at 10 milliamperes for 12 hours. After electrophoresis, the proteins were transferred onto Gene Screen (New England Nuclear) hybridization transfer membrane, by electroblotting according to the procedure of Burnette (1981). Transfer buffer contained 0.02 M Tris, 0.15 M Glycine and 20 % Methanol. Electroblotting was carried out using a constant current of 200 milliamperes for 3-5 hours. After transfer the membrane was sprayed with EnHance (New England Nuclear), dried and exposed to Cronex X-Ray film (Dupont) at -70°C.

In order to compare the relative amounts of each viral protein in a sample, the autoradiographs of the SDS-polyacrylamide gels were scanned with the gel scanning system of a DU-8B spectrophotometer (Beckman). The area of each viral protein peak was calculated and this was compared to the total area of all the protein peaks.

2.2 Study of the Structure of Human Parainfluenza Virus 3.

2.2.1 Cells and virus

The continuous monkey kidney cell line, designated LLC-MK2, was obtained from Flow laboratories. Human parainfluenza virus 3 strain C-243

was obtained from D.A. McLeod of the Laboratory Centre for Disease Control, Ottawa. Strain C-243 was originally obtained from the American Type Culture Collection (strain 47885). The identification of this virus was confirmed by a neutralization test. The HPIV3 specific horse antisera used in the neutralization test were obtained from both Flow laboratories and The Center for Disease Control, Atlanta.

2.2.2 Human parainfluenza virus 3 plaque assay

Approximately $1x10^6$ LLC-MK2 cells were seeded into each $60mm^2$ culture dish 18 hours before inoculation. Serial 10 fold dilutions of the virus were prepared. An aliquot of each serial dilution (100 ul) was inoculated onto monolayer cultures of LLC-MK2 cells and allowed to adsorb for one hour. DMEM containing 0.9% noble agar (4ml) was added to each culture dish. The infected cultures were incubated at 37^oC for 4 days. One milliliter of a 1/8000 dilution of neutral red in phosphate buffered saline was added to each culture. The cultures were then incubated for an additional 24 hours before the plaques were visualized and counted.

2.2.3 Radiolabeling of human parainfluenza virus 3 proteins

To label HPIV3 proteins, radioactive precursors in DMEM were added to the cultures immediately after the adsorption period of one hour. Viral proteins were labeled with either 10 uCi/ml of [^{35}S]-methionine (1,295 Ci/mmol; Amersham), 5 uCi/ml of [^{14}C]-amino acids mixture (300 mCi/mmol; Amersham), 50 uCi/ml of [^{32}P]-orthophosphate (10 mCi/mmol, carrier free, Amersham) or 5 uCi/ml of [^{14}C]-glucosamine hydrochloride (309 mCi/mmol; Amersham). Virus in the culture medium was harvested 48 hours after infection. First, cellular debris was removed by centrifugation at 3000 x g for 10 minutes. The virus was then pelleted by high speed centrifugation (113,000 x g, 2 hours, 4^oC). The virus pellet was resuspended in 200 ul of

sample buffer (0.00625 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.02% bromophenol blue) with or without 4% dithiothreitol. The viral proteins were then analyzed by SDS-polyacrylamide gel electrophoresis (section 2.1.6).

2.2.4 Two dimensional polyacrylamide gel electrophoresis of viral proteins

Two dimensional polyacrylamide gel electrophoresis was carried out using essentially the same methods as Smith and Hightower (1981). Briefly, [^{14}C]-glucosamine hydrochloride (309 mCi/mmol, Amersham) labeled virion proteins were electrophoresed under non-reducing conditions (sample buffer did not contain dithiothreitol). The gel composition and electrophoretic conditions were as detailed in section 2.1.6. A single lane was cut from the gel and soaked in buffer containing 0.5 M Tris-HCl [pH 6.8], 0.4% SDS, and 2% mercaptoethanol for 30 minutes. The equilibrated gel section was then positioned on top of a 15% polyacrylamide gel containing 0.1% SDS and electrophoresed in the second dimension. The electrophoretic conditions were as detailed in section 2.1.6. The proteins in the gel were then electroblotted onto Gene Screen (New England Nuclear), sprayed with EnHance (New England Nuclear) and exposed to Cronex X-Ray Film (Dupont) at -70 ^{o} C.

2.2.5 Triton X-100 Fractionation of the Viral Proteins

Triton X-100 fractionation of the virion proteins was carried out as described previously for simian virus 5 and Newcastle disease virus (Scheid et al., 1972; Scheid and Choppin, 1973; Scheid and Choppin, 1975a). Virus was prepared as in Section 2.2.3 except the viral pellet was resuspended in 200 ul of TE buffer (0.02 M Tris-HCl [pH 7.4], 0.001 M EDTA) containing 0.002 M benzamidine hydrochloride (Sigma) and 0.002 M phenylmethylsulfonyl-fluoride (Sigma). The sample was aggressively vortexed to break up any virus aggregates and then centrifuged (10,000 x g, 5 minutes) to pellet any remaining aggregates. Triton X-100 and KCl were added to the supernatant to

bring the concentration to 2% and 1 M respectively. The soluble components were separated from the insoluble components by centrifugation at 100,000 x g for 30 minutes at 5° C. The pellet was resuspended in 40 ul of TE buffer. The supernatant was layered onto a 5 ml 10-25% sucrose gradient in TE buffer containing 2% triton X-100 and 1M KCl and centrifuged at 200,000 x g for 24 hours at 5 °C. The gradient was fractionated by puncturing the bottom of the tube and collecting 200ul aliquots. Proteins were precipitated from aliquots of each fraction with trichloroacetic acid and counted as detailed in section 2.1.4. Two peaks of radioactivity were located. An aliquot of the lower peak was dialyzed against TE buffer containing no KCl. The aggregate formed during dialysis was pelleted by centrifugation at 100,000 x g for 1 hour at 5 ° C. The samples were analyzed by SDS-polyacrylamide gel electrophoresis as outlined in section 2.1.6.

2.2.6 Extraction and electrophoresis of viral RNA

To label HPIV3 RNA, [^{3}H]-uridine (10 uCi/ml, 20-30 Ci/mmol, Amersham) was added to the culture medium at 12 hours after infection. At 24 hours after infection the virus was pelleted from clarified culture medium. The pellet was then resuspended in 200 ul of buffer containing 0.05 M NaOAc [pH 5.1], 0.1 M NaCl, 0.01 EDTA, 0.5% SDS, and 200 ug/ml of proteinase K (Sigma). Proteinase K digestion was carried out at 37° C for 30 minutes. The virion RNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 V:V) and once with chloroform-isoamyl alcohol (24:1 V:V). Two volumes of ethanol were added to the sample and RNA was precipitated overnight at -20° C. RNA was pelleted by centrifugation at 10,000 x g for 30 minutes at 4° C. The pellet was resuspended in distilled H_2O to give a concentration of 1 ug/ml. HPIV3 RNA (5ul) was denatured with 1M deionized

glyoxal in 0.010 M NaH_2PO_4 and 66% dimethyl sulfoxide at 60°C for 15 minutes. Electrophoresis was carried out in a 1.0% agarose gel with 0.01 M NaH_2PO_4 as the electrophoresis buffer (McMaster and Carmicheal, 1977). The gel was run at 100 volts constant current for 3-4 hours. The gel was impregnated with 2,5-diphenyloxazole, dried, and exposed to Cronex X-ray film (Dupont) at -70°C (Bonner and Laskey, 1974).

2.3 Human Parainfluenza Virus 3 Specific Intracellular Components

2.3.1 Analysis of mRNA From Virus Infected Cells

LLC-MK2 cells were infected at a MOI of 3 and labeled with [^{3}H]-uridine (10 uCi/ml, 20-30 Ci/mmol, Amersham) in the presence or absence of 5 ug/ml of actinomycin D. At 24 hours after infection the culture medium was removed. The cell monolayers were scraped from the plates and resuspended in 5 ml of 0.05 M Tris-HCl [pH 7.2], 0.005 M $MgCl_2$, 0.025 M NaCl, 0.25 M sucrose, 25 ug/ml polyvinylsulfate (Sigma), and 30 ug/ml spermine (Sigma). A 10% solution of triton X-100 and sodium deoxycholate was added to the suspension to give a final concentration of 1% for each detergent. The cells were lysed by Dounce homogenization 5 x on ice. The sample was centrifuged at 10,000 x g for 5 minutes at 4°C. After centrifugation an equal volume of 0.1 M NaOAc, 0.2 M NaCl, 0.02 M EDTA, 1% SDS and 300 ug/ml proteinase K was added to the supernatant. The sample was incubated at 37 °C for 30 minutes. The sample was extracted with phenol-chloroform-isoamyl alcohol as outlined in section 2.2.6. After precipitation with ethanol the RNA was separated into poly $(A)^+$ and poly $(A)^-$ fractions using oligo (dT) cellulose chromatography as detailed by Maniatis et al. (1982). The RNA species were then analyzed on glyoxal gels using the methods outlined in section 2.2.6.

2.3.2 Analysis of viral specific intracellular proteins

LLC-MK2 cells were infected with HPIV3 at a MOI of 10. Sixteen hours after infection the culture medium was replaced with fresh DMEM containing 10 uCi/ml of [^{35}S]-methionine (1000 Ci/mmol, Amersham). The cells were incubated in the presence of the radioactive label for 2 hours. The medium was removed from the cultures. The cell monolayers were scraped from the plates, and pelleted by centrifugation at 3,000 x g for 10 minutes. The cell pellets were resuspended in RIPA buffer (0.05 M Tris-HCl [pH 7.2], 0.15 M NaCl, 0.1% SDS) containing 1% sodium deoxycholate and 1% triton X-100. Immediately after the addition of the RIPA buffer the nuclei were pelleted by centrifugation (10,000 x g, 1 minute) in a microfuge. A 10 ul aliquot of the supernatant was analyzed on a SDS-polyacrylamide gel (Section 2.1.6). In some experiments 0.5 ug/ml of tunicamycin, an inhibitor of glycosylation, was added to the culture medium at the time of infection. The tunicamycin was maintained throughout the infection period. Labeling was carried out for two hours as detailed at the beginning of this section.

2.4 Cloning of the Human Parainfluenza Genes and Analysis of Genome Organization

2.4.1 Construction of human parainfluenza virus 3 specific cDNA clone bank.

The template used for synthesis of double stranded cDNA was poly $(A)^+$ RNA isolated from HPIV3 infected cells 18 hours after infection. The reaction mixture for synthesis of the first cDNA strand contained 5 ug of poly$(A)^+$ RNA, 6 ug of oligo $(dT)_{12-18}$ (Pharmacia/PL), 0.13 M KCl, 0.1 M Tris-HCl [pH 8.3], 0.010 M $MgCl_2$, 0.0025 M dithiothreitol, 0.001 M deoxyadenosine triphosphate, 0.001 deoxycytidine triphosphate, 0.001 M deoxyguanosine triphosphate, 0.001 M deoxythymidine triphosphate, 50 uCi [^{32}P]

deoxycytidine triphosphate (800 Ci/mmol, Amersham) and 34 units of reverse transcriptase (Life Sciences). Synthesis of the first cDNA strand was carried out at 37°C for 30 minutes. Synthesis of the second cDNA strand was carried out as described elsewhere (Gubler and Hoffman, 1983) with the modification that the incubation was carried out at 15°C for 2 hours. The products were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 V:V) and then extracted once with chloroform-isoamyl alcohol (24:1 V:V). The unincorporated label was removed from the sample by passing it over a Sephadex G-50 column. The cDNA was precipitated with 2 volumes of ethanol at -20 °C for 12 hours. An aliquot of the double stranded cDNA (approximately 200 ng) was blunt end ligated into the Sma I site of pUC 8. Aliquots of the ligation mixture were transformed into E. coli HB 101. HPIV3-specific cDNA clones , pPI 3, 10, 14, 28, and 47 were generously provided by Dr. K. Dimock, University of Ottawa. HPIV3-specific cDNA clones, pPIg 10-5, 10-6, 10-7 and 40-1 were generously provided by Marie-Jose Côté, University of Ottawa.

2.4.2. Northern blot analysis of human parainfluenza virus 3 mRNA

RNA from HPIV3 infected cells was isolated from the cells and fractionated into poly $(A)^+$ RNA and poly $(A)^-$ RNA fractions as outlined in section 2.3.1. Approximately 5 ug of poly $(A)^+$ RNA was denatured with glyoxal and electrophoresed as described in section 2.2.6. The RNA was then transferred from the gel to a sheet of nitrocellulose following the procedures of Thomas (1980).

Plasmid or insert DNA was labeled with [^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol, Amersham) by nick translation following the procedures of Maniatis et al. (1975). The labeled DNA was passed over a Sephadex G-50 column to remove the unincorporated label. Labeled DNA was denatured by heating in a boiling water bath for 10 minutes prior to

hybridization.

HPIV3 genomic RNA was labeled following the procedures of Maizels, (1976). Briefly, 2.5 ug of genomic RNA was heated for 60 minutes at 90°C in 25 ul of 0.05 M Tris-HCl [pH 9.5]. The partially degraded RNA was then added to 100 uCi of gamma-[^{32}P]-adenosine triphosphate (3000 Ci/mmol, Amersham) which had been dried under vacuum. The labeling reaction mix consisted of the viral RNA, gamma-[^{32}P]-adenosine triphosphate, 2.5 ul of 10x Kinase buffer (0.050 M Tris-HCl [pH 9.5], 0.01 M $MgCl_2$, 0.002 M dithiothreitol) and approximately one unit of T_4 polynucleotide kinase (Pharmacia/PL). The reaction was carried out at 37°C for 30 minutes. The labeled RNA was precipitated with ethanol and separated from the unincorporated label using a Sephadex G-100 column.

The labeled RNA or DNA molecules were hybridized for 16-20 hours at 65°C to RNA fixed onto nitrocellulose strips. Hybridization buffer contained: 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate), 100ug/ml denatured salmon sperm DNA, 0.1% each of polyvinylpyrrolidone, bovine serum albumin, Ficoll 400 and SDS. After hybridization the nitrocellulose strips were washed three times in 2 x SSC containing 0.1% SDS, followed by three washes in 0.2 x SSC containing 0.1% SDS. Finally the nitrocellulose strips were washed in 0.003 M Tris-base. All washes were of 15 minutes duration and were carried out at room temperature.

2.4.3 Colony Hybridizations

Colony hybridizations were carried out using the procedure described by Grunstein and Hogness, (1975). The colonies were transferred onto Colony/Plaque Screen (New England Nuclear). Plasmid or insert DNA was labeled by nick translation as described in section 2.4.2. The hybridization buffer contained 50% deionized formamide (Sigma), 1% SDS, 1 M

NaCl, 10% dextran sulfate (Pharmacia) and 100 ug/ml denatured salmon sperm DNA. Hybridizations were carried out at 65°C for 18 hours with constant agitation.

2.4.4 Hybrid selection and in vitro translation of human parainfluenza virus 3 mRNA's

HPIV3-specific mRNA's were hybrid-selected as described by Cooper et al. (1981). Recombinant plasmids representing each non-homologous group of clones were digested with Bam HI to release the inserts and filtered onto 5mm nitrocellulose discs (2.5 ug of DNA per disc) in buffer containing 3 M NaCl and 0.3 M Na citrate. The DNA was fixed onto the nitrocellulose discs by baking for 2 hours at 80°C. The hybridization mixtures (200 ul) contained at least 2 nitrocellulose discs, 60-100 ug of cytoplasmic RNA, 80% deionized formamide, 0.04 M Pipes (Sigma) pH 6.4, 0.4 M NaCl, 0.001 M EDTA, 0.1% SDS and 30 ug wheat germ tRNA (Sigma). Hybridization was carried out for 18 hours at 37°C. After hybridization the filters were washed ten times in 0.15 M NaCl/ 0.015 M Nacitrate/ 0.5 % SDS at 60°C followed by two washes in 0.002 M EDTA pH 7.0. The final wash was carried out in 0.0002 M EDTA pH 7.9 at 60°C for 5 minutes. The selected RNA was eluted into 200 ul of H_2O. To elute the RNA the tube containing the discs was placed in a boiling water bath for 60 seconds and then placed into a methanol dry ice bath. The eluted RNA was transferred to another tube and 2 ug of wheat germ tRNA was added. The RNA was precipitated with ethanol and resuspended in H_2O at a concentration of 1 ug/ul.

The hybrid selected mRNA was translated in vitro in either wheat germ extracts (BRL) or rabbit reticulocyte lysates (Promega Biotec) in the presence of 50 uCi of [^{35}S]-methionine (1000 Ci/mmol,NEN) according to the procedures of the suppliers. The translation products were electrophoresed on SDS-polyacrylamide gels as outlined in section 2.1.6.

2.5 Nucleotide Sequencing of cDNA Clones

2.5.1 Isolation of cDNA fragments from plasmid DNA

The plasmid containing the cDNA fragment was digested with an appropriate restriction enzyme(s). The DNA fragments were then separated in a horizontal gel consisting of 0.8% agarose, 0.1 M Tris base, 0.1 M boric acid and 0.003 M Na_2EDTA. The electrophoresis buffer contained 0.1 M Tris base, 0.1 M boric acid, and 0.003 Na_2EDTA. The gel was electrophoresed for 2 hours at a constant voltage of 100 volts. The DNA fragments in the gel were stained with 0.5 ug/ml of ethidium bromide. Staining and all subsequent steps were carried out in the dark. The DNA fragments were located using an ultraviolet lamp. A slot was cut in the gel just below the DNA fragment and DEAE membrane was inserted into the slot. The DNA fragment was then transferred onto the membrane by electrophoresis at a constant voltage of 150 volts for 10 minutes. The membrane was removed from the gel and washed twice with 1 ml of H_2O. The DNA was eluted from the membrane in 200 ul of buffer containing 0.01 M Tris-HCl [pH 7.2], 0.001 M EDTA, and 1.5 M NaCl at 37°C for 12 hours. The buffer was then replaced with 200 ul of fresh buffer and the remaining DNA eluted at 56°C for 1 hour. Finally the DNA was extracted once with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.

2.5.2 Ligation of DNA fragments into M13 phage DNA

The replicative form of M13 DNA (RF DNA) was prepared according to the procedures of Messing et al., (1983). The M13 RF DNA was cleaved at the desired site(s) using the appropriate restriction enzyme(s). When necessary the digested M13 DNA was treated with 6-8 units of alkaline phosphatase (Boehringer Mannheim) for the last 30 minutes of the restriction enzyme

digestion. Usually ligations were carried out with a 1:1 ratio of insert DNA to plasmid (DNA concentration:10 ug/ml). The ligation reaction mix contained 0.05 M Tris-HCl [pH 7.8], 0.01 M $MgCl_2$, 0.02 M dithiothreitol, 0.001 M adenosine triphosphate and 50 ug/ml of bovine serum albumin. One unit of T_4 DNA ligase (Bethesda Research Laboratories) was used for each ligation. The reactions were carried out at 16°C for 12 hours. The plasmid DNA was then used to transfect either E.coli JM 101 or JM 109 using the procedures of Messing (1983).

2.5.3 Generation of sequential overlapping clones.

HPIV3 specific DNA fragments were ligated into M13mp11 or M13mp19 using the procedure outlined in section 2.5.2. These clones were used to sequence the entire HN gene and flanking regions. Sequentially shorter clones were generated using the Cyclone System for rapid deletion subcloning (IBI) and the methods of Dale et al. (1985). Briefly, single-stranded DNA M13 templates were prepared according to the procedures of Messing (1983). An oligomer RD 20 (5'-CGACGGCCAGTGAATTCCCC-3') was annealed to the single-stranded DNA template and the hybrid was cleaved with Eco RI. The reaction buffer contained 0.01 M Tris-HCl [pH 7.4], 0.01 M $MgCl_2$, and 0.005 M dithiothreitol. An exonuclease digestion was used to digest the single-stranded DNA. The exonuclease digestion was carried out at 37°C in the above reaction mix with the addition of 1 ug of bovine serum albumin, 6 units of T_4 DNA polymerase and a dithiothreitol concentration of 0.010 M. The exonuclease reactions were stopped by heating to 65°C for 10 minutes. These conditions removed approximately 200 bases during a 5 minute incubation. A reaction containing 15 units of terminal transferase (IBI) and deoxyguanosine triphosphate (0.005 M) was carried out on the single-stranded DNA at 37°C for 10 minutes. The reaction was stopped by heating

the mix to 65°C for 10 minutes. The RD 20 oligomer was annealed to the single-stranded DNA and the oligomer-DNA hybrid was ligated at room temperature for 2 hours or longer. The ligation mix contained 0.003 M adenosine triphosphate and 1.5 units of T_4 DNA ligase. The recircularized plasmid DNA was used to transfect E. coli JM 101 using the procedures of Messing (1983).

2.5.4 DNA sequencing

DNA sequencing was done using the dideoxynucleotide chain termination method of Sanger et al. (1977). The M13 sequencing kit and [^{35}S]-deoxyadenosine triphosphate were purchased from Amersham. Single-stranded DNA templates were prepared according to the procedures of Messing (1983). DNA templates were annealed with the M13 sequencing primer (5'-GTAAAACGACGGCCAGT-3') at 60°C for one hour. To the annealed template 15 uCi of [^{35}S]-deoxyadenosine triphosphate (600 Ci/mmol, Amersham) and 1 unit of Klenow fragment (Amersham) were added. This sample was split into four aliquots and one aliquot was added to each of the four deoxynucleotide triphosphate/dideoxynucleotide triphosphate mixes. The sequencing reaction volume was 4.5 ul. The composition of the four mixes are outlined in Appendix 2. The sequencing reactions were carried out at room temperature for 15 minutes at which time 2 ul of chase solution (Appendix 2) was added. The reactions were terminated after a 15 minute incubation.

Samples were electrophoresed using a 37 cm x 40 cm x 0.02 cm sequencing gel (IBI). The gel consisted of 7 M urea, 8% acrylamide, 0.4 % bis-acrylamide made up in electrophoresis buffer (0.1 M Tris base, 0.1 M boric acid, 0.003 M EDTA). To catalyze the polymerization of the gel 60 ul of 10 % ammonium persulphate (Biorad) and 20 ul of N,N,N',N'-tetramethylethylenediamine (Biorad) were added to 40 ml of the gel mix. Electrophoresis was carried out for two hours at a constant power of 50

watts.

2.5.5 Computer Analysis of HN Gene sequence and Gene Product

Computer analysis was done using an IBM XT computer and "DNA/Protein Sequence Analysis Software" (IBI) as described by Pustell and Kafatos (1982a; 1982b; 1984). The sequence analysis program was used to manipulate the nucleotide sequence of the HN gene, to generate an amino acid sequence from the nucleotide sequence and to search for homologies between HN genes of different paramyxoviruses. In addition, this program was used to evaluate the hydropathy of the HN proteins of HPIV3, Sendai virus and simian virus 5. The methods used in the evaluation of protein hydropathy were those of Kyte and Doolittle (1982). The program used a hydropathy scale in which each amino acid was assigned a value that reflected the relative hydrophilicity or hydrophobicity of the amino acid. The hydropathy plot was simply a running average of the hydropathy scale for each 9 consecutive amino acids at each position in the protein (Kyte and Doolittle, 1982). The midpoint line printed on each plot represented the grand average of the hydropathy of the amino acid compositions found in most sequenced proteins (Kyte and Doolittle, 1982).

CHAPTER 3: RESULTS

3.1 HOST CELL-VIRUS INTERACTIONS: A study of cell fusion induced by vesicular stomatitis virus (VSV).

3.1.1 Cytopathic effects caused by vesicular stomatitis virus

VSV infections of most cell lines produce a rapid cytolytic response, which includes rounding and degeneration of infected cells. The cytopathic effects caused by VSV are to some extent a reflection of the infected host. For example, L-cells, BHK-21 cells, and R(B77) cells showed a typical cytolytic effect when infected with VSV (Figure 1). In contrast, Rat-1 cells formed polykaryocytes and chick embryo fibroblasts formed vacuolar ghosts when infected with VSV (Figure 1). R(B77) cells are avian sarcoma virus transformed Rat-1 cell, yet R(B77) and Rat-1 cells differed in their response to VSV infection. R(B77) and BHK-21 cells were selected for this study because they showed a typical cytolytic response when infected by VSV.

3.1.2 Vesicular stomatitis virus-induced cytopathic effects in cells grown in amino acid deficient medium.

Initially it was observed that VSV infected cells grown in amino acid deficient medium formed polykaryocytes. To examine this effect, BHK-21 and R(B77) cells were grown, before and after infection, in normal medium, or medium containing 1/5, 1/10, 1/20, or 1/30 of the normal amino acid concentration. As a control uninfected BHK-21 and R(B77) cells were also grown in amino acid deficient medium. Neither of these cell lines spontaneously formed polykaryocytes when grown in amino acid deficient medium. VSV (IND-HR) infected cells grown in medium containing the normal concentrations of amino acids showed the typical CPE of rounding and degeneration (Figure 2). However, VSV (IND-HR) infected cells grown in

Figure 1. Cytopathic effects caused by vesicular stomatitis virus in various cell lines. The control cultures (Cont.) were not infected. The infected cultures (Infect.) were infected at an MOI of 1. Note the polykaryocyte formation in vesicular stomatitis virus infected Rat-1 cells and the lack of polykaryocyte formation in infected BHK-21 and R(B77) cells. Magnification in all photographs was 200 x.

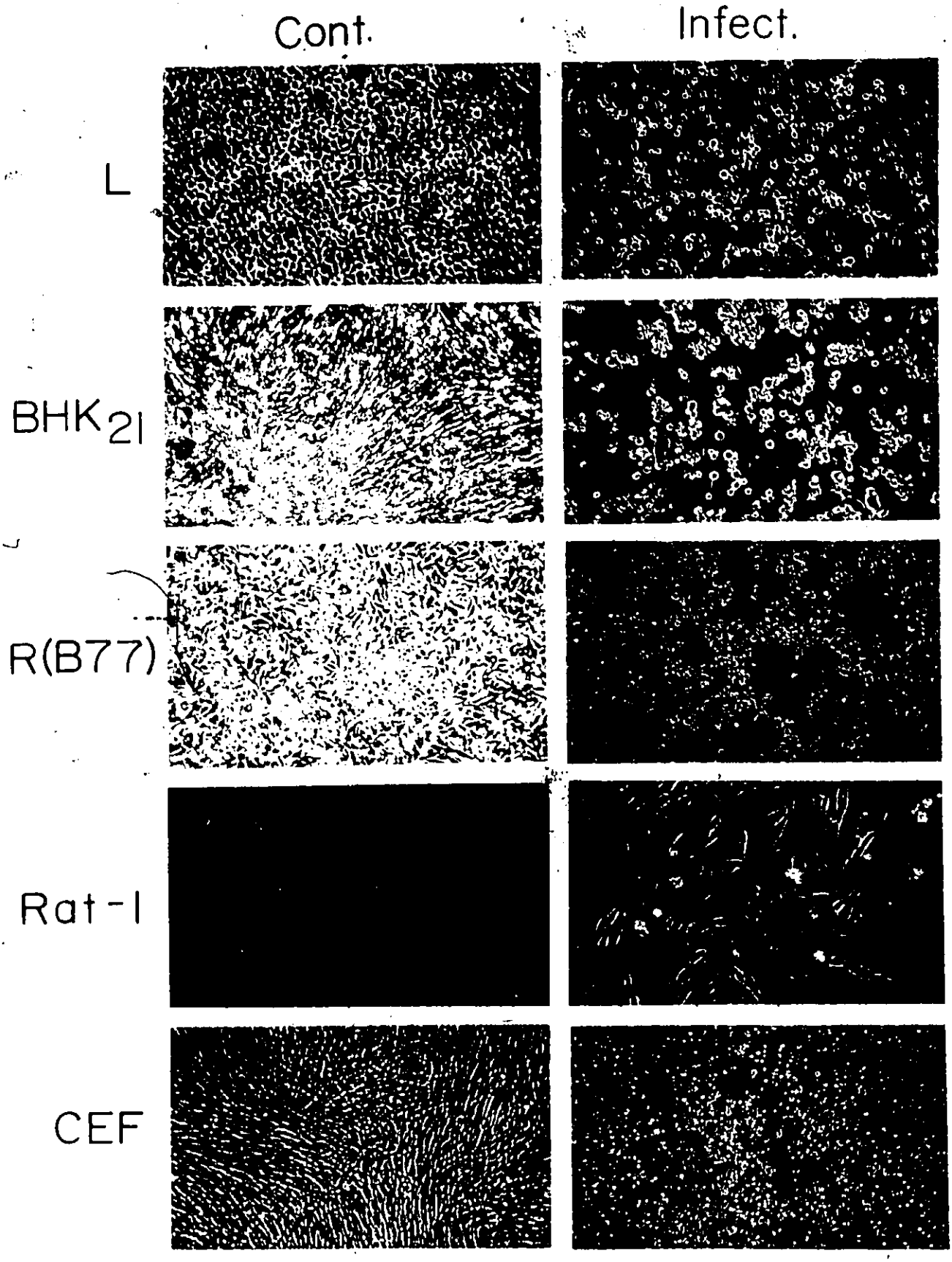
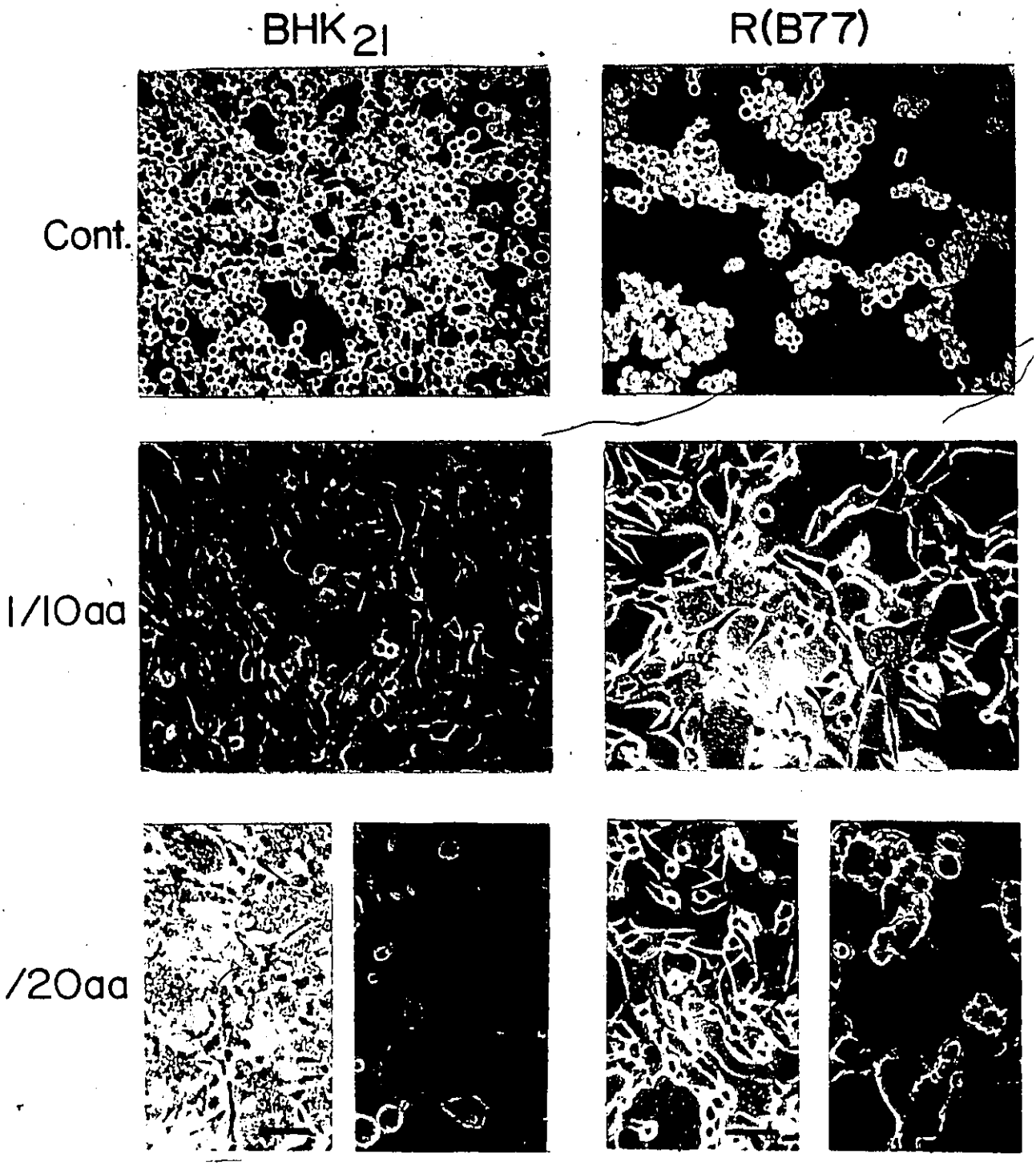

Cont.
Infect.
L
BHK21
R(B77)
Rat-1
CEF

Figure 2. Cytopathic effects caused by vesicular stomatitis virus when infected cells were grown in amino acid deficient media. Vesicular stomatitis virus-infected cells were grown in either normal DMEM or DMEM containing 1/10 or 1/20 of the normal amino acid concentration. The second photographs in lower panels were taken at higher magnification. The bar represented 0.5 um in all photographs.


BHK21
R(B77)
Cont.
1/10aa
1/20aa

medium containing 1/5 to 1/30 of the normal concentrations of amino acids formed polykaryocytes (Figure 2). The size and number of polykaryocytes formed varied according to the concentration of amino acids. For example, when VSV infected cells were grown in medium containing 1/5 of the normal concentration of amino acids, 30-40% of the cells fused. In contrast, when VSV infected cells were grown in medium containing 1/30 of the normal concentration of amino acids, 80-90% of the cells fused.

To demonstrate that cell fusion was not an unique cytopathic effect of the Indiana serotype of VSV (IND-HR), the experiment was repeated with another serotype (VSV N.J. [Concan]) and different strains of VSV (VSV IND [HR, New Mexico, Glasgow]). All serotypes and strains tested induced cell fusion in either BHK-21 or R(B77) cells grown in medium containing 1/20 of the normal concentration of amino acids. Thus, VSV-induced cell fusion was neither strain nor serotype dependent.

Defective interfering (DI) particles are known to interfere with the replication of standard virus (Cooper and Bellett, 1959). Thus, the presence of DI particles in the stock virus or generation of DI particles may alter the VSV-induced cytopathic effects. To determine whether DI particles in the viral stocks were responsible for cell fusion, BHK-21 cells grown in amino acid deficient medium, were infected with virus containing no detectable level of DI particles (i.e. low MOI progeny of VSV[IND-HR]). The same level of cell fusion was observed in cultures infected with either the progeny or parent virus. This indicated that defective interfering particles were not involved in VSV-induced cell fusion.

To determine if synthesis of host proteins was involved in VSV-induced cell fusion, BHK-21 cells were grown in amino acid deficient medium

containing 1 ug/ml of actinomycin D. A 12 hour treatment with actinomycin D (an inhibitor of DNA dependent RNA synthesis) should inhibit over 90% of the synthesis of host proteins. Actinomycin D treatment did not affect the formation of VSV-induced polykaryocytes when the infected cells were grown in medium containing 1/20 of the normal concentration of amino acids. In addition, when VSV infected cells were grown in medium containing normal concentrations of amino acids and actinomycin D, the cells rapidly rounded up and degenerated. Thus, the synthesis of host cell proteins was not required for the formation of VSV-induced polykaryocytes.

3.1.3 Induction of polykaryocytes by vesicular stomatitis virus infection as a result of inhibition of viral protein synthesis.

The effect that inhibition of viral protein synthesis had on VSV-induced polykaryocytosis was determined by growing the cells in the presence of either cycloheximide or puromycin. VSV infected BHK-21 cells formed polykaryocytes when treated with as little as 1 ug/ml of either cycloheximide or puromycin (Table 3, Figure 3). R(B77) cells treated with either inhibitor also fused when infected with VSV, but a higher concentration of inhibitor was required for fusion to occur (Table 3). Uninfected cells, however, did not fuse as a result of treatment with either puromycin or cycloheximide. Thus, inhibition of viral protein synthesis played a role in VSV-induced cell fusion. To determine the level of protein synthesis at each concentration of inhibitor, duplicate cultures were labeled with a mixture of [^{3}H]-amino acids at the time of infection. At 12 hours after infection the amount of label incorporated was determined. A comparison of the level of [^{3}H]-amino acid incorporation in VSV infected BHK-21 and R(B77) cells is shown in Table 3. R(B77) and BHK-21 cells responded differently to the two inhibitors. BHK-21 cells were inhibited to a greater extent than were R(B77) cells (Table 3). The level

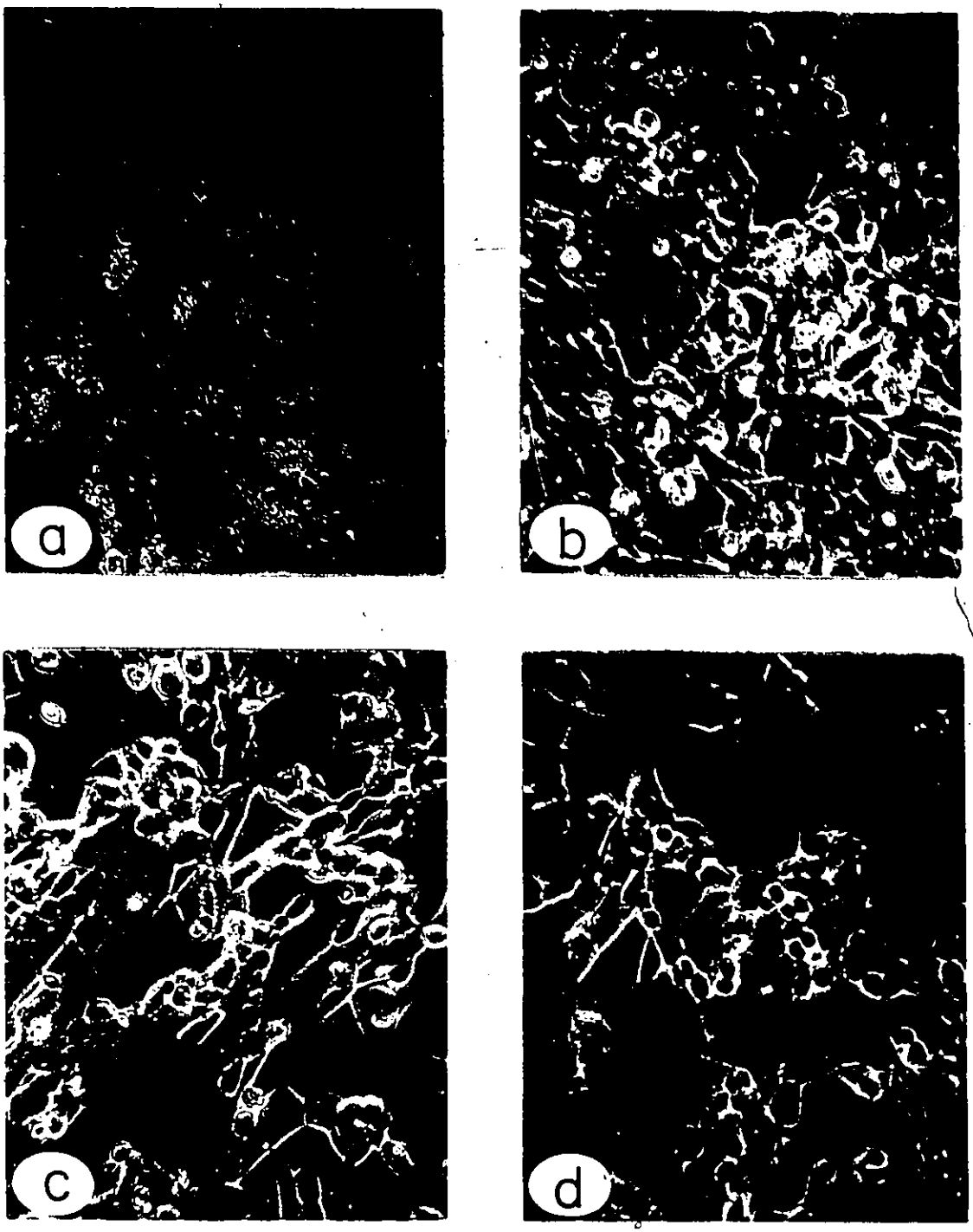
TABLE THREE: The effect of inhibition of protein synthesis on vesicular stomatitis virus infected cells

Inhibitor	cell line	Percentage of ^{3}H-amino acid incorporation in the presence of of inhibitors (ug/ml) relative to untreated cultures[a]				
		0	1	10	50	100
Puromycin	BHK-21	100 (R+D)[b]	62 (PK)	ND	ND	10 (PK)
Puromycin	R(B77)	100 (R+D)	71 (R+D)	ND	ND	10 (PK)
Cycloheximide	BHK-21	100 (R+D)	38 (PK)	18 (PK)	12 (PK)	9 (PK)
Cycloheximide	R(B77)	100 (R+D)	71 (R+D)	40 (R+D)	25 (PK)	18 (PK)

[a] Cells were grown in the presence of inhibitor for 15 hours prior to infection. The cells were infected at an MOI of 1. Immediately after the 1 hour adsorption, the cells were labeled for 15 hours in the presence of 5 uCi/ml of [^{3}H]-amino acids. The percentage of incorporation was calculated by dividing amount of incorporation found in the treated sample by amount of incorporation found in the untreated sample and multiplying by 100.

[b] Cytopathic effects: R+D, rounding and degeneration; PK, polykaryocyte formation; ND, not determined.

Figure 3. Cytopathic effects caused by vesicular stomatitis virus when BHK-21 cells were grown in the presence of cycloheximide. A: infected cells grown in the absence of cycloheximide. B, C, and D: infected cells grown in presence of 1, 10, and 100 ug/ml of cycloheximide respectively. All photographs were at the same magnification. The bar in photograph D represented 0.5 um.

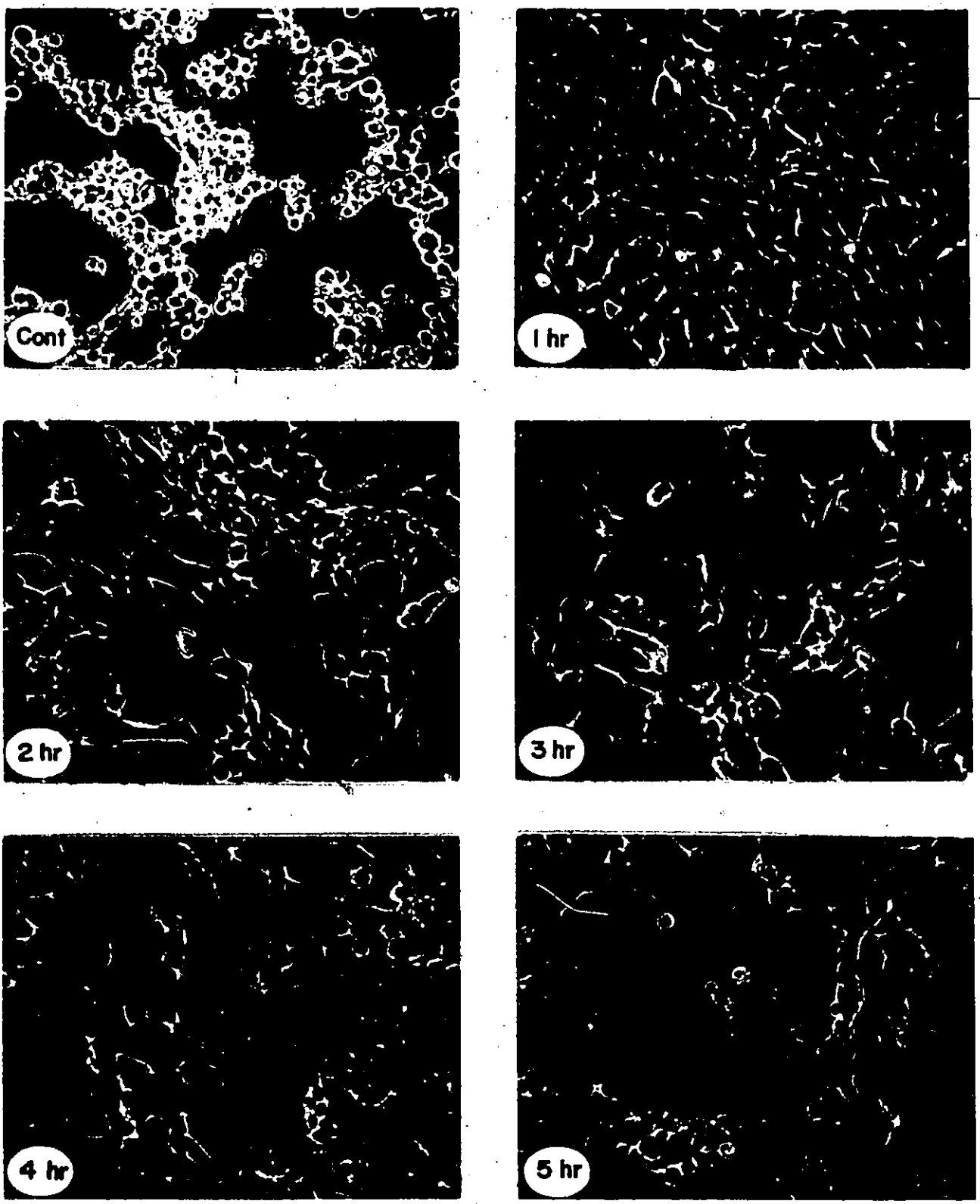

a
b
c
d

of inhibition of protein synthesis at which VSV-induced polykaryocytosis also varied. In BHK-21 cells, VSV infection caused cell fusion when protein synthesis was inhibited by as little as 38% (Table 3:1 ug/ml of puromycin). In contrast, VSV-induced cell fusion occurred in R(B77) cells only when protein synthesis was inhibited by greater than 60% (Table 3: 50 ug/ml cycloheximide, 100 ug/ml puromycin). VSV-induced polykaryocytosis occurred in both cell lines when protein synthesis was inhibited by 90% (Table 3). Thus, cell fusion occurred even at a low level of viral protein synthesis. As the concentration of inhibitor increased, the size and number of polykaryocytes increased (Figure 3). Clearly, VSV-induced polykaryocytosis was dependent on the level of viral protein synthesis.

3.1.4 Effect of total inhibition of protein synthesis on vesicular stomatitis virus-induced cytopathic effects

Having established that inhibition of viral protein synthesis was involved in VSV-induced cell fusion it was important to determine if the inhibition must occur before, during or after adsorption. To explore this problem, induction of cell fusion after VSV infection was examined when 100 ug/ml of cycloheximide was added at various intervals before and after infection (Figure 4). Cells treated with cycloheximide at 1 or 2 hours before infection or at 1 hour after infection showed only minor cytopathic effects (Figure 4). Thus, early viral protein synthesis was required for the infection to progress. When cycloheximide was added at 2, 3, 4, or 5 hours after infection, the majority of the cells fused (Figure 4). Indeed, if viral protein synthesis was inhibited at 2 to 5 hours after infection the predominant cytopathic effect was cell fusion.

Figure 4. Vesicular stomatitis virus-mediated cytopathic effect when cyloheximide was added at various times during infection. All photographs were taken at 12 hours after infection. The control culture (CONT) did not receive cycloheximide. The cytopathic effect of vesicular stomatitis virus on BHK-21 cells that received cycloheximide 1 and 2 hour before infection was identical to that shown for the culture that received cycloheximide at 1 hour after infection.


Cont
1 hr
2 hr
3 hr
4 hr
5 hr

3.1.5 Intracellular synthesis and extracellular appearance of virus specific proteins.

To determine which viral protein might be most affected by inhibition of protein synthesis the rates of synthesis and transport of each viral protein was studied in a pulse-chase experiment. VSV infected BHK-21 cells were grown at 30°C for 1 hour, labeled with [^{35}S] methionine for 3 hours and the label was chased with an excess of unlabeled methionine for 3 hours. After the addition of the unlabeled methionine hourly samples were taken. The profiles of intracellular viral proteins present in the cells at each hour during the chase period are shown in Figure 5 (lanes 6 to 9). To quantitate the amount of [^{35}S]-methionine labeled protein represented by each band, the autoradiograph of the gel was scanned with a Beckman DU-8B spectrophotometer gel scanning system. For each sample the area of the all viral protein peaks was determined. This area represented the total amount of viral protein in the sample. The area of individual viral protein peaks were compared to the total area to determine the percentage that each viral protein contributed to the total viral protein (Table 4). At the beginning of the chase period the most prominent intracellular viral protein was the N protein, accounting for 71% of the total intracellular viral protein (Figure 5, lane 6, Table 4). During the chase, the actual amount of the N protein remained constant, but its percentage of the total intracellular viral protein increased to 87% (Table 4). The percentage of the total intracellular viral protein remained constant for the G and NS proteins during the chase period at approximately 3% and 1%, respectively. As a percentage of intracellular viral protein, both the L and the M protein dropped during the chase period. The reduction in the percentage of M protein was the most dramatic, dropping from 19% to 8% of the total intracellular viral protein (Table 4).

Figure 5. SDS-polyacrylamide gel electrophoresis of pulse labeled virion-associated and intracellular proteins of vesicular stomatitis virus (IND-HR). Cells were pretreated for 18 hours with actinomycin D and infected at a MOI of 1. At 1 hour after adsorption the cells were labeled with [^{35}S]-methionine for 3 hours, washed and the radioactivity was chased with non-radioactive methionine. Lanes: 1, standard vesicular stomatitis virus (IND-HR) proteins; 2, 3, 4, and 5, virion associated proteins at 0, 1, 2 and 3 hours after the pulse respectively; 6, 7, 8, and 9, intracellular proteins at 0, 1, 2, and 3 hours after the pulse respectively.

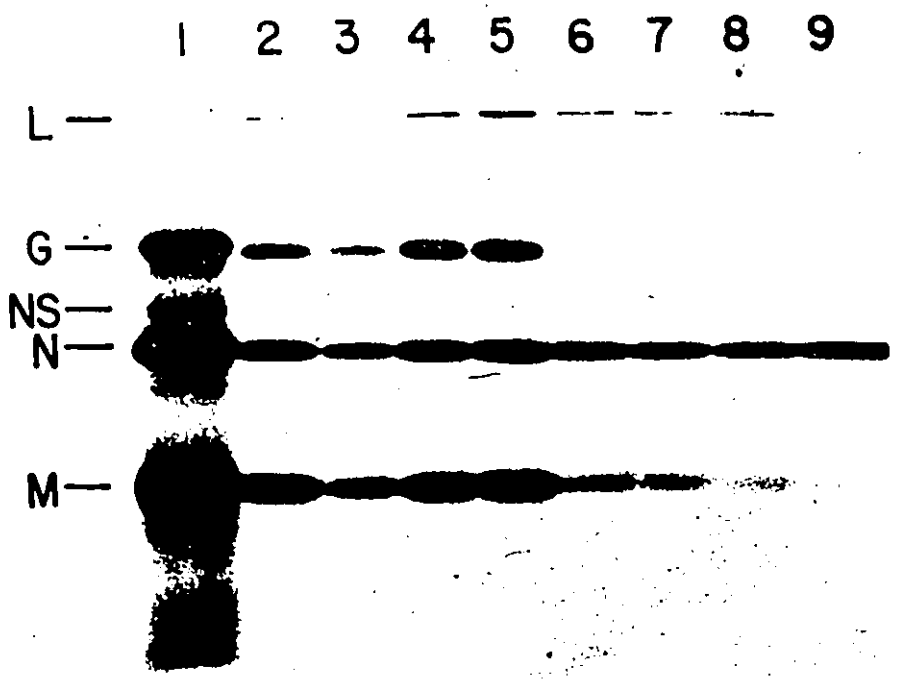

1 2 3 4 5 6 7 8 9
L
G
NS
N
M

TABLE FOUR: Pulse-chase analysis of vesicular stomatitis virus proteins labeled with [^{35}S]-methionine.

	Percentage of [^{35}S]-methionine incorporated into viral proteins[a]							
	Virion-associated proteins				Intracellular viral-specific proteins.			
Time of chase (hr) / Viral protein	0	1	2	3	0	1	2	3
L	1.5	1.4	2.3	4.2	6.2	7.2	8.4	0.2
G	11.6	11.1	18.0	18.2	3.1	5.1	7.7	3.3
NS	0.4	0.4	0.5	0.9	0.7	4.0	2.6	1.1
N	41.1	46.3	40.4	37.2	71.3	65.0	69.5	86.8
M	45.4	40.8	38.8	39.5	18.7	18.7	11.8	8.6

[a] The autoradiograph from Figure 6 was scanned with the gel-scanning system of a Beckman DU-8B spectrophotometer. At each time point the area of all the viral protein peaks was determined. The percentage incorporation into each viral protein was determined from the area of an individual peak divided by the total area and multiplied by 100.

The virion-associated proteins at 0, 1, 2 and 3 hours of chase are shown in Figure 5 (lanes 2 to 5). At the beginning of the chase period all the structural proteins were represented (Figure 5 lane 2). However, the M protein accounted for the highest percentage (45%) of the total virion-associated proteins (Table 4). During the chase, the percentage of M protein dropped significantly from 45 to 39% (Table 4). These findings indicated that the actual amount of M protein remained approximately the same during the chase period, whereas the amounts of the other proteins increased.

The combination of a rapid disappearance of intracellular M protein and the rapid appearance of M protein in the virion confirmed the findings of Kang and Prevec, (1971) that the M protein was the protein most rapidly packaged into the progeny virions. Consequently, compared with the other viral proteins, inhibition of viral protein synthesis would first affect the intracellular pool of M protein.

3.1.6 Formation of polykaryocytes by temperature sensitive mutants of vesicular stomatitis virus at the non-permissive temperature.

To determine if a specific viral protein was directly involved in VSV-induced cell fusion, temperature sensitive (ts) mutants from each complementation group were used to infect BHK-21 cells grown at either the permissive (31° C) or the non-permissive (40° C) temperature. The mutant ts G31 (III) induced cell fusion in cells grown at either the permissive or the non-permissive temperatures (Table 5). However, a much greater percentage of the cells fused at the non-permissive temperature (Table 5). None of the other temperature sensitive mutants induced cell fusion at either temperature (Table 5). Furthermore, at the non-permissive temperature only infection with ts G31 (III) resulted in any detectable

TABLE FIVE: Cytopathic effects in BHK-21 cells caused by temperature sensitive mutants of vesicular stomatitis virus

VSV Mutant	Complementation group	Cytopathic effect[a] 31^0C(Permissive) 12 h PI	31^0C(Permissive) 24 h PI	40^0C(Non-permissive) 12 h PI	40^0C(Non-permissive) 24 h PI
G114	I	50% R	R+D	NC	NC
G23	II	50% R	R+D	NC	NC
G31	III	50% R	5% PK	30% PK	70% PK
G44	IV	50% R	R+D	NC	NC
O45	V	80% R	R+D	NC	NC
IND-HR	WT[b]	70% R	R+D	80% R	R+D

[a] R+D, rounding and degeneration; NC, no cytopathic effect; PK, polykaryocyte formation.

[b] PI, post-infection; WT, wild type.

cytopathic effect (Table 5). The mutant ts G31 is a complementation group III mutant which has a temperature sensitive defect in the M protein (Lafay, 1974; Knipe et al., 1977b; Hughes et al., 1979b).

Figure 6 shows an analysis of intracellular and virion-associated proteins of ts G31 (III) produced at permissive (31° C) and non-permissive (40° C) temperatures. Intracellularly, less viral protein was made at the non-permissive temperature compared to the permissive temperature (Figure 6, lanes 3 and 4). However, the G, NS, N and M proteins accounted for approximately the same percentage of the total viral proteins at both the permissive and non-permissive temperatures (Figure 6, lanes 3 and 4, Table 6). Only the percentage of total intracellular viral protein of the L protein dropped at the non-permissive temperature (Figure 6, Table 6). Less virion-associated protein was produced at the non-permissive temperature than at the permissive temperature (Figure 6, lanes 5 and 6). As a percentage of the total virion-associated proteins, the G protein remained approximately the same at the permissive and non-permissive temperatures (Table 6). The N protein produced at the non-permissive temperature accounted for a greater percentage of the virion-associated proteins than at the permissive temperature (Table 6). The percentage of the total virion-associated proteins dropped for three proteins (M, NS, and L) at the non-permissive temperature (Table 6). Clearly, at the non-permissive temperature the L protein of ts G31 (III) was not synthesized as efficiently as at the permissive temperature. Moreover, although synthesis of the NS and M proteins was occurring at the non-permissive temperature these proteins were not as efficiently assembled into the progeny virus.

3.1.7 Summary of the study on cell fusion induced by vesicular stomatitis virus.

Five highly cytolytic strains of both Indiana and New Jersey serotypes

Figure 6. Analysis of intracellular and virion-associated proteins of ts G31 (III). BHK-21 cells were pretreated with actinomycin D and infected at a MOI of 1. The infected cultures were incubated at 31°C for 3 hours. After this period, the cultures were divided into two groups. One group was incubated at 40°C and the other group was incubated at 31°C. The cells were incubated for an additional hour and then were labeled with 3.5 uCi/ml of [^{35}S] methionine. The cells were labeled for 4 hours, harvested, fractionated into viral or intracellular components and then electrophoresed on a 12% SDS-polyacrylamide gel. Lanes: 1, vesicular stomatitis virus (IND-HR) standard proteins; 2, mock-infected BHK-21 cells; 3, ts G31 (III) intracellular proteins generated at non-permissive temperature; 4, ts G31 (III) intracellular proteins generated at the permissive temperature; 5, virion associated proteins of ts G31 (III) produced at non permissive temperature; 6, virion associated proteins of ts G31 (III) produced at permissive temperature.

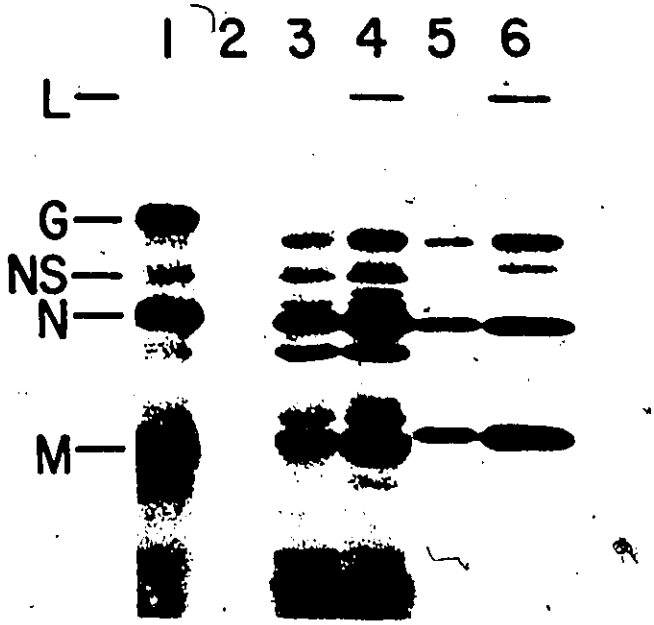

1 2 3 4 5 6
L
G
NS
N
M

TABLE SIX: Analysis of the viral proteins of ts G31 (III) produced in BHK-21 cells grown at either the permissive or non-permissive temperatures

	Percentage [^{35}S]-methionine incorporated into viral protein[a]			
	Intracellular viral-specific proteins		Virion-associated proteins	
Incubation temperature / Viral Protein	40^{0}C	31^{0}C	40^{0}C	31^{0}C
L	0.3	4.8	0.4	6.3
G	13.2	12.5	12.7	11.5
NS	12.0	8.1	1.2	3.6
N	46.4	47.3	52.1	38.4
M	28.1	27.3	33.6	40.2

[a] The autoradiograph from Figure 7 was scanned with the gel-scanning system of a Beckman DU-8B spectrophotometer. The percentage incorporation into each viral protein was determined from the area of an individual peak divided by the total area and multiplied by 100.

of VSV were shown to induce cell fusion in BHK-21 and R(B77) cells. This change in cytopathic effect occurred when viral protein synthesis was inhibited after the eclipse period of viral replication. Pulse-chase experiments showed that inhibition of protein synthesis led to a reduction in the intracellular pool of M protein in comparison to the other viral proteins. A temperature sensitive mutant defective in the M protein (ts G31) was the only mutant of the five VSV complementation groups to spontaneously induce polykaryocytes at the non-permissive temperature. Therefore, M protein influences the membrane fusion activity of VSV.

3.2 STRUCTURE OF HUMAN PARAINFLUENZA VIRUS 3.

The involvement of the M protein in VSV-induced membrane fusion highlights the necessity of studying all the viral components involved in a biological function rather than concentrating on a single component. The complexity of virus-host cell interactions is sometimes lost as we try to focus in on the elements involved. Thus, a necessary first step in the study of virus-host cell interaction is the characterization of all the viral components. The second part of this thesis deals with the characterization of the components of human parainfluenza virus 3.

3.2.1 Selection of growth parameters for human parainfluenza virus 3

A difficulty encountered when working with HPIV3 has been low virus yields from cultured cells (Guskey and Bergtrom, 1981). To overcome this problem it was necessary to find a cell line which yielded high virus titres. Previously, primary rhesus monkey kidney cell cultures have been the most widely used for detection and quantitation of parainfluenza viruses (Frank et al., 1979; Hawthorne and Albrecht, 1981). However, primary cell cultures were not used in this study because they are

expensive and difficult to maintain. Continuous lines of monkey kidney cells (LLC-MK2, GMK, AH-1, BSC-1 and VERO) have been used to grow parainfluenza viruses (Hawthorne and Albrecht, 1981; Orstavik, 1981). To identify a cell line which produces high yields of HPIV3, four cell lines, all continuous monkey kidney cells (LLC-MK2, RE2A, E6 and VERO) were infected with HPIV3. Of the four cell lines tested, HPIV3 infected LLC-MK2 cells continually gave the highest titre of between $7.6X10^6$ and $6x10^8$ P.F.U./ml. Accordingly, LLC-MK2 cells were selected as the cell line for production of HPIV3 on the basis of the high virus titres produced.

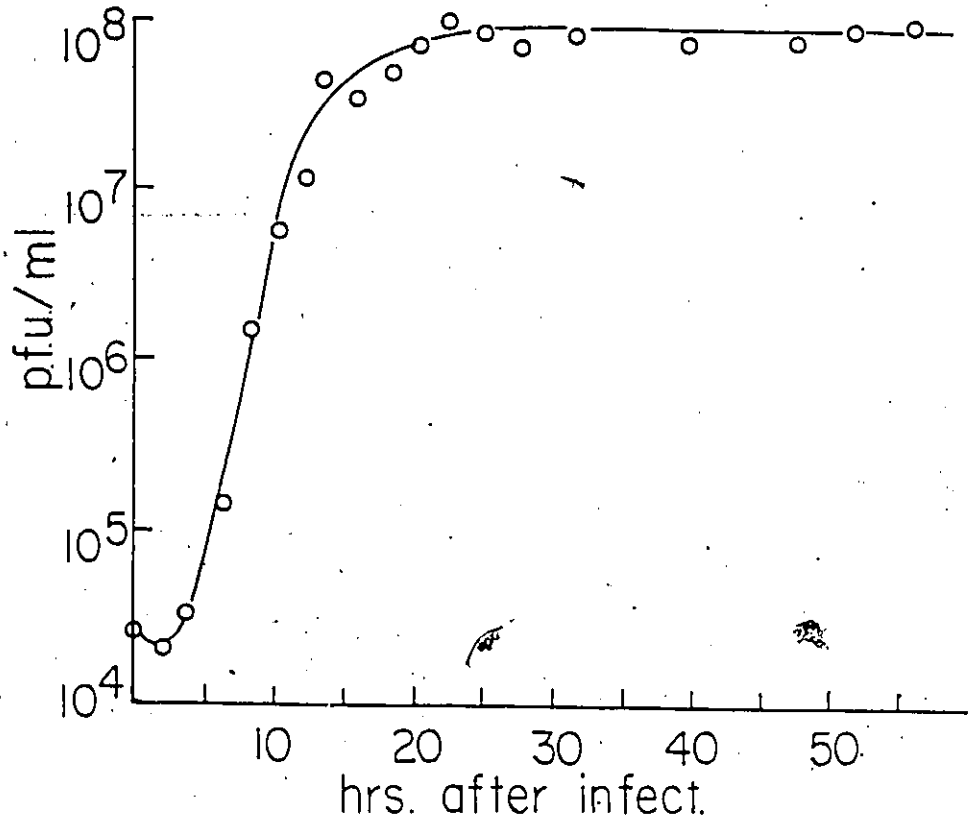
To characterize the growth kinetics of HPIV3 in LLC-MK2 cells, a growth curve was established. Figure 7 shows the growth curve of HPIV3 in LLC-MK2 cells infected at a multiplicity of infection (MOI) of 3. A titre of $9x10^7$ P.F.U./ml was achieved 24 hours after infection. With a lower MOI of 0.1 P.F.U./cell, higher viral yields of between $2x10^8$ and $6x10^8$ were obtained. Once the maximum titre was reached at approximately 24 hours after infection, it was maintained until 55-72 hours after the infection.

The cytopathic effects produced by HPIV3 in LLC-MK2 cells did not become apparent until 10 hours after infection. Polykaryocytes were first observed in these cultures at 12 hours after the infection. The number and size of the polykaryocytes increased until 55 hours after infection at which time the cells started to round up. The nuclear membranes in the polykaryocytes remained distinct until 18 hours after infection at which time they also began to fuse. The predominant cytopathic effect observed between 55 and 72 hours after infection was rounding and degeneration of the cells.

3.2.2 Human parainfluenza virus 3 genomic RNA

To determine the molecular weight of HPIV3 genomic RNA, infected cells were labeled with [^{3}H]-uridine between 12 and 24 hours after the

Figure 7. Growth curve of human parainfluenza virus 3 in LLC-MK2 cells. Cell cultures ($7x10^6$ cells) were infected at a MOI of 3. Samples were assayed for infectivity by plaque assay on LLC-MK2 cells.


10^8
10^7
10^6
10^5
10^4
p.f.u./ml
10
20
30
40
50
hrs. after infect.

infection. The virus was isolated. Genomic RNA was extracted from the isolated virus, denatured with glyoxal and analyzed in a 1% agarose gel (Figure 8). The molecular weight of the genomic RNA was determined using ribosomal RNA's and vesicular stomatitis virus genomic RNA as markers. The molecular weight of the genome of HPIV3 was estimated to be 4.6×10^6.

3.2.3 Structural proteins of human parainfluenza virus 3

HPIV3 proteins were labeled with [^{35}S]-methionine, harvested 48 hours after infection, and analyzed by SDS-polyacrylamide gel electrophoresis (Figure 9). Proteins of known molecular weights (^{14}C labeled high molecular weight standards, Bethesda Research Laboratories, Inc.) were electrophoresed in the same gel to determine the molecular weights of the virion proteins. The virions of HPIV3 contained seven major proteins with molecular weights of 195,000, 87,000, 69,000, 67,000, 46,000, 44,000, and 35,000 (Figure 9, lane 2). On longer exposure of the autoradiograph two minor proteins with molecular weights of 60,000 and 20,000 were found in the virion. Three proteins with molecular weights of 69,000, 60,000 and 46,000 were labeled with (^{14}C)-glucosamine. These proteins were considered to be glycoproteins (Figure 9, lane 4).

Since the electrophoretic profile of HPIV3 was similar to that of other mammalian parainfluenza viruses (Shibuta et al. 1979; Morein et al., 1983; Scheid and Choppin, 1974), the six major proteins were tentatively designated as L, P, HN, NP, F and M, as indicated in Figure 9. The nature of the protein of M.W. 44,000 is unknown. Cellular actin, with a similar molecular weight, has frequently been reported to be associated with virions of other paramyxoviruses (Lamb et al., 1976; Guiffre et al., 1982; Cowley and Barry, 1983; Morein et al., 1983). It is likely that the minor 60,000 protein labeled with [^{14}C]-glucosamine corresponds to the F_0 protein

Figure 8. Agarose gel electrophoresis of genomic RNA of human parainfluenza virus 3 after glyoxal denaturation. (A) Autoradiograph of [^{3}H] uridine labeled vesicular stomatitis virus genomic RNA (lane 1) and human parainfluenza virus 3 genomic RNA, isolated from two different preparations of virus (lanes 2 and 3). (B) Ethidium bromide stained gel. Agarose gel electrophoresis of ribosomal markers (lanes 1 and 4), human parainfluenza virus 3 genomic RNA (lane 2) and vesicular stomatitis virus genomic RNA (lane 3).

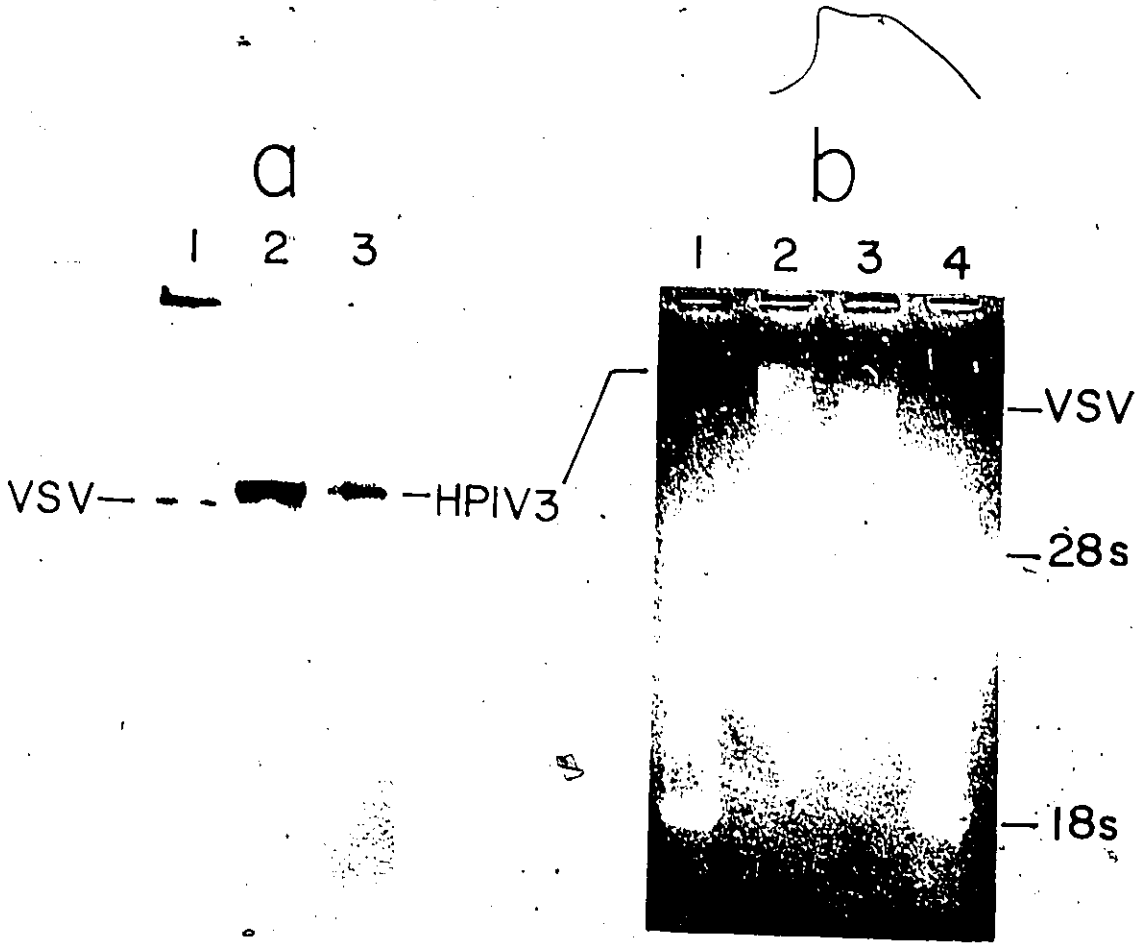
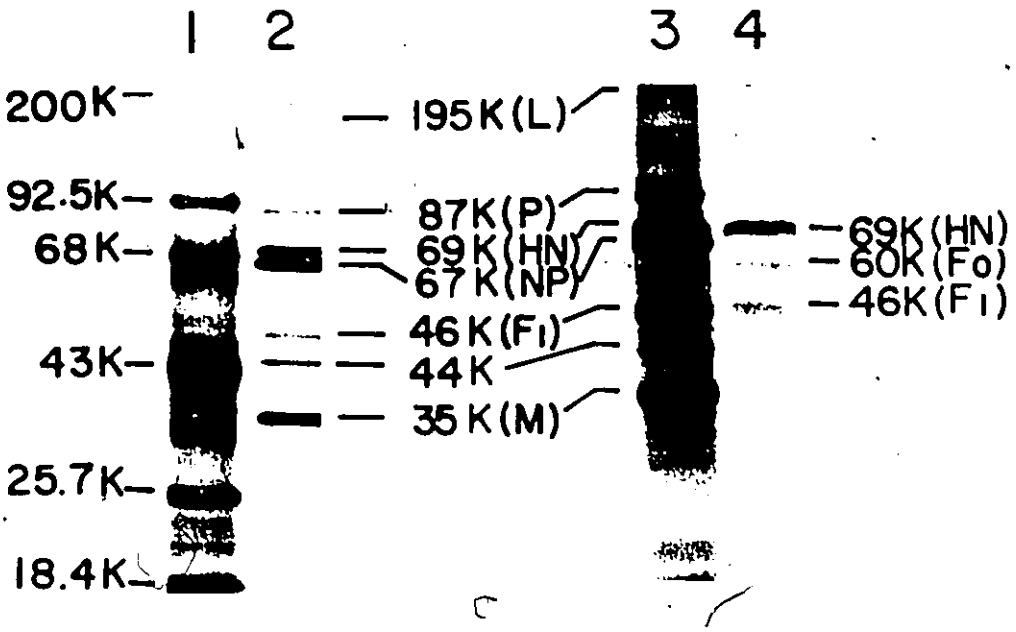

a
1 2 3
VSV
HPIV3
b
1 2 3 4
VSV
28s
18s

Figure 9. Polyacrylamide gel analysis of human parainfluenza virus 3 proteins. Lanes: 1, ^{14}C-amino acid labeled myosin (M.W. 200,000), phosphorylase B (M.W. 92,500), bovine serum albumin (M.W. 68,000), ovalbumin (M.W. 43,000), chymotrypsinogen (M.W. 25,700) and B-lactoglobulin (M.W. 18,400); 2 and 3, [^{35}S]-methionine labeled human parainfluenza virus 3 proteins; 4, [^{14}C]-glucosamine labeled human parainfluenza virus 3 proteins. Electrophoresis was carried out in 15% polyacrylamide gels containing 0.1% SDS. The molecular weights of the proteins are indicated on the gel (K denotes x 10^3).


1
2
3
4
200K
92.5K
68K
43K
25.7K
18.4K
195K(L)
87K(P)
69K(HN)
67K(NP)
46K(F1)
44K
35K(M)
69K(HN)
60K(Fo)
46K(F1)

of other paramyxoviruses.

HPIV3 infected LLC-MK2 cells were grown in the presence of [^{32}P]-orthophosphate to determine if any of the HPIV3 proteins were phosphorylated. Five of the seven viral proteins were labeled with [^{32}P]-orthophosphate, the P, HN, NP, F and M proteins (Figure 10). Presumably, phosphorylated proteins are involved in genome replication. However, the significance of these phosphorylated proteins has not been explored.

3.2.4 Disulfide bonding of the viral proteins

To examine which HPIV3 proteins participate in intramolecular disulfide bonding, [^{35}S]-methionine or [^{14}C]-glucosamine labeled virion proteins were separated by SDS-polyacrylamide gel electrophoresis under either reducing conditions or non-reducing conditions (Figure 11). On the basis of protein band intensity, it appeared that the NP protein (protein c) migrated to the same position under reducing and non-reducing conditions (Figure 11a, lanes 1 and 2). This suggested that intramolecular disulfide bonds did not occur in the structure of NP protein. In contrast, intramolecular disulfide bonds occurred in the M and P proteins because they migrated to different positions under non-reducing conditions than under reducing conditions (Compare lanes 1 and 2 in Figure 11a). Likely, the M protein corresponded to protein e under non-reducing conditions (Figure 11a, lane 1). The migration of P protein under non-reducing conditions was not firmly established. The P protein under non-reducing conditions may have corresponded to protein b or one of the faintly labeled proteins between proteins b and e. Protein a did not correspond to the P protein under non-reducing conditions because protein a is glycosylated (Figure 11a, lane 1 and Figure 11b, lane 1).

To facilitate the identification of the glycoprotein species, both one and two dimensional non-reducing gel systems were used. Under non-reducing

Figure 10. SDS-polyacrylamide gel electrophoresis of the phosphorylated proteins of human parainfluenza virus 3. [^{32}P]-orthophosphate labeled human parainfluenza virus 3 proteins were analyzed in 15% polyacrylamide gels. Human parainfluenza virus 3 proteins labeled with [^{35}S]-methionine were co-electrophoresed as molecular weight markers.

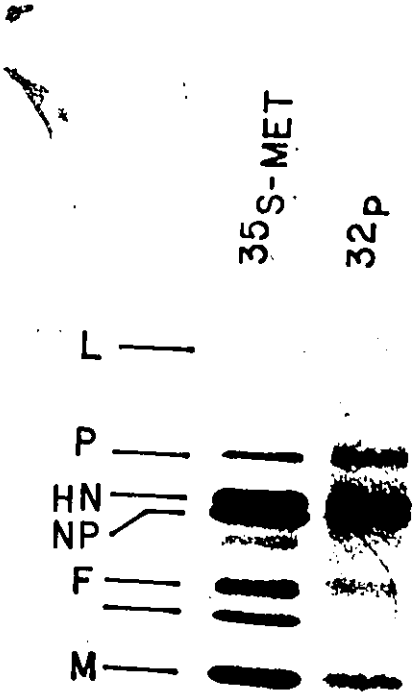
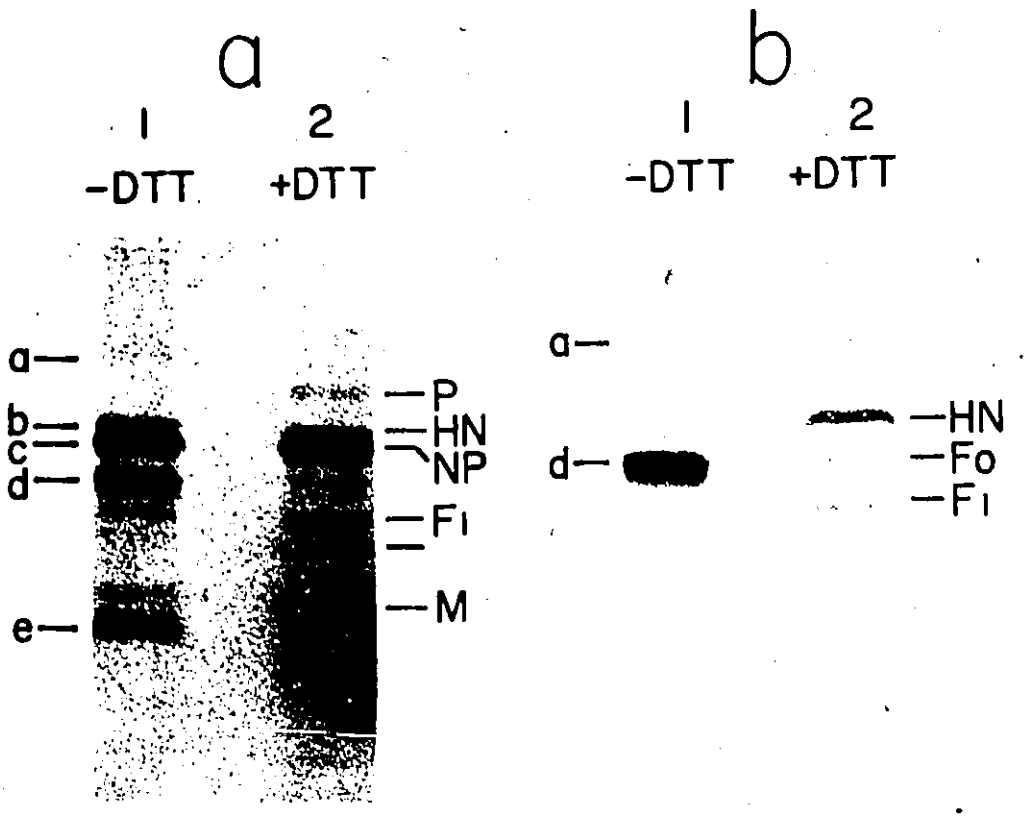

35S-MET
32P
L
P
HN
NP
F
M

Figure 11. SDS-polyacrylamide gel electrophoresis of human parainfluenza virus 3 proteins in the presence or absence of a reducing agent. Gel a: [^{35}S]-methionine labeled proteins. Gel b: [^{14}C]-glucosamine-labeled proteins. Lanes: 1, proteins electrophoresed without dithiothreitol in sample buffer (non-reducing conditions); 2, dithiothreitol was added to sample buffer (reducing conditions). Predominant bands in the samples electrophoresed without dithiothreitol have been designated a through e.


a
b
1
2
-DTT
+DTT
1
2
-DTT
+DTT
a
b
c
d
e
P
HN
NP
F1
M
a
d
HN
Fo
F1

conditions HPIV3 glycoproteins resolved into three proteins (Figure 11b, lane 1), labeled protein a and protein d. Protein band d contained a major slower migrating protein and a minor faster migrating protein. In the two dimensional gel system (Figure 12), protein band d migrated in the reducing dimension as a major triangular-shaped spot and two minor faster migrating spots. The molecular weight of the triangular-shaped spot corresponded to that of HN protein. Furthermore, the two faint spots migrated to the same position as F_0 and F_1 under reducing conditions (Figure 12). These findings demonstrated that protein band d (Figure 11a and 11b) was composed of HN, F_0 and $F_{1,2}$ with HN being the major slower migrating protein. This implied that both F_0 and cleaved F_1 proteins were present in the virion. It also indicated that HN was not present as a disulfide linked dimer on the HPIV3 virion. The nature of the high molecular weight protein (Figure 11b, lane 1) represented by protein band a was unknown. Protein a was not a glycoprotein dimer linked by disulfide bonds because protein with the molecular weight of HN or F_0 did not appear following reduction. Furthermore, in two dimensional gels, protein a was located on the diagonal, which indicated that disulfide bonds did not effect the migration of this protein.

3.2.5 Location of the structural proteins in the virion.

The envelope proteins of Sendai virus, simian virus 5 (SV5) and Newcastle disease virus (NDV) have been separated from nucleocapsid proteins by disrupting virions with Triton X-100 in the presence of 1 M KCl (Scheid et al., 1972; Scheid and Choppin, 1973; Hosaka et al., 1974; Lamb and Mahy, 1975). A similar procedure was used to determine the location of each of the proteins in the HPIV3 virion. Figure 13 (lane 1) shows the electrophoretic pattern of virion proteins of HPIV3 labeled with [^{35}S]-

Figure 12. Two dimensional polyacrylamide gel analysis of human parainfluenza virus 3 proteins labeled with [^{14}C]-glucosamine. Radioactively labeled virion proteins were electrophoresed in the first dimension (horizontal) under non-reducing conditions. A single lane was cut from the gel, soaked for 30 minutes in buffer containing 0.5 M Tris-HCl pH 6.8, 0.4% SDS, 2% mercaptoethanol, positioned on a 15% polyacrylamide gel containing 0.1% SDS, and electrophoresed in the second dimension (vertical). The black dot indicates the origin. The upper gel shows the position of proteins after electrophoresis under non-reduced conditions in the first dimension. Marker lanes in the second dimension indicate the positions of proteins after electrophoresis under reduced conditions. Both [^{14}C]-glucosamine and [^{35}S]-methionine labeled proteins have been used as markers.

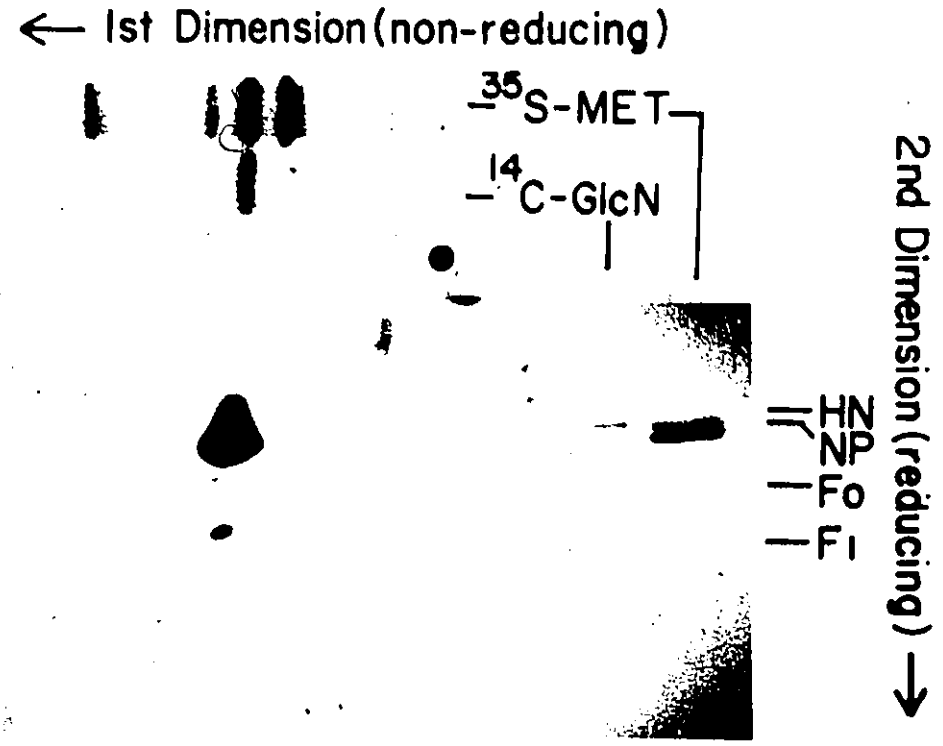
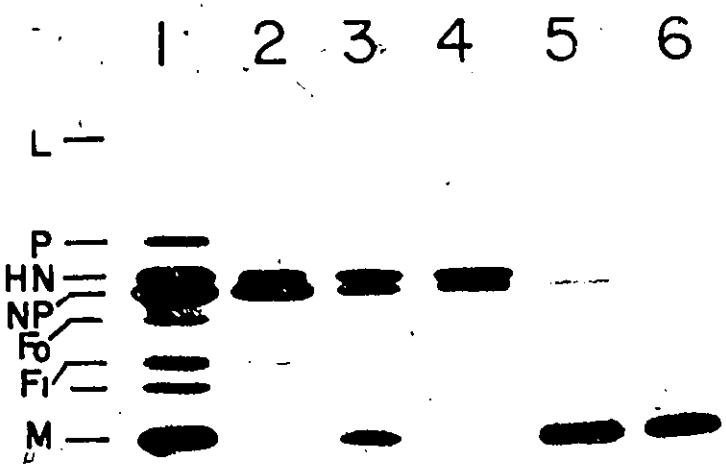

← 1st Dimension (non-reducing)
^{35}S-MET
^{14}C-GlcN
HN2
N2
F0
F1
2nd Dimension (reducing) →

Figure 13. Location of human parainfluenza virus 3 proteins in the virion. [^{35}S]-methionine labeled human parainfluenza virus 3 proteins were analyzed by SDS-polyacrylamide gel electrophoresis after various treatments. Lanes: 1, total virion proteins; 2, pelleted nucleocapsid proteins after treatment with 2% Triton X-100 and 1 M KCl; 3, soluble envelope components after treatment with 2% Triton X-100 and 1 M KCl; 4, Upper peak fraction after 10 to 25% sucrose gradient velocity ultracentrifugation; 5, lower peak fraction after sucrose gradient velocity sedimentation; 6, low salt aggregate pelleted from lower peak fraction.


1
2
3
4
5
6
L
P
HN
NP
Fo
F1
M

methionine. To solubilize the envelope components, virions were disrupted with 2% Triton X-100 and 1 M KCl. After ultracentrifugation, the proteins associated with the soluble portion and the pellet were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins in the pellet (Figure 13, lane 2) resembled those in the intact virus except for a large reduction in the amounts of HN and M relative to other proteins. Both F_0 and F_1 proteins were associated with the pelleted nucleocapsid material and not the soluble envelope fraction. The predominant protein bands in the soluble portion (Figure 13, lane 3) were the HN and M protein bands. Some NP protein was found in the soluble fraction (Figure 13, lane 3) but was greatly reduced in amount compared with either the pelleted nucleocapsid material or the total NP protein. The envelope components were further separated by sucrose gradient velocity sedimentation. Two peaks were obtained. The upper peak (Figure 13, lane 4) contained predominantly HN protein with some NP protein and a trace of M protein. The lower peak contained mainly M protein, with a trace of HN and NP proteins (Figure 13, lane 5). When the fractions in the lower peak were pooled, dialyzed to remove the salt, and ultracentrifuged, only the M protein was pelleted (Figure 13, lane 6).

3.2.6 Summary of the structural components of human parainfluenza virus 3.

The genomic RNA and virion proteins of HPIV3 were characterized. The genome of human parainfluenza virus 3 was found to be a linear RNA molecule with a molecular weight of approximately 4.6×10^6. Seven major and two minor proteins were associated with the virus particles of HPIV3. Three proteins of molecular weights 195,000, 87,000, and 67,000 were associated with the nucleocapsid of the virion. These proteins were designated L, P, and NP, respectively. Three proteins labeled with [^{14}C]-glucosamine had molecular weights of 69,000, 60,000, and 46,000. These proteins were designated HN, F_o and F_1 respectively. The HN protein had intramolecular

disulfide bonds, but did not participate in disulfide bonding to form dimers. A protein of M.W. 35,000 associated with the envelope components of the virion and aggregated in low salt buffer. This protein was designated M protein.

3.3 VIRAL SPECIFIC INTRACELLULAR COMPONENTS

3.3.1 Virus specific intracellular RNA's.

To identify HPIV3-specific intracellular RNA's, infected LLC-MK2 cells were labeled with [^{3}H]-uridine in the presence or absence of actinomycin D. RNA was extracted and fractionated by oligo(dT) cellulose chromatography into poly(A)$^+$ and poly(A)$^-$ fractions. RNA from each fraction was denatured with glyoxal and electrophoresed on a 1.5% agarose gel. A strong RNA band corresponding in size to HPIV3 genomic RNA (M.W. 4.6×10^6, 15Kb) was readily detected in the poly(A)$^-$ RNA fraction from infected cells (Figure 14). Relatively less genomic RNA was found in HPIV3 infected cells which were not treated with actinomycin D (Figure 14). In the poly(A)$^+$ fraction of HPIV3 infected cell RNA, several RNA species were found which presumably represented HPIV3 mRNA's (Figure 14, 15). Figure 15 shows the poly(A)$^+$ fraction of infected cell RNA that was electrophoresed for an extended period to enhance the separation of the RNA species. Varying amounts of HPIV3 genomic size RNA (M.W. 4.6×10^6, 15 Kb) were always found associated with the poly (A)$^+$ RNA fractions even after several cycles of heating and oligo(dT) cellulose chromatography (Figures 14, 15). This RNA is likely present due to a sandwich effect in which the poly (A)$^+$ RNA binds to the column and the genomic RNA binds to the poly (A)$^+$ RNA. Following transfer to nitrocellulose, all of these polyadenylated RNA species hybridized with [^{32}P]-labeled HPIV3 genomic RNA (Figure 16, lane 1). Clearly, the five RNA species shown in Figure 16 (lane 1) are all polyadenylated HPIV3 specific

Figure 14. Agarose gel electrophoresis of RNA isolated from human parainfluenza virus 3-infected cells. Human parainfluenza virus 3-infected LLC-MK2 cells were labeled with ^{3}H-uridine in the presence or absence of actinomycin D. Cytoplasmic RNA was isolated and separated into Poly $(A)^{+}$ and Poly $(A)^{-}$ fractions. Aliquots of each fraction, containing equal number of counts, were treated with glyoxal and electrophoresed on a 1.5% agarose gel. Vesicular stomatitis virus (IND-HR) mRNA's were included in a parallel lane as molecular weight markers. The molecular weights of the human parainfluenza virus 3 RNA species are indicated.

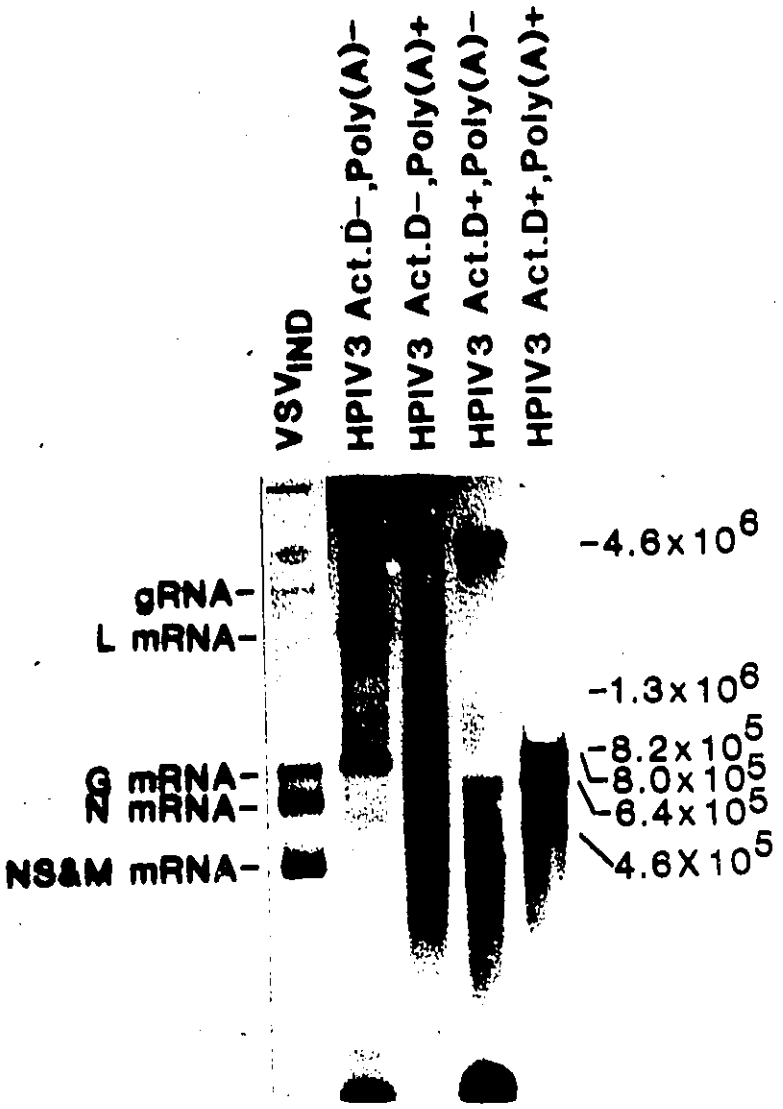

VSV$_{IND}$
HPIV3 Act.D−,Poly(A)−
HPIV3 Act.D−,Poly(A)+
HPIV3 Act.D+,Poly(A)−
HPIV3 Act.D+,Poly(A)+
gRNA−
L mRNA−
G mRNA−
N mRNA−
NS&M mRNA−
−4.6x10^6
−1.3x10^6
−8.2x10^5
−8.0x10^5
−6.4x10^5
−4.6X10^5

Figure 15. Agarose gel electrophoresis of polyadenylated RNA isolated from human parainfluenza virus 3 infected LLC-MK2 cells. The sizes of human parainfluenza virus 3 RNA species are indicated in kilobases (Kb).

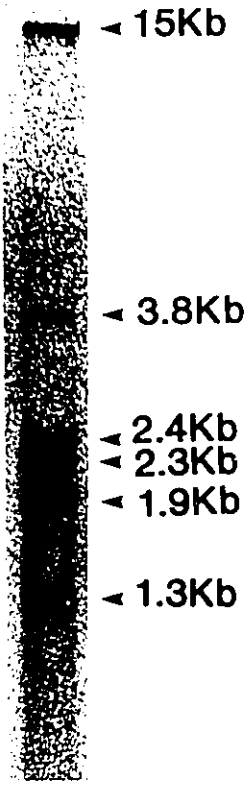
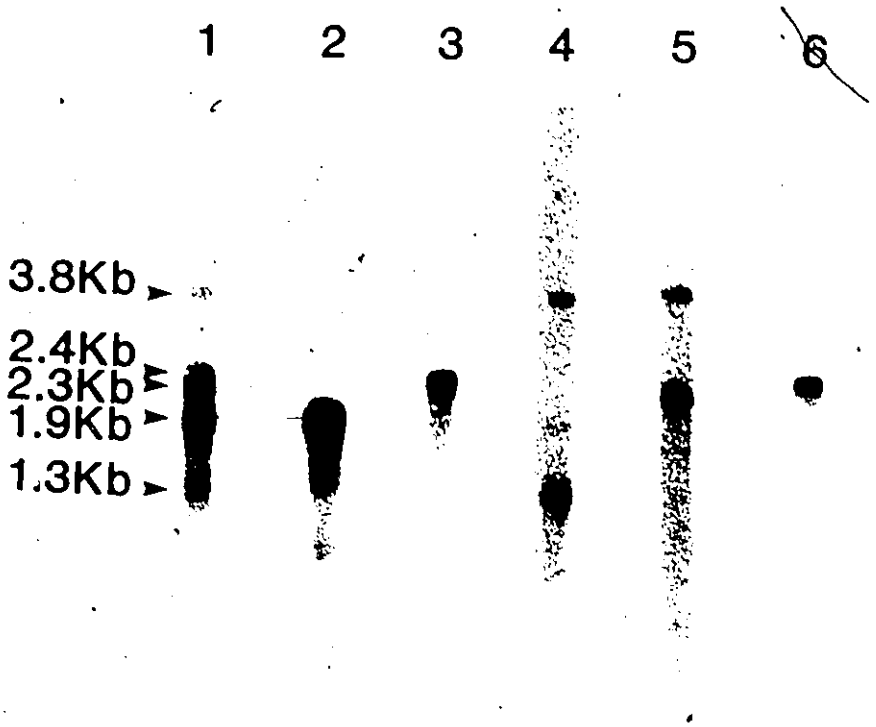

15Kb
3.8Kb
2.4Kb
2.3Kb
1.9Kb
1.3Kb

Figure 16. Northern blot analysis of human parainfluenza virus 3 polyadenylated RNA. Poly $(A)^+$ RNA was isolated from actinomycin D treated LLC-MK2 cells infected with human parainfluenza virus 3. The RNA samples were denatured with glyoxal, electrophoresed on a 1.2% agarose gel and transferred to nitrocellulose. Individual strips were hybridized with ^{32}P-labeled genomic RNA (lane 1) or with nick-translated recombinant DNA's: pPI 47 (lane 2), pPI 28 (lane 3), pPI 3 (lane 4), pPI 14 (lane 5) and pPI 10 (lane 6).


1
2
3
4
5
6
3.8Kb
2.4Kb
2.3Kb
1.9Kb
1.3Kb

RNA species and likely mRNA's.

3.4 CLONING OF HUMAN PARAINFLUENZA VIRUS 3 GENES AND STUDY OF THE GENOME ORGANIZATION

3.4.1 Construction of cDNA libraries

A bank of HPIV3-specific clones used in my study was provided by Dr. K. Dimock, University of Ottawa. This bank of clones was generated using polyadenylated RNA isolated from LLC-MK2 cells 18-24 hours after infection. Oligo (dT) was used to prime cDNA synthesis and established methods were followed for the synthesis of double stranded cDNA (Maniatis et al. 1982). Fractions of cDNA with an average size of 1.2-1.3 Kilobase pairs (Kbp) were selected by Sepharose 4B chromatography. The cDNA was ligated into plasmid pBR322 using Bam HI linkers and the ligation mixture was used to transform E. coli RR1. Another bank of HPIV3 specific clones was constructed by the author. The second bank of HPIV3 mRNA-specific cDNA clones was constructed by direct blunt end ligation of double-stranded cDNA into the Sma I site of pUC 8.

HPIV3 specific clones from both banks were identified by colony hybridization with ^{32}P-labeled fragments of genomic HPIV3 RNA. Roughly 5% of the transformants contained viral-specific sequences. HPIV3 specific inserts from the first cDNA clone bank were isolated, nick-translated and used to identify related clones by cross-hybridization (Dimock and Côté, personal communication). In this manner, five distinct groups of clones which contained non-homologous viral sequences were identified. Representatives of each of these five groups were used to screen the second bank of clones. Each clone in the second library hybridized uniquely to a representative of one of the five non-homologous groups of clones from the first bank. These results indicated that none of the five groups

contained cloned fragments of the same HPIV3 gene, generated by internal Bam HI cleavage of cDNA during linker digestion. In all, 112 HPIV3 specific clones from both mRNA derived clone banks, were screened in this manner. The total number of clones in each group is listed in Table 7.

3.4.2 Coding assignments of viral-specific mRNA's.

To correlate each group of clones to a specific HPIV3 mRNA species, a nick-translated plasmid representing each group (A-E, Table 7) was hybridized to Northern blots of polyadenylated RNA isolated from HPIV3 infected cells. Each plasmid hybridized strongly to a different monocistronic species of mRNA (Figure 16, Lanes 2-6). In addition, all the representative plasmids hybridized to genomic RNA (data not shown). Two species of bicistronic transcripts were also detected. One of these transcripts (M.W. $1.3x10^6$ or 3.8 Kb) contained sequences which corresponded to clones pPI 3 (Figure 16, lane 4) and pPI 14 (Figure 16, lane 5). This bicistronic transcript was relatively abundant in infected cells. The molecular weight of this transcript ($1.3x10^6$) approximated the sum of the two monocistronic transcripts (M.W. $4.6x10^5$, $8.0x10^5$) which hybridized to pPI 3 and pPI 14 (Table 7). A second larger bicistronic transcript was detected only upon prolonged exposure of the autoradiograph (data not shown). This RNA species hybridized to both clone pPI 47 (group A, Table 7) and clone pPI 28 (group B, Table 7). The molecular weight of this transcript ($1.5x10^6$) again approximated the sum of the smaller mRNA's (M.W. $6.4x10^5$, $8.2x10^5$) which hybridized specifically to clone pPI 47 and clone pPI28 (Table 7). Bicistronic transcripts similar to these are detected in cells infected with other paramyxoviruses (Collins and Wertz, 1983; Paterson et al., 1984a; Udem and Cook, 1984; Wilde and Morrison, 1984; Bellini et al., 1985).

To assign a viral gene product to both the mRNA species and the

TABLE SEVEN: Assignments of HPIV3 mRNA clones[1]

Clone[2] group	Number of clones	Representative clone	Approximate size of insert (Kbp)	Size of major mRNA(s) hybridized (Kb)	Size of major mRNA(s) hybridized ($M.W. \times 10^{-6}$)	Gene assignment[3]
A	59	pPI47	1.5	1.9	0.64	NP
B	21	pPI28	1.0	2.4	0.82	P
C	16	pPI10	1.0	2.4	0.82	HN
D	9	pPI3	1.0	1.3	0.46	M
E	7	pPI14	1.6	2.3	0.80	F
D+E[2]	-	pPI3+pPI14	-	3.8	1.3	M/F
A+B	-	pPI47+pPI28	-	4.3	1.5	NP/P

[1] Analysis of two banks of cDNA clones derived from Human Parainfluenza Virus 3 mRNA are presented in this summary. One clone bank was generously provided by Dr. K.Dimock, University of Ottawa.

[2] Determined by cross-hybridization using two independent clone banks; one constructed in pBR322 and the other in pUC8.

[3] Determined by hybrid selection and *in vitro* translation.

[4] Non-homologous groups of clones hybridizing to mRNA of the same size.

groups of clones, in vitro translation of hybrid-selected mRNA's was carried out. Representative plasmids (pPI 3, 10, 14, 28, and 47) from each group of clones (A-E) were digested with Bam HI and the DNA fixed to nitrocellulose discs. Total cytoplasmic RNA from HPIV3 infected cells was hybridized to the plasmid DNA fixed on the nitrocellulose discs. After several washes, bound RNA was eluted and translated in vitro in both wheat germ extracts and rabbit reticulocyte lysates in the presence of ^{35}S-methionine. The translation products were electrophoresed on SDS-polyacrylamide gels and visualized by fluorography. The products of the in vitro translations of hybrid selected RNA are shown in Figure 17a. Clone pPI 47 (group A) contains sequences which selected a mRNA that directed synthesis of the NP protein. Similarly, clones pPI 28 (Group B) and pPI 3 (Group D) were assigned to P and M genes respectively. NP, P and M mRNA's were translated efficiently in both in vitro translation systems. Translation of the P mRNA in wheat germ extracts yielded not only the 87,000 molecular weight P protein but also at least 2 smaller polypeptides of molecular weights 33,000 and 45,000. These proteins may have occurred as a result of proteolytic cleavage of the P protein. Alternatively these proteins may have resulted from translation initiated at alternate sites in the same reading frame as P, as reported for SV5 V protein (Paterson et al., 1984a) or in a different reading frame, as with Sendai virus C protein (Giorgi et al., 1983). The latter possibility was considered unlikely because the largest open reading frame not in frame with P protein would code for a protein of molecular weight 23,000 (Luk et al., 1986). In vitro translations were carried out using two concentrations of hybrid-selected RNA in order to determine if the products varied according to the quantity of RNA added. Three groups of hybrid-selected RNA (pPI 3, pPI 28 and pPI 47) were used. Figure 17b shows the results of these in vitro

Figure 17a. Analysis of the translation products of hybrid selected human parainfluenza virus 3 mRNA's. Translations were carried in wheat germ extracts containing [^{35}S]-methionine. The translation products were electrophoresed on a 12.5% /SDS-polyacrylamide gel. Virion proteins were electrophoresed in a parallel lane as markers (M.W. (P) 87,000, (HN) 69,000, (NP) 67,000, (F) 46,000, and (M) 35,000). A number of control translations are also shown. These include translations to which no RNA, RNA from uninfected LLC-MK2 cells, or increasing amounts of RNA from infected LLC-MK2 cells were added. In addition, a control hybrid selection was done with pBR322 alone. The last lane is a longer exposure of the pPI 14 lane. Plasmid pPI 40 is a member of the same group as pPI 10(HN).

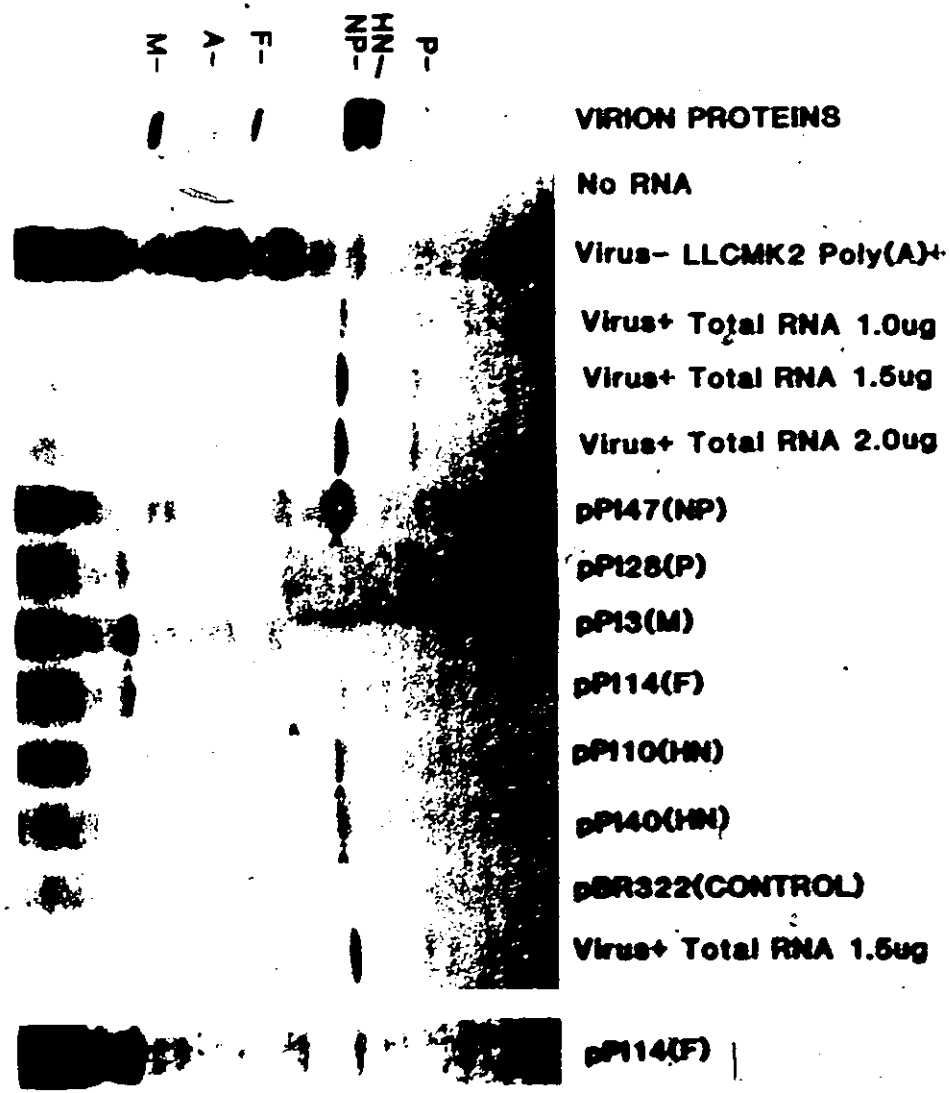
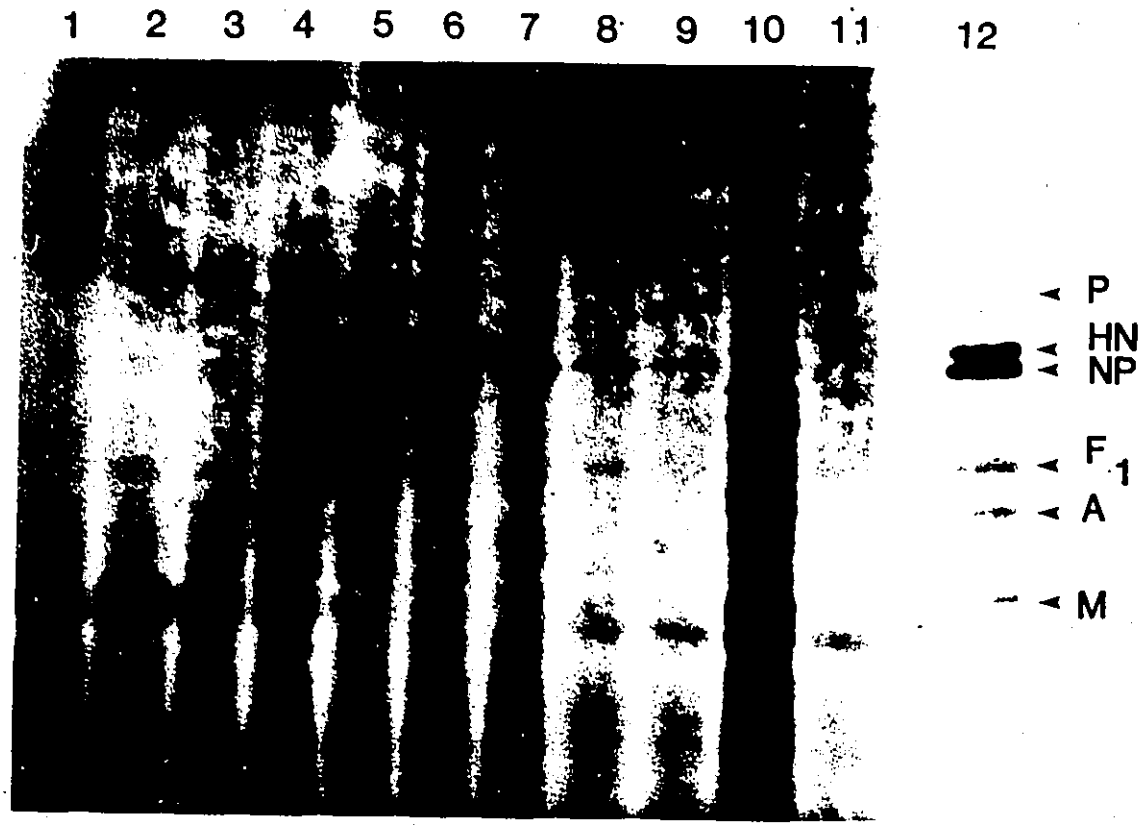

M
F
HN/NP
P
VIRION PROTEINS
No RNA
Virus- LLCMK2 Poly(A)+
Virus+ Total RNA 1.0ug
Virus+ Total RNA 1.5ug
Virus+ Total RNA 2.0ug
pP147(NP)
pP128(P)
pP13(M)
pP114(F)
pP110(HN)
pP140(HN)
pBR322(CONTROL)
Virus+ Total RNA 1.5ug
pP114(F)

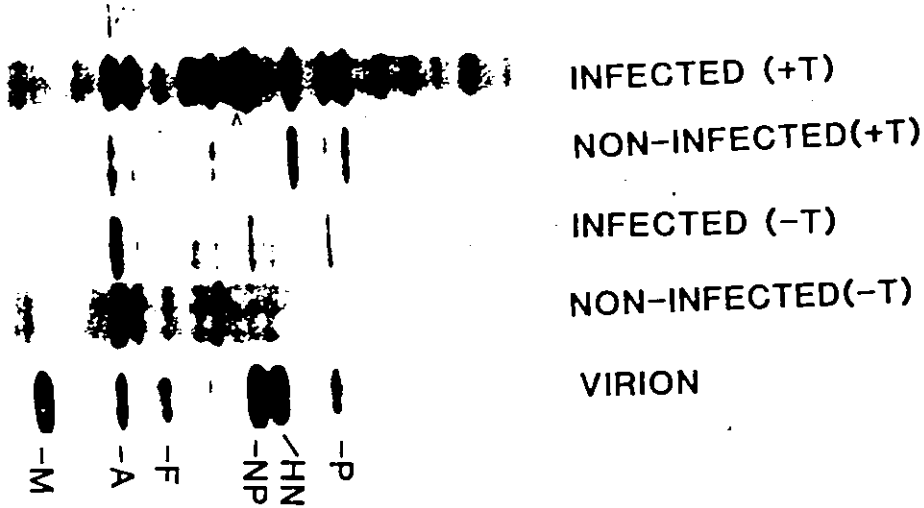
Figure 17b: Analysis of translation products of hybrid selected human parainfluenza virus 3 mRNA's. Three clones (pPI 3, 28, and 47) were used to hybrid select RNA. Two different concentrations of the selected mRNA (1.0 ug and 1.5 ug) were used to direct translation in wheat germ extracts. Lanes: 1, translation products from 1.5 ug of pBR 322 hybrid-selected RNA; 2 and 3, translation products from 1.5 and 1.0 ug of pPI 3 hybrid-selected RNA respectively; 4 and 5, translation products from 1.5 and 1.0 ug of pPI 28 hybrid-selected RNA respectively; 6 and 7, translation products from 1.5 and 1.0 ug of pPI 47 hybrid-selected RNA respectively; 8 and 9, translation products from 1.5 and 1.0 ug of total mRNA species respectively; 10, translation products from 1.0 ug of uninfected cellular RNA; 11, translation products with no RNA added; 12, human parainfluenza virus 3 virion proteins.


1 2 3 4 5 6 7 8 9 10 11 12
P
HN
NP
F1
A
M

translations. For all three hybrid-selected RNA species, as the concentration of RNA increased so did the amount of translation product (Figure 17b).

The mRNA's selected by pPI 10 (Group C) and pPI 14 (Group E) were not translated efficiently by either in vitro translation system. When the amount of hybrid selected mRNA added to the reaction mixtures was increased tenfold, translation products from wheat germ extracts could be readily detected. Plasmid pPI 10 selected an mRNA coding for a protein with a molecular weight of 64,000 which migrated just below the position of the NP protein. A protein of this size was also found in HPIV3-infected LLC-MK2 cells treated with tunicamycin (Figure 18) whereas a HN protein of molecular weight 69,000 was not found. The opposite situation arises in HPIV3-infected LLC-MK2 cells grown in the absence of tunicamycin, where the HN protein was found but not the protein with a molecular weight of 64,000 (Figure 18). These results indicated that the 64,000 molecular weight protein was the unglycosylated form of the HN protein. Thus, Group C clones were assigned to the HN gene. Plasmid pPI 14 hybridized with mRNA's which directed the synthesis of two proteins. A protein with a molecular weight of 54,000 (migrating just above the F_1 protein) likely was the non-glycosylated form of the HPIV3 $F_{1,2}$ protein precursor F_0. The other protein electrophoresed to the same position as the HPIV3 M protein. As indicated by Northern blot analysis (Figure 16), both pPI 3 (M gene clone) and pPI 14 hybridized with a larger (M.W. 1.3×10^6, 3.8 Kb) relatively abundant polyadenylated HPIV3 transcript. This transcript could be a bicistronic M/F mRNA, which was hybrid selected by pPI 14 and translated into M protein in vitro. Translation of the M protein would be favored because the M protein coding sequences would be 5' proximal. On the other hand, the F protein would not be translated from the bicistronic mRNA because internal

Figure 18. Synthesis of human parainfluenza virus 3 proteins in infected LLC-MK2 cells. Cells were either infected with an MOI of 10 or were mock infected. Tunicamycin treated cells were grown in DMEM containing 0.5 ug/ml of tunicamycin. Eighteen hours after infection the DMEM was aspirated and MEM containing 10 uCi/ml of [^{35}S]-methionine as the only source of methionine was added. The cells were labeled for 2 hours, lysed and subjected to SDS-polyacrylamide gel electrophoresis. Virion proteins were co-electrophoresed to serve as markers. The arrow indicates migration of the unglycosylated form of the HN protein.


INFECTED (+T)
NON-INFECTED(+T)
INFECTED (-T)
NON-INFECTED(-T)
VIRION
-M
-A
-F
-NP
-HN
-P

initiation sites are not generally used by eukaryotic translational machinery. Thus, the F protein is not seen in the M protein translation (pPI 3). These results are consistent with the proposed M/F gene order for the related paramyxoviruses Sendai virus (Dowling et al., 1983; Hidaka et al., 1984) and SV5 (Paterson et al., 1984a).

The coding assignments of the clones and HPIV3 mRNA's are summarized in Table 7.

3.5 CHARACTERIZATION OF THE CLONED HN GENE OF HUMAN PARAINFLUENZA VIRUS 3

3.5.1 Analysis of the nucleotide sequence of the HN mRNA

Six HPIV3-specific clones were used to sequence the coding and flanking regions of the HPIV3 HN gene. Two clones (pPI 10,40), provided by Dr. K. Dimock of the University of Ottawa, were shown to code for HN sequences by *in vitro* translations of hybrid selected mRNA (Figure 17a). Four clones (pPIg 10-6, 10-7, 14-1, 40-1) derived from genomic RNA were provided by M.J. Côté, University of Ottawa. The genomic clones pPIg 10-7 and pPIg 10-6 hybridized to HN mRNA and to the putative L mRNA (M.W. 2.0×10^6)(Côté, personal communication). These clones thus appeared to contain sequences from both the HN and L gene. Southern blot analysis showed that genomic clone pPIg 14-1 hybridized to clones pPI 14 and pPI 40 (Côté, personal communication). Clone pPIg 14-1 therefore contained sequences covering the F-HN flanking region. The last two clones, pPIg 40-1 and pPIg 10-5 were shown to hybridize to each other and to clone pPI 40 and pPI 10 respectively (Côté, personal communication). Therefore, these clones covered the entire coding region of the mRNA of the HN protein.

Each of the six HN clones was inserted into M13 phage vectors in both orientations and sequenced using the dideoxynucleotide chain termination method of Sanger et al. (1977). Four of the clones (pPI 10, pPI 40, pPIg

10-5, pPIg 10-7) in M13 vectors were prepared for sequencing using the technique of Dale et al.(1985) for the generation of sequential overlapping clones. The HN clones and their derivatives are shown in Figure 19.

3.5.2 Nucleic acid sequence of the mRNA of HN protein.

Figure 20 shows the nucleic acid sequence of the HPIV3 HN gene in its cDNA form complementary to the genome (i.e. message sense). There is only one large open reading frame from base 73 to base 1789. This region codes for 572 amino acids which have a combined M.W. of 64,178. The codon beginning at base 73 was likely the initiation codon because it was preceded by a G residue 3 bases upstream and was immediately followed by another G residue (Kozak, 1983). There were four potential glycosylation sites (asn-x-ser/thr) at amino acids 308, 351, 485 and 523. The four potential glycosylation sites are boxed in Figure 20.

The polymerase recognition site of the HN gene (AGGAGTAAAG) had only a single nucleotide difference from the polymerase recognition site of the L gene AGGAGCAAAG, (Figure 20) and only a two nucleotide difference from that of the NP gene, AGGATTAAAG (Dimock et al.,1986b). The polymerase binding site for the HN gene of Sendai, AGGGTGAAAG, was also similar to that of HPIV3 with 3 nucleotide differences (Shioda et al., 1986).

3.5.3. Comparison of deduced amino acid sequences for HN genes of human parainfluenza virus 3, Sendai virus and simian virus 5

There were a number of similarities between the amino acid sequences of the HN proteins of Sendai virus, SV5 and HPIV3. Figure 21 shows a comparison of the hydropathy plots of the HN proteins of HPIV3, Sendai virus (Blumberg et al., 1985) and SV5 (Hiebert et al., 1985a). An overall similarity existed between the gene products of HPIV3 and Sendai virus especially in the distribution of long stretches of hydrophilic or hydro-

Figure 19. Restriction enzyme map of the HN gene of human parainfluenza virus 3 and cDNA clones of human parainfluenza virus 3 RNA used to sequence the HN gene. The orientation from left to right is 3' to 5' relative to the genomic RNA. Arrows indicate direction that the clones were sequenced.

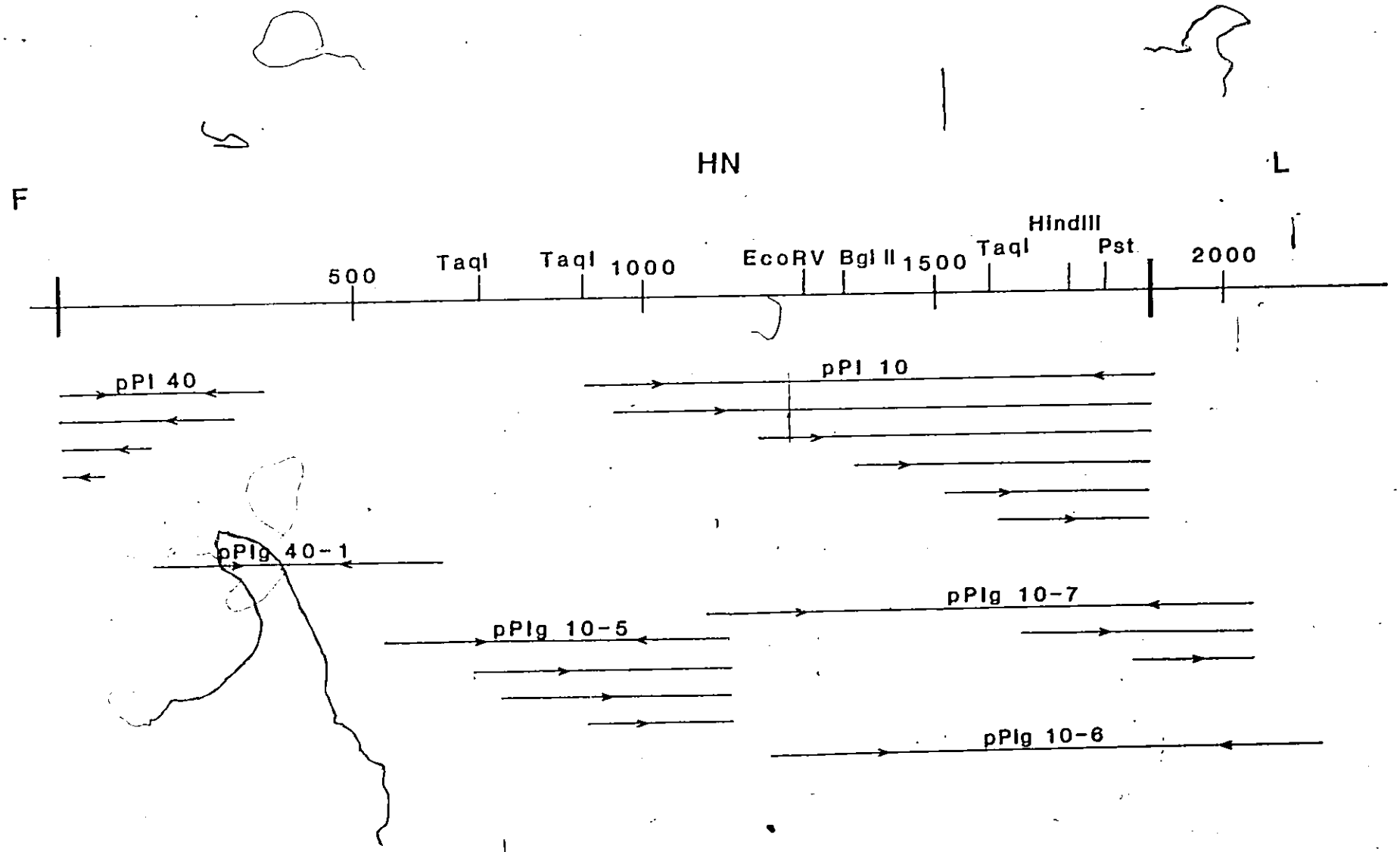

HN
L
F
500
TaqI
TaqI
1000
EcoRV
Bgl II
1500
TaqI
HindIII
Pst
2000
pPI 40
pPI 10
pPIg 40-1
pPIg 10-7
pPIg 10-5
pPIg 10-6

Figure 20. Nucleotide sequence of the HN mRNA 5' to 3' and the predicted amino acid sequence of the HN protein. Nucleotide 1 (marked by the large dot) is the 5' terminal nucleotide of the polymerase binding site of the HN gene. Polymerase recognition sites are underlined. Potential glycosylation sites are boxed. (---) indicates location of putative stop codon.

```
AAC TTA GGA GTA AAG TTA CGC AAT TCA ACT CTA CTC ATA AAA TTG AGA AAG AAC CCA ACA GAC AAA TCC AAA TCC GAG   73

ATG GAA TAC TGG AAG CAC ACC AAT CAC GGG AAA GAT GCT GGT AAT GAG CTG GAA ACA TCC ATG GCT ACT CAT GGC AAC  151
Met Glu Tyr Trp Lys His Thr Asn His Gly Lys Asp Ala Gly Asn Glu Leu Glu Thr Ser Met Ala Thr His Gly Asn   26

AAG ATC ACC AAC AAG ATA ACA TAT ATA TTA TGG ACA ATA ATC CTG GTG TTA TTA TCA ATA GTC TTC ATC ATA GTG CTA  229
Lys Ile Thr Asn Lys Ile Thr Tyr Ile Leu Trp Thr Ile Ile Leu Val Leu Leu Ser Ile Val Phe Ile Ile Val Leu   52

ATT AAT TCC ATC AAA AGT GAA AAA GCC CAT GAA TCA TTG CTA CAA GAC GTA AAC AAT GAG TTT ATG GAA GTT ACA GAA  307
Ile Asn Ser Ile Lys Ser Glu Lys Ala His Glu Ser Leu Leu Gln Asp Val Asn Asn Glu Phe Met Glu Val Thr Glu   78

AAG ATC CAA ATG GCA TCG GAT AAT ATT AAT GAT CTA ATA CAG TCA GGA GTG AAT ACA AGG CTT CTT ACA ATT CAG AGT  385
Lys Ile Gln Met Ala Ser Asp Asn Ile Asn Asp Leu Ile Gln Ser Gly Val Asn Thr Arg Leu Leu Thr Ile Gln Ser  104

CAT GTC CAG AAT TAT ATA CCA ATA TCA TTG ACA CAA CAA ATG TCG GAT CTT AGG AAA TTC ATT AGT GAA ATT ACA ATT  463
His Val Gln Asn Tyr Ile Pro Ile Ser Leu Thr Gln Gln Met Ser Asp Leu Arg Lys Phe Ile Ser Glu Ile Thr Ile  130

AGG AAT GAT AAT CAA GAA GTG CCA CCA CAA AGA ATA ACA CAT GAT GTG GGC ATA AAA CCT TTA AAT CCA GAT GAT TTT  541
Arg Asn Asp Asn Gln Glu Val Pro Pro Gln Arg Ile Thr His Asp Val Gly Ile Lys Pro Leu Asn Pro Asp Asp Phe  158

TGG AGA TGC ACG TCT GGT CTT CCA TCT TTA ATG AAA ACT CCA AAA ATA AGG TTA ATG CCG GGG CCG GGA TTA TTA GCT  619
Trp Arg Cys Thr Ser Gly Leu Pro Ser Leu Met Lys Thr Pro Lys Ile Arg Leu Met Pro Gly Pro Gly Leu Leu Ala  182

ATG CCA ACG ACT GTT GAT GGC TGT GTT AGA ACT CCG TCC TTA GTT ATA AAT GAT CTG ATT TAT GCT TAT ACC TCA AAT  697
Met Pro Thr Thr Val Asp Gly Cys Val Arg Thr Pro Ser Leu Val Ile Asn Asp Leu Ile Tyr Ala Tyr Thr Ser Asn  208

CTA ATT ACT AGA GGT TGC CAG GAT ATA GGA AAA TCA TAT CAA GTA TTA CAG ATA GGG ATA ATA ACT GTA AAC TCA GAG  775
Leu Ile Thr Arg Gly Cys Gln Asp Ile Gly Lys Ser Tyr Gln Val Leu Gln Ile Gly Ile Ile Thr Val Asn Ser Asp  234

TTG GTA CCT GAC TTA AAT CCT AGG ATC TCT CAT ACT TTC AAC ATA AAT GAC AAT AGA AAG TCA TGT TCT CTA GCA CTC  853
Leu Val Pro Asp Leu Asn Pro Arg Ile Ser His Thr Phe Asn Ile Asn Asp Asn Arg Lys Ser Cys Ser Leu Ala Leu  260

CTA AAT ACA GAT GTA TAT CAA CTG TGT TCG ACT CCC AAA GTT GAT GAA AGA TCA GAT TAT GCA TCA TCA GGC ATA GAA  931
Leu Asn Thr Asp Val Tyr Gln Leu Cys Ser Thr Pro Lys Val Asp Glu Arg Ser Asp Tyr Ala Ser Ser Gly Ile Glu  286

GAT ATT GTA CTT GAT ATT GTC AAT CAT GAT GGT TCA ATC TCA ACA ACA AGA TTT AAG AAC AAT AAT ATA AGT TTT GAT 1009
Asp Ile Val Leu Asp Ile Val Asn His Asp Gly Ser Ile Ser Thr Thr Arg Phe Lys Asn Asn Asn Ile Ser Phe Asp  312

CAA CCA TAT GCG GCA TTA TAC CCA TCT GTT GGA CCA GGG ATA TAC TAC AAA GGC AAA ATA ATA TTT CTC GGG TAT GGA 1087
Gln Pro Tyr Ala Ala Leu Tyr Pro Ser Val Gly Pro Gly Ile Tyr Tyr Lys Gly Lys Ile Ile Phe Leu Gly Tyr Gly  338

GGT CTT GAA CAT CCA ATA AAT GAG AAT GCA ATC TGC AAC ACA ACT GGG TGT CCC GGG AAA ACG CAG AGA GAC TGC AAT 1165
Gly Leu Glu His Pro Ile Asn Glu Asn Ala Ile Cys Asn Thr Thr Gly Cys Pro Gly Lys Thr Gln Arg Asp Cys Asn  364

CAG GCA TCT CAT AGT CCC TGG TTT TCA GAC AGA AGG ATG GTC AAC TCC ATT ATT GTT GTT GAC AAG GGC TTA AAC TCA 1243
Gln Ala Ser His Ser Pro Trp Phe Ser Asp Arg Arg Met Val Asn Ser Ile Ile Val Val Asp Lys Gly Leu Asn Ser  390

ATT CCA AAA CTG AAG GTA TGG ACG ATA TCC ATG AGA CAA AAT TAC TGG GGG TCA GAA GGA AGG CTA CTT CTA CTA GGT 1321
Ile Pro Lys Leu Lys Val Trp Thr Ile Ser Met Arg Gln Asn Tyr Trp Gly Ser Glu Gly Arg Leu Leu Leu Leu Gly  416

AAC AAG ATC TAT ATA TAT ACA AGA TCT ACA AGT TGG CAT AGC AAG TTA CAA TTA GGA ATA ATT GAT ATT ACT GAT TAC 1399
Asn Lys Ile Tyr Ile Tyr Thr Arg Ser Thr Ser Trp His Ser Lys Leu Gln Leu Gly Ile Ile Asp Ile Thr Asp Tyr  442

AGT GAT ATA AGA ATA AAA TGG ACA TGG CAT AAT GTG TTA TCA AGA CCA GGA AAC AAT GAA TGT CCA TGG GGA CAT TCA 1477
Ser Asp Ile Arg Ile Lys Trp Thr Trp His Asn Val Leu Ser Arg Pro Gly Asn Asn Glu Cys Pro Trp Gly His Ser  468

TGT CCA GAT GGA TGT ATA ACA GGA GTA TAT ACT GAT GCA TAT CCG CTC AAT CCC ACA GGA AGC ATT GTG TCA TCT GTC 1555
Cys Pro Asp Gly Cys Ile Thr Gly Val Tyr Thr Asp Ala Tyr Pro Leu Asn Pro Thr Gly Ser Ile Val Ser Ser Val  494

ATA TTA GAC TCG CAA AAA TCG AGA GTA AAC CCA GTC ATA ACT TAC TCA GCA GCA ACT GAA AGG GTA AAC GAG CTG GCC 1633
Ile Leu Asp Ser Gln Lys Ser Arg Val Asn Pro Val Ile Thr Tyr Ser Thr Ala Thr Glu Arg Val Asn Glu Leu Ala  520

ATC CGA AAC AAA ACA CTC TCA GCT GGA TAT ACA ACA ACG AGC TGC ATT ACA CAC TAT AAC AAA GGA TAT TGT TTT CAT 1711
Ile Arg Asn Lys Thr Leu Ser Ala Gly Tyr Thr Thr Thr Ser Cys Ile Thr His Tyr Asn Lys Gly Tyr Cys Phe His  546

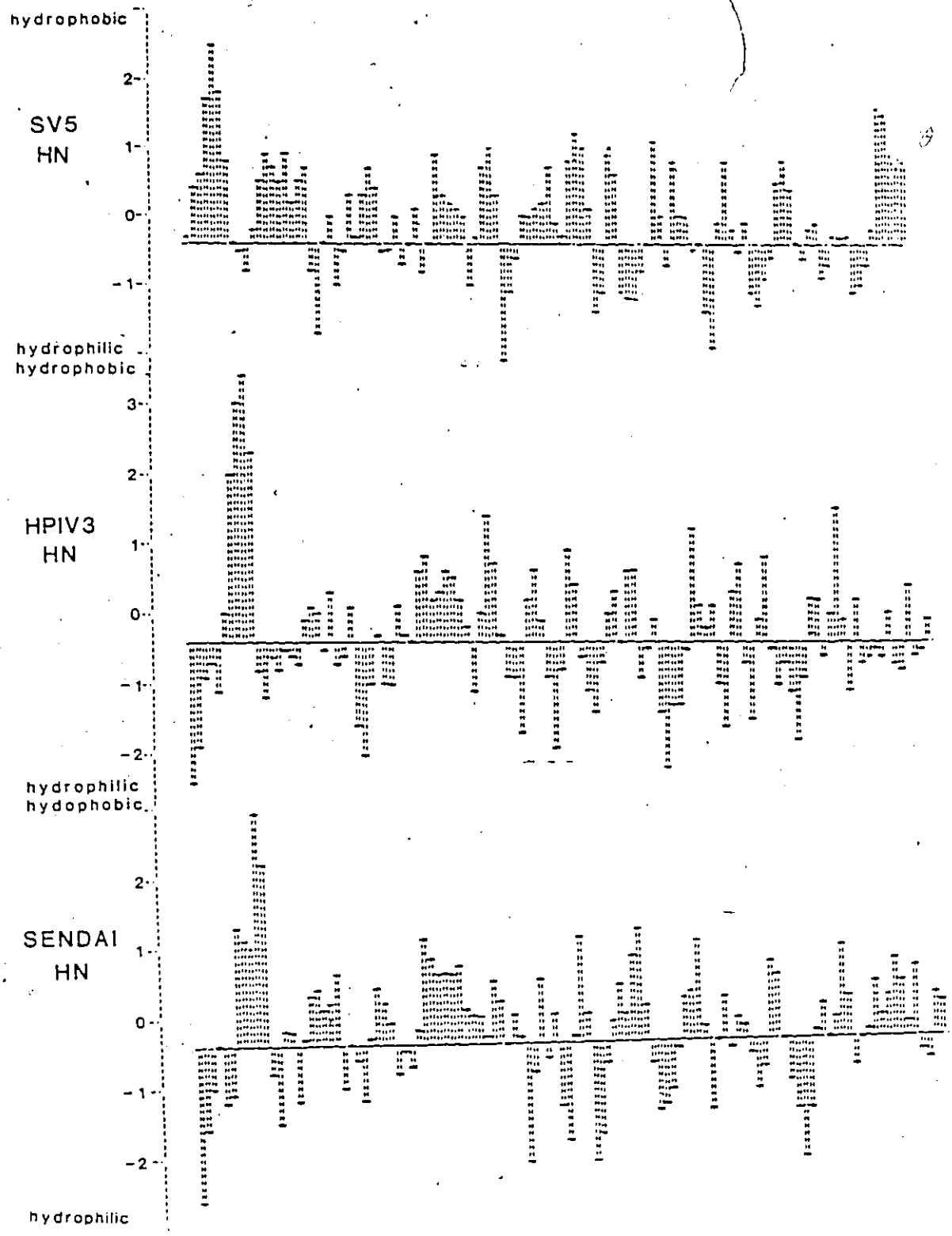
ATA GTA GAA ATA AAT CAT AAA AGC TTA GAC ACA TTC CAA CCT ATG TTG TTC AAA ACA GAG ATT CCA AAA AGC TGC AGT 1789
Ile Val Glu Ile Asn His Lys Ser Leu Asp Thr Phe Gln Pro Met Leu Phe Lys Thr Glu Ile Pro Lys Ser Cys Ser  572

TAA TCA TAA TTA ACC ATA ATA TGT ATT AAC CTA TCT ATA ATA CAA GTA TAT GAT AAG TAA TCA GCA ATC AGA CAA TAG 1867
---

ATA AAA GAG AAA TAT AAA AAA CTT AGG AGC AAA GCG TGC TCG AAA ATG GAC ACT GAA TCT AAC AAT GGC GCT GTA TCT 1945
                                                Met Asp Thr Glu Ser Asn Asn Gly Ala Val Ser  11

GAC ATA CTC TAT CCT GAG TGT GAC GTT AAT TCT GTT ATC GTT AGG                                  1993
Asp Ile Leu Tyr Pro Glu Cys His Leu Asn Ser Pro Ile Val Arg                                   28
```

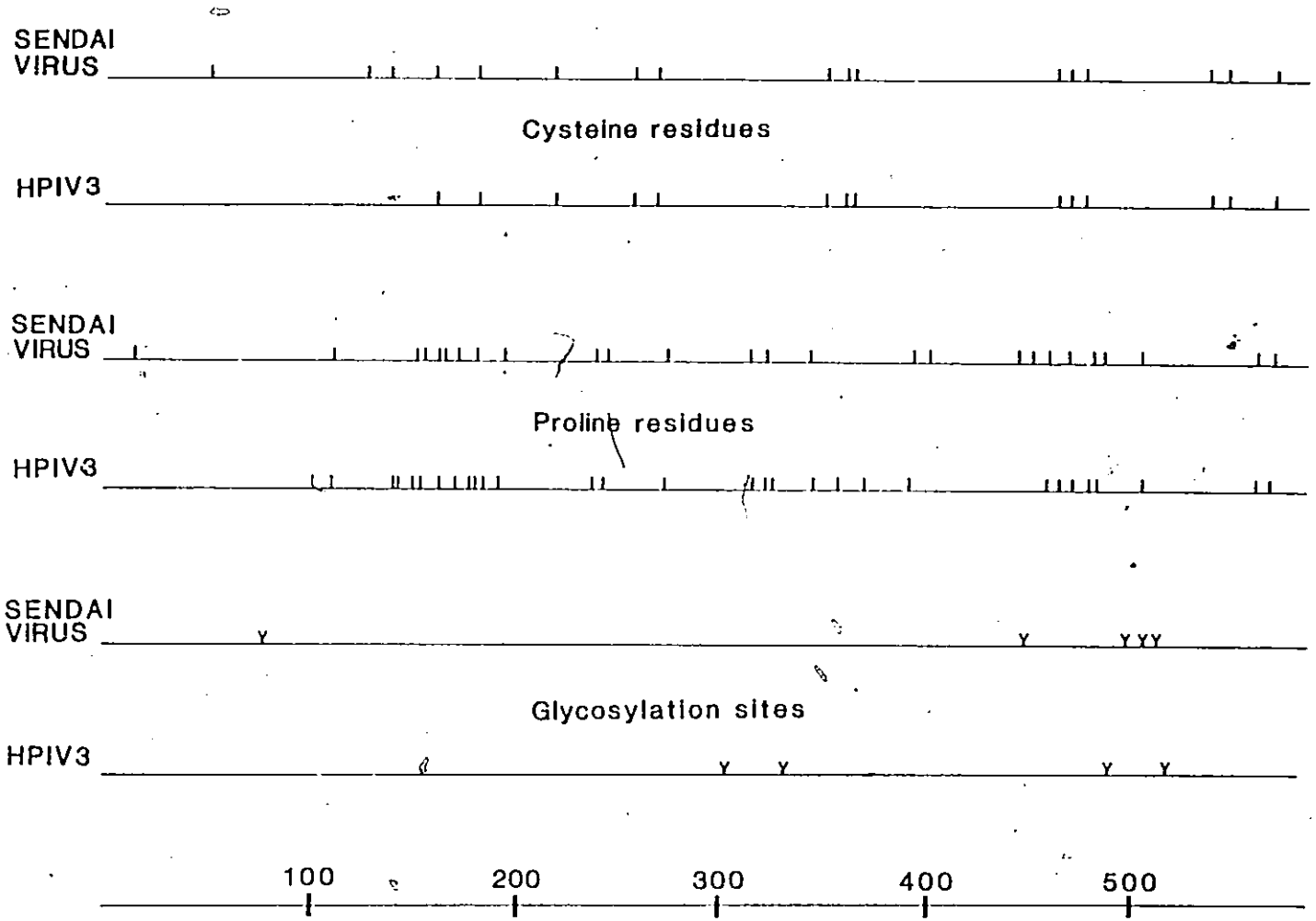
Figure 21. Hydropathy plots of the HN proteins of human parainfluenza virus 3, Sendai virus and SV5. Hydropathy plots were determined by the methods of Kyte and Doolittle, (1982). The plot of the HN protein of Sendai virus was generated from the sequence data of Blumberg et al. (1985). The plot of the SV5 HN protein was generated from the sequence data of Heibert et al. (1985a). The plots are presented with the amino-terminus on the left hand side and the carboxyl-terminus on the right hand side.


hydrophobic
SV5
HN
2
1
0
-1
hydrophilic
hydrophobic
HPIV3
HN
3
2
1
0
-1
-2
hydrophilic
hydophobic
SENDAI
HN
2
1
0
-1
-2
hydrophilic

phobic regions. There was much less similarity between the hydropathy profiles of SV5 and those of either Sendai virus or HPIV3 HN gene products (Figure 21). All three viral HN gene products had a long hydrophobic stretch of amino acids located near the N-terminal end of the protein. However, SV5 lacked the hydrophilic N-terminal amino acids that were found in both HPIV3 and Sendai virus HN gene products. The HN gene product of HPIV3 had a potential transmembrane region near the N-terminal end of the molecule and lacked a similar region near the C-terminal end of the molecule. HPIV3 HN therefore followed the pattern of the HN gene products of Sendai virus, SV5 and Newcastle disease virus in having only one potential signal and anchor region located at the N-terminus of the polypeptide.

In addition to the similarities between the hydropathy plots of Sendai virus and HPIV3 HN gene products, there was a close correlation in the positions of certain amino acids. All the cysteine residues of the HPIV3 HN protein were located within 2 amino acids of those found in the Sendai virus HN protein (Figure 22). Only 3 cysteine residues, located at the N-terminal end of Sendai virus HN protein, were not represented in the HN protein of HPIV3 (Figure 22). The positions of the cysteine residues in the HN molecules of SV5 and Sendai virus were similar if the sequence was positioned for best fit (Shioda et al., 1986), but the relationship was not as close as with HPIV3 and Sendai virus. Three cysteine residues were conserved in all three amino acid sequences at positions 159, 363 and 535 along the HPIV3 HN protein. The positions of the proline residues in the HN proteins of HPIV3 and Sendai virus also corresponded, although not to the same extent as the cysteine residues (Figure 22). Nonetheless 72% of the HPIV3 proline residues were within 2 amino acids of those found in Sendai virus (Figure 22). In comparison, 58% of the SV5 proline residues

Figure 22. Locations of the cysteine residues, proline residues and potential glycosylation sites within the HN proteins of Sendai virus and human parainfluenza virus 3. Locations of the cysteine and proline residues are denoted by vertical bars. Locations of the potential glycosylation sites are denoted by Y's. Sendai virus HN protein gene sequence data from Blumberg et al., (1985). The maps are presented with the amino-terminus on the left hand side and the carboxyl-terminus on the right hand side.


SENDAI VIRUS
Cysteine residues
HPIV3
SENDAI VIRUS
Proline residues
HPIV3
SENDAI VIRUS
Glycosylation sites
HPIV3
100
200
300
400
500

were located within 8 amino acids of those found in Sendai virus and 41% of HPIV3 proline residues were located within 6 amino acids of those in SV5 HN protein.

Given the high degree of homology between the HN proteins of Sendai virus and HPIV3 it was interesting that only one of the HPIV3 glycosylation sites was within 20 amino acids of a counterpart in Sendai virus (Figure 22). In general, four of the five Sendai virus and four out of six SV5 potential glycosylation sites occurred in the C-terminal end of the protein. This corresponded to the location of the glycosylation sites in the HN of HPIV3 in that all four sites are located in the C-terminal half of the polypeptide.

3.5.4. Highly conserved regions of the HN proteins of human parainfluenza virus 3, Sendai virus and simian virus 5

Shioda et al., (1986) identified 2 regions that are highly conserved between the HN proteins of Sendai virus and SV5, A comparison of the homologous regions of the Sendai virus and SV5 HN proteins to those found in the HN protein of HPIV3 (Figure 23) showed these two regions were also highly conserved in HPIV3. The degree of homology between Sendai virus and HPIV3 was 5-15% greater than between Sendai virus and SV5. In addition to these two regions, two other regions of high homology between Sendai virus and HPIV3 were identified (Figure 23). Region C was 72% homologous and region D, the C-terminal end of the polypeptide, was 78% homologous to the corresponding sequence in Sendai virus HN (Figure 23).

3.5.5 Highly conserved regions of the L proteins of human parainfluenza virus 3 and Sendai virus.

In the course of sequencing the flanking regions of the HPIV3 HN gene some of the sequence the L gene was determined. Figure 24 shows the highly

Figure 23. Highly conserved regions of the HN proteins of human parainfluenza virus 3 and Sendai virus. Numbers indicate the amino acid positions. Asterisks denote identical amino acid residues in the sequence. Sendai HN amino acid sequence from Shioda et al., (1986).

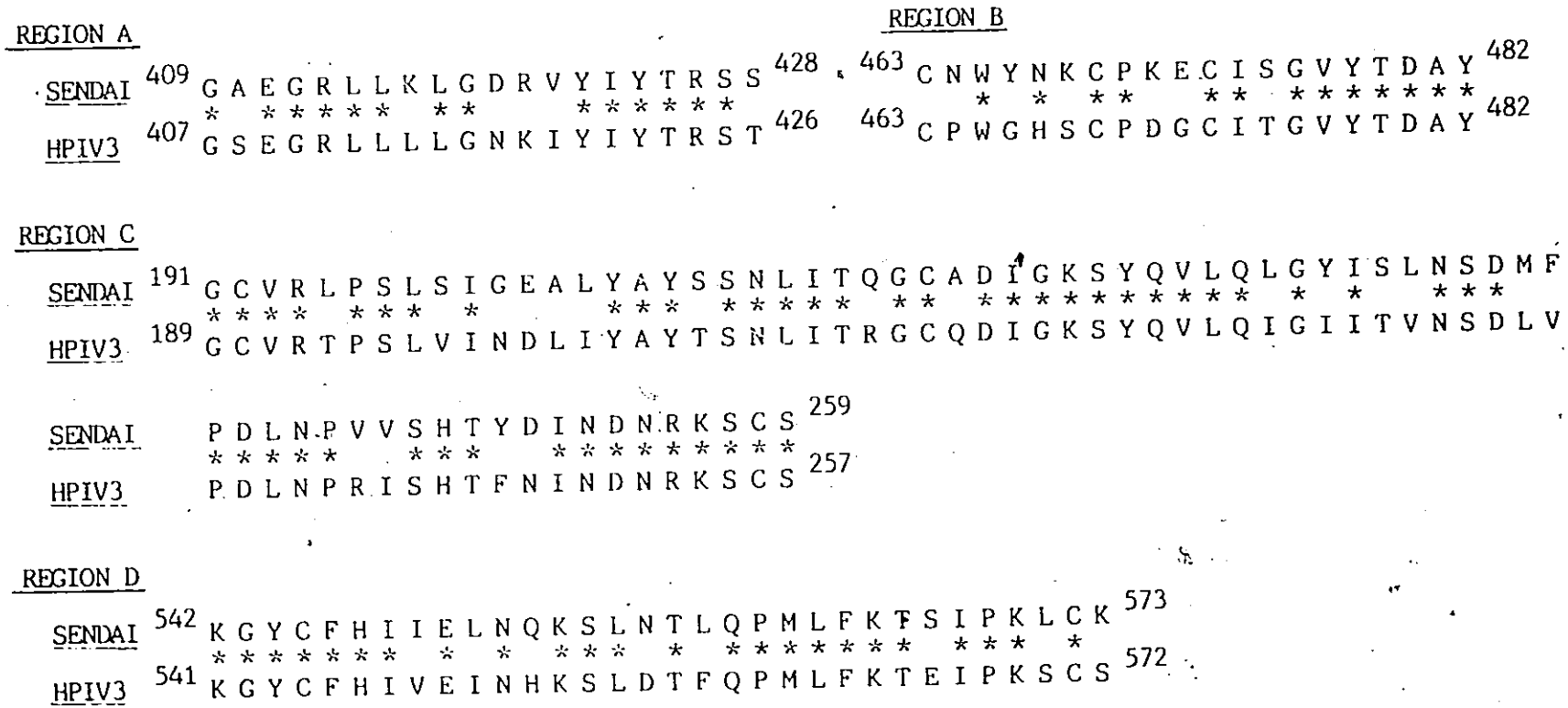

REGION A
REGION B
SENDAI 409 G A E G R L L K L G D R V Y I Y T R S S 428 463 C N W Y N K C P K E C I S G V Y T D A Y 482
HPIV3 407 G S E G R L L L L G N K I Y I Y T R S T 426 463 C P W G H S C P D G C I T G V Y T D A Y 482
REGION C
SENDAI 191 G C V R L P S L S I G E A L Y A Y S S N L I T Q G C A D I G K S Y Q V L Q L G Y I S L N S D M F
HPIV3 189 G C V R T P S L V I N D L I Y A Y T S N L I T R G C Q D I G K S Y Q V L Q I G I I T V N S D L V
SENDAI P D L N P V V S H T Y D I N D N R K S C S 259
HPIV3 P D L N P R I S H T F N I N D N R K S C S 257
REGION D
SENDAI 542 K G Y C F H I I E L N Q K S L N T L Q P M L F K T S I P K L C K 573
HPIV3 541 K G Y C F H I V E I N H K S L D T F Q P M L F K T E I P K S C S 572

Figure 24: Nucleotide sequence of the 5' end of the human parainfluenza virus 3 L gene and deduced amino acid sequence. Underlined amino acids are identical to the amino acids in the N-terminal end of Sendai virus L protein. The Sendai virus L protein amino acid sequence used for this comparison, was from the data of Shioda et al., 1986). Nucleotide 1 (marked by an asterisk) is the 5' terminal nucleotide of the polymerase binding site.

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                                        *
ATA AAA GAG AAA TAT AAA AAA CTT AGG AGC AAA GCG TGC TCG AAA ATG GAC ACT  30
                                                            Met Asp Thr  L3

GAA TCT AAC AAT GGC ACT GTA TCT GAC ATA CTC TAT CCT GAG TGT CAC CTT AAT  84
Glu Ser Asn Asn Gly Thr Val Ser Asp Ile Leu Tyr Pro Glu Cys His Leu Asn  L21

TCT CTT ATC GTT AGG                                                     99
Ser Pro Ile Val Arg                                                     L26
```

conserved regions between HPIV3 and Sendai virus which occurred at the amino terminal end of the L proteins. From amino acid 11 to 26 there was 100% homology. Interestingly, this region was preceded by a completely non-homologous stretch of 8 amino acids. The polymerase binding sites of the HPIV3 L gene (AGGAGCAAAG) differed from Sendai virus L gene polymerase binding site (AGGGTGAATG) by four nucleotides (Shioda et al.,1986) and from the NDV L gene polymerase binding site (AGTGGCAATG) by only three nucleotides (Chambers et al., 1986).

3.5.6 Summary of the sequence of the HN protein gene

The nucleotide sequence of the HPIV3 HN protein gene was determined using cDNA clones derived from both HPIV3 genomic RNA and mRNA. The HN mRNA contained 1882 nucleotides not including the poly (A) tail. The HPIV3 mRNA had one large open reading frame that coded for 572 amino acids with a deduced molecular weight of 64,178. Potential polymerase recognition signals for the HN and L genes were located in the flanking regions. The HN protein shared some common features with the previously sequenced HN proteins of Sendai virus and simian virus 5. These features included: a N-terminal membrane anchor, four regions of highly conserved amino acid sequence and strong conservation in the positions of the cysteine residues. The hydropathy profile of the HPIV3 HN protein indicated that it may well be a transmembrane protein.

CHAPTER 4: DISCUSSION

In this project two systems were explored to gain insight into virus-host cell interactions. In the first part of this study the molecular mechanism involved in VSV-induced cell fusion was investigated. In the second part of this work the components of the HPIV3 virion were analyzed with special emphasis on the glycoproteins. These two seemingly distinct lines of investigation yielded complementary results which demonstrated the importance of the molecular configuration of the virion glycoproteins in virus-host cell interactions.

VSV is among the most extensively studied animal viruses. Its structure and replication are understood in great detail. However, there are a number of unanswered questions concerning the early stages of infection and the chemical nature of the receptor(s) which bind VSV (Choppin and Scheid, 1980). VSV contains a single glycoprotein which, like the glycoprotein(s) of other enveloped viruses, forms spike-like projections on the surface of the virion and is embedded in the viral membrane by a hydrophobic anchor. The G protein is the only protein located on the external surface of the virion and is involved in adsorption (Kelly et al., 1972; Wiktor et al., 1972). The mechanism of penetration is a subject of controversy. VSV virions enter cells by either phagocytosis (Simpson et al., 1969; Dahlberg, 1974; Fan and Sefton, 1978) or by fusion of the viral and cell membranes (Heine and Schnaitman, 1969). The current hypothesis is that VSV virions are engulfed in phagocytic vesicles and enter the cell by receptor-mediated endocytosis (Matlin et al., 1981; Schlegel et al., 1982). The pH in the vesicle drops, triggering the membrane fusion activity of the G protein (Handa et al., 1982; Hughes et al., 1979a; Florkiewicz and Rose, 1984; Riedel et al., 1984). This fusion event merges the membranes of the virus and vesicle (Mifune et al., 1982).

Through this process the genome of VSV gains entry into the cytoplasm of the target cell. Support for the pH dependence of VSV-induced membrane fusion comes from the observation that agents known to increase the pH in lysosomal vesicles, inhibit the uncoating of the virus (White et al., 1981). Furthermore, at the cell membrane, expression of the G protein will not induce cell fusion unless the pH is below 5.5 (Florkiewicz and Rose, 1984; Riedel et al., 1984). However, highly cytolytic strains of VSV, induced cell fusion when the viral protein synthesis was inhibited after infection. Thus, the membrane fusion activity of VSV is active at the surface of the target cell at physiological pH. It was also shown that both the M protein and the G protein were involved in VSV-induced cell fusion.

It has been observed that VSV induced cell fusion is strictly dependent on both the strain of VSV used and the host involved (Chany-Fournier et al., 1977; Handa et al., 1982). However, it was shown in this project that when protein synthesis was modified, cell fusion was not dependent on the strain of VSV used. Thus, VSV has cell fusion activity under certain conditions. Differences between R(B77) and BHK-21 cells in their tendency to form polykaryocytes (Figure 2 and Table 3) indicated that the host cell influences VSV-induced cell fusion. However, what role the host cell plays is not clear. Previous studies of VSV-mediated cell fusion also show that host-associated factors are involved, with susceptibility of the host membrane to fusion being the most important feature (Poste, 1970; 1972). Inhibition of the synthesis of host proteins, by treatment with actinomycin D, did not affect the VSV-mediated cell fusion or other cytopathic effects. Clearly, the fusion potential of the host cell is not affected by host proteins. It should be emphasized that the differences in VSV-induced cell fusion between R(B77) and BHK-21 cells, reported in this

study, are minor compared with the all-or-none response reported previously (Nishiyama et al., 1976; Chany-Fournier et al., 1977; Handa et al., 1982).

A series of experiments were performed to determine when and how much, inhibition of protein synthesis was needed for VSV to induce cell fusion. Figure 4 reveals that total inhibition of protein synthesis before or during the eclipse period of viral replication prevented polykaryocyte formation. When cycloheximide is added immediately after infection and maintained during infection, viral replication will be blocked (Chany-Fournier et al., 1977; Hughes et al., 1979a). Thus, the lack of cytopathic effects in cells treated with cycloheximide one hour after infection (Figure 4) could be due to lack of viral replication. On the other hand, if protein synthesis was blocked after the eclipse period, VSV-infected cells were predisposed to polykaryocyte formation (Figure 4; Nishiyama et al., 1976). An interpretation of these results was that some viral protein synthesis was required for VSV-induced cell fusion. Furthermore, it seemed that proteins synthesized for the assembly of the virus were critical in the mechanism of cell fusion.

There have been a number of reports on the pH dependence of VSV-induced cell fusion (White et al., 1981; Florkiewicz and Rose, 1984; Riedel et al., 1984). The threshold of pH dependence for cell fusion as a result of VSV infection is reported to be pH 6 (White et al., 1981). Likely there was little pH dependence on the VSV-induced cell fusion observed in my study because the experiments were conducted in media of physiological pH. However, in these experiments one cannot completely rule out a pH dependence because amino acid starvation or inhibition of protein synthesis may result in localized alterations in pH of the medium or the cell surface. VSV may inherently have the ability to cause cell fusion if certain conditions are met. Likely, low pH has a direct chemical effect on

synthesis of viral proteins such that VSV infection results in cell fusion.

It was important to establish whether individual viral proteins were involved in cell fusion induced by VSV. Glycoproteins of a number of enveloped viruses have fusion activities, specifically the F protein of the paramyxoviruses (Choppin and Scheid, 1980) and the B_2 glycoprotein of herpes simplex virus (Manservigi et al., 1977). There is solid evidence that the G glycoprotein of VSV mediates cell fusion. Hughes et al. (1979a) found that when glycosylation is inhibited by 0.01 M D-glucosamine, the process of VSV-induced cell fusion is blocked. This block of VSV-induced polykaryocytosis occurs even when the glucosamine is added at a concentration that inhibits neither host nor viral protein synthesis. Monospecific antibody directed against G protein also inhibits VSV-induced cell fusion (Hughes et al., 1979a, Handa et al., 1982). Antibodies directed against M and N proteins did not block polykaryocyte formation as a result of VSV infection (Handa et al., 1982). It is noteworthy that anti-M antibody may in fact promote cell fusion. However, this possibility was not tested. Finally, expression of the G protein of VSV in the absence of the other viral proteins induces cells to fuse when the pH of the culture medium is reduced (Florkiewicz and Rose, 1984; Riedel et al., 1984). Clearly, the G protein is required for cell fusion to take place.

In a typical VSV infection, G protein is abundantly expressed on the surface of infected cells, yet cell fusion does not take place. This may well be due to a rapid destruction of the cells by insertion of the M and G proteins into the membrane and the sequential budding of the virus from the cell. Indeed, cell surface G protein in the presence of M protein or other viral proteins will not promote fusion. The results presented in this thesis support this hypothesis. It was found that viral protein synthesis

must be inhibited after the eclipse period for cell fusion to take place (Figure 3 and 4, Table 3), at which time much of the G protein has already been synthesized. Thus, a reduction in the amount of another protein must be required for VSV to cause polykaryocytosis. Support for the involvement of another protein comes from experiments carried out on temperature sensitive mutants from the five complementation groups of VSV (Table 5). Cell fusion was induced at the non-permissive temperature by tsG31, a mutant of complementation group III (Table 5). The mutant ts G31 grown at the non-permissive temperature produces a smaller amount of M protein compared to the wild type VSV (Little and Huang, 1978). In addition, the M protein of ts G31 had an aberrant electrophoretic mobility (Figure 6). At the non-permissive temperature ts G31 does not produce infectious virus (Hughes et al. 1979a). At the permissive temperature ts G31 induces cell fusion in two neuroblastoma cell lines, mouse EAT cell, rat XC cells, R(B77) cells and BHK-21 cells (Hughes et al., 1979a; Handa et al., 1982; Table 5). The properties of ts G31 support the hypothesis that if the M protein produced during a VSV infection is non-functional, then normal maturation cannot occur and the cells undergo fusion mediated by G protein.

It seemed that when non-functional M protein was present in infected cells the G protein promoted cell fusion. But could reduced levels of M protein affect the cytopathic effects produced by VSV? There are significant differences among the rates of transport of the five VSV proteins into virions (Wagner et al., 1970; Kang and Prevec, 1971; Figure 5). VSV M protein is transported rapidly from the cells and incorporated into virions (Kang and Prevec, 1971; Knipe et al., 1977b; Figure 5). If protein synthesis was inhibited early in the infection, the intracellular pool of M protein would be the most drastically reduced. However, G protein is not rapidly transported from the cell into virions. Indeed, if protein

synthesis was inhibited early in infection, M protein would be lost from the cells, but G protein would slowly accumulate in the cell membrane. Thus, if protein synthesis is inhibited during an infection, the ratio of G protein to M protein is disturbed and G protein mediates cell fusion.

Two models for VSV-induced cell fusion can be proposed. First, VSV infection could lead to cell fusion when the intracellular molar ratio of G protein to M protein is increased. Specifically, when G protein accumulates on the membrane in the absence of functional M protein, the cells will fuse. An alternative model is that the total amount of cell surface G protein or the amount of time required for the cells to remain in contact with G protein on the cell surface may determine whether the cells will fuse. In this model any effects M protein would have might be indirect since M protein is required for budding, which would reduce the level of cell surface G protein. The pH dependence of cell fusion as induced by expressed G protein (Florkiewicz and Rose, 1984; Riedel et al., 1984) argues in favor of the first model. If we accept the first model, then we must accept that M protein found on the inner surface of the membrane influences the molecular configuration of the G protein located outside of the membrane. The influence of the M protein on the G protein could be mediated by an interaction between a domain on the M protein and the membrane anchor of the G protein. During an infection, if the M protein is not functional, is absent or is present in low amounts this interaction cannot take place. Thus, the molecular configuration of the G protein is altered allowing the G protein to mediate cell fusion. An acidic environment may also alter the interaction between G and M proteins and/or may alter the molecular configuration of the G protein itself allowing expression of its fusion potential. An analogous situation exists with the

M protein of Sendai virus. Markwell and Fox (1980) showed that in the mature virion there was a close contact between the M protein and the nucleocapsid core. During maturation, the M protein mediates an alignment of the core to areas of the host cell membrane into which the viral glycoproteins have been inserted (McSharry et al., 1971; Yoshida et al., 1976). Heggeness et al. (1982) point out that in the absence of underlying nucleocapsids the viral glycoproteins, on the surface of infected cells are only detected with immune electron microscopy. However, once the M protein mediates the alignment of the nucleocapsids under the virus-modified plasma membrane then the glycoproteins are seen as their characteristic spikes (Heggeness et al., 1982). Clearly, an interaction between the M protein, nucleocapsid and host membrane is important for the correct positioning of the glycoprotein.

In summary, VSV-induced cell fusion occurred if viral protein synthesis was inhibited 2-5 hours after infection. Inhibition of protein synthesis altered the intracellular molar ratio of G protein to M protein due to different rates of transport of these two proteins. An alteration in the ratio between the two proteins resulted in an accumulation of the G protein on the surface of the plasma membrane, in an absence or a reduced amount of M protein. The accumulation of G protein on the membrane in an absence of M protein led to membrane fusion. A similar situation occurred if the M protein was non-functional rather than reduced in amounts.

Parainfluenza viruses are important respiratory tract pathogens of infants and children (Chanock and McIntosh, 1985). These viruses are also increasingly important as pathogens in people over the age of 50 years (van der Logt et al., 1985). Despite the clinical importance of HPIV3 infections, no effective immunoprophylaxis is currently available. The

failure of formalin-inactivated paramyxovirus vaccines (Chanock and McIntosh, 1985) and the consequences of their failure made the study of the molecular biology of HPIV3 a priority. An understanding of the molecular biology of HPIV3 could be used to gain insight into the nature of the virus-host cell interaction and the antigenic properties of the viral components. This information could then be used to devise a strategy for the prevention of HPIV3 infections or disease by immunological or antiviral means. At the start of this work little was known about the composition of the HPIV3 virions and nothing was known about its genetic make up. During the course of this project it was shown that the virion was composed of 6 structural proteins. Three structural proteins (M, HN and $F_{1,2}$) were associated with the viral membrane and three proteins (L, NP and P) were associated with the nucleocapsid complex. Two of the membrane-bound proteins are glycosylated. The virion proteins were shown to have counterparts in virus infected cells. This was conjecture for the L protein whose presence intracellularly was not firmly established. A number of features of the replication strategy of HPIV3 were also elucidated including coding assignments of the mRNA's, polymerase binding sites, polyadenylation signals and translation patterns of the proteins.

The genomic RNA of HPIV3 (M.W. 4.6×10^6)(Figure 8) was similar in size to that of the other paramyxoviruses. Measles virus RNA has a molecular weight of 4.8×10^6 (Udem and Cook, 1984), RSV RNA has a molecular weight of 5×10^6 (Huang and Wertz, 1982) and Sendai virus genomic RNA has a molecular weight of 5×10^6 (Kolakofsky et al., 1974a;b; Shibuta et al., 1979). Shibuta et al. (1979) found that bovine parainfluenza virus 3 (BPIV3) genomic RNA has an approximate molecular weight of 4.5×10^6. In addition to the size similarity between the genomes of the paramyxoviruses there is

also a similarity in the genetic maps of the viruses in this family. The deduced gene order of HPIV3 is 3'-NP-P-M-F-HN-L-5' (Dimock et al., 1985, 1986a, Spriggs and Collins, 1986). Three other paramyxoviruses have a similar gene order, Sendai virus (Dowling et al., 1983), SV5 (Paterson et al., 1984a) and Newcastle disease virus (NDV) (Chambers et al., 1986). Although the genetic map of canine distemper virus (CDV) has not been completely determined it appears to be similar to the others with the order 3'-NP-P-M-70,000-65,000-L-5' (Russell et al., 1985). There are, however, a few differences in the genetic content of the paramyxoviruses. As yet a counterpart to the SV5 short hydrophobic (SH) protein gene, located between the F and the HN gene, (Heibert et al., 1985a) has not been identified in the other paramyxoviruses. For example, this gene is not found in Sendai virus even though the entire genome has been sequenced (Shioda et al., 1983; 1986). Furthermore, the flanking regions between F and HN of HPIV3 were sequenced and a gene like the SH of SV5 was not found. Major differences are found in the gene order of RSV (3'-14,000-11,000-N-P-M-95,000-G-F-24,000-L-5') (Collins et al., 1984) suggesting that this virus may not belong in the same family as the other paramyxoviruses. However these results indicate that among the paramyxoviruses SV5, Sendai, NDV and HPIV3, the gene order is remarkably conserved.

The L (large) protein of HPIV3 was associated with the nucleocapsid complex of the virion (Figure 13). Transcription of the L gene yields a 6Kb mRNA (Cote, personal communication). Presumably, the L mRNA translates to yield a protein of M.W. 195,000. The L protein was identified in HPIV3 virions (Figure 9) but was not identified in virus infected LLC-MK2 cells (Figure 18). Using BSC-1 or CV-1 cells, the HPIV3 L protein is detected intracellularly (Sanchez and Banerjee, 1985a; Wechsler et al., 1985). Synthesis of the host proteins in BSC-1 and CV-1 cells is inhibited to a

greater extent by HPIV3 infection than in LLC-MK2 cells, which may allow a less abundant viral protein to be detected and identified. The L mRNA was not translated in vitro since the L mRNA is not abundant intracellularly (Figure 14; 15) and large proteins (M.W. >90,000) do not translate well in either wheat germ extracts or reticulocyte lysates. This has left the identification of L mRNA to be determined by the size of the transcript and by elimination. However, 100% homology in the N-terminal regions of the amino acid sequence between Sendai virus and HPIV3 L protein lend further support to the designation of the L mRNA (Figure 24). The large size of the L protein and its low abundance seem to be common among all the paramyxoviruses. In Sendai virus, the L protein also has a high molecular weight (M.W. >160,000) and is the least abundant protein, being present in the virion with an average of 40 molecules (Lamb et al., 1976). The L proteins of measles virus and CDV have been estimated to be between the molecular weights of 160,000 and 200,000 (Rima, 1983). In addition Rima (1983) reports that the L mRNA occurs in morbillivirus infected cells in low abundance. The L protein of NDV is also the largest and least abundant protein in the virion (Hightower et al., 1975).

The role of the L protein in paramyxovirus RNA synthesis has not been firmly established. A number of reports have implicated the L protein in RNA transcription of Sendai virus, NDV and SV5 (Robinson, 1971; Huang et al., 1971; Stone et al., 1972; Buetti and Choppin, 1977; Hamaguchi et al., 1983). Likely, the P and L proteins function as the RNA polymerase for paramyxoviruses. Evidence for this comes from studies on NDV in which the P and L proteins were dissociated from the nucleocapsids, and then reconstituted to form the active transcription complex (Hamaguchi et al., 1983). Neither protein alone formed an active transcriptase complex

(Hamaguchi et al., 1983).

While sequencing the flanking regions of the HN gene the extreme 5' end of the L gene was also sequenced. This sequence revealed the location of the HPIV3 L gene, the polymerase binding site, an open reading frame and a region of high homology to the Sendai virus L gene. Interestingly, the HPIV3 L protein amino acid sequence shows 100% homology to the L protein of Sendai virus from the amino acid at position 11 to the amino acid at position 26 (Figure 24; Shioda et al., 1986). Further work is needed to complete the sequencing of the HPIV3 L gene. However, the high homology between the Sendai virus and HPIV3 genes might imply that this region has an important biological function and has been conserved.

The second largest protein in the virion of HPIV3 was the P protein (Figure 9) which was found associated with the nucleocapsid complex of the virus (Figure 13). This protein was phosphorylated (Figure 10), non-glycosylated (Figure 9) and had intramolecular disulfide bonds (Figure 11). These features correspond with those of the P proteins of other members of the Paramyxoviridae, Sendai virus, SV5, NDV, measles virus and CDV (Marx et al., 1974; Stone et al., 1972; Buetti and Choppin, 1977; Smith and Hightower, 1981; Rima, 1983). The HPIV3 P gene was transcribed as a mRNA of M.W. 8.2×10^5 with a size of 2.4Kb (Figure 16). When translated in vitro this mRNA yielded the P protein of molecular weight 87,000 (Figure 17a).

The P protein is required for transcription of both NDV (Hamaguchi et al., 1983) and Sendai virus (Deshpande and Portner, 1985). However, whether the L protein must complex with P protein for transcription to take place is not known. Molecules of the P protein of both NDV and Sendai virus form trimers linked by disulfide bonds (Markwell and Fox, 1980; Smith and Hightower, 1981). Whether these trimers represent an active P protein complex has not been established. In fact the monoclonal antibody work of

Deshpande and Portner (1985) suggests that the active site is located on a protease resistant fragment of M.W. 40,000 and likely P protein complexes are not necessary for the protein to be biologically active. Trimers of the P protein linked by disulfide bonds were not found in HPIV3 virions (Figure 11).

The P mRNA's of Sendai virus, measles virus and SV5 produce a second protein product (Bellini et al., 1985; Giorgi et al., 1983; Paterson et al., 1984a). For Sendai virus and measles virus this product is the C protein and for SV5 this product is the V protein. The second protein arises from a downstream AUG codon. For SV5 V protein this AUG codon is in frame with the P protein and peptide maps of these two proteins show that the V protein is a subset of peptides from the P protein (Paterson et al., 1984a). In the case of Sendai virus and measles virus, the second reading frame (for the C protein) is out of frame from that of the P protein (Giorgi et al., 1983; Bellini et al., 1985). It has also been reported that there is a third open reading frame in Sendai virus P mRNA, coding for C' protein (Giorgi et al., 1983; Gupta and Kingsbury, 1985; Shioda et al., 1983). The hypothesis put forward by Gupta and Kingsbury (1985) is that the ribosomes scan the P mRNA from its 5' end to find initiation codons. The ribosomes could potentially initiate at any site but the selection of the alternate initiation codons is influenced by the secondary structure of the mRNA.

In vitro translation of the HPIV3 P mRNA yielded the P protein of molecular weight 87,000 and a smaller product of M.W. 33,000 (Figure 17a; b). Sanchez and Banerjee (1985a) also found that HPIV3 P mRNA directed synthesis, in vitro, of several polypeptides in addition to the P protein. The HPIV3 protein of M.W. 33,000 is found in virus infected LLC-MK2 cells

as well as a protein of M.W. 21,000 (Dimock, personal communication) and both appear to be viral specific proteins. The identities of these proteins were not established. However, the possibilities are that: 1) the protein of molecular weight 33,000 is equivalent to the C protein of Sendai and Measles virus, 2) the protein of M.W. 33,000 is a breakdown product of the P protein, or 3) the protein of M.W. 33,000 initiates out of frame and well downstream from the P protein AUG codon. It is likely that the protein with a molecular weight of 21,000 found intracellularly is the equivalent of the C protein of Sendai virus. Sequence analysis of the P protein reveals an open reading frame that would allow translation of a protein of M.W. 23,000 (Luk et al., 1986). In addition a protein with an approximate M.W. of 23,000 has been translated in vitro from the P mRNA (Sanchez and Banerjee, 1985a,b). When poly $(A)^+$ RNA from infected cells is used to direct protein synthesis, in vitro, the ratio of P and NP proteins remained constant and about in equal proportion to that found intracellularly (Figures 17a, 18). This suggested that the P mRNA is relatively stable during in vitro translation. Furthermore, when different concentrations of hybrid selected P mRNA are translated in vitro the ratio of the protein of M.W. 33,000 and the P protein remained remarkably constant (Figure 17b). This experiment was repeated using different preparations of RNA and these did not show any variation in the ratio of the protein of M.W. 33,000 and the P protein produced. Note that in both Figure 17a and Figure 17b the smaller protein of M.W. 33,000 was present as a much stronger band than the P protein. This indicated that this protein was translated, in vitro, much more efficiently than P protein. These results also indicated that the protein was not a breakdown product of the P protein and in fact must be translated from the P mRNA. Presumably, the protein of M.W. 33,000 initiates at a downstream AUG and could use the same

termination codon as the P protein. A protein with similar characteristics to the protein of M.W. 33,000 is found in measles virus (R. Lazzarini, personal communication). The measles protein in fact initiates at an AUG near the middle of the P mRNA and terminates at the same stop codon as the P protein. Definitive proof that HPIV3 P mRNA has an alternative reading frame will come when the protein of M.W. 33,000 is either peptide-mapped or identified by immunoprecipitation.

The nucleocapsid protein (NP) is directly associated with the RNA genome of paramyxoviruses, is the major viral protein both in the virion and infected cells, and confers helical symmetry to the ribonucleoprotein core (Lamb et al., 1976; Mountcastle et al., 1970; Rima 1983). The NP protein of HPIV3 was the most abundant protein in the virion (Figure 9), was associated with the nucleocapsid complex (Figure 13) and was phosphorylated (Figure 10). The NP gene of HPIV3 is the 3' proximal gene (Dimock et al., 1986b). Transcription of the NP gene yielded a mRNA of M.W.6.4×10^5 (Figures 14; 15; 16). When NP mRNA was hybrid selected and translated in vitro, a protein of M.W. 67,000 was synthesized which corresponded exactly in size to the native NP protein. Recently, the NP gene of HPIV3 has been completely sequenced (Galinski et al., 1986; Sanchez et al., 1986). The NP gene codes for a predicted 515 amino acids with an estimated M.W. of 57,800 (Galinski et al., 1986; Sanchez et al., 1986). This calculated molecular weight was well below that calculated by polyacrylamide gel electrophoresis of the NP protein (Figure 9). This discrepancy could result from anomalous migration of the NP protein in SDS-polyacrylamide gels due to intrinsic features of the protein such as an abundance of proline residues. An alternative explanation of the difference could be a post-translational modification of the NP protein,

possibly a phosphorylation. Phosphorylated proteins are known to migrate anomalously in SDS-polyacrylamide gels (Sokol and Clark, 1973) and this may account for the discrepancy in the molecular weight of the native protein.

The amino acid sequence from position 1 through 420 of the HPIV3 NP protein shows extensive homology with the corresponding amino acids of Sendai virus NP protein (Sanchez et al., 1986; Galinski et al., 1986). Little homology occurs in the C-terminal 95 amino acids (Sanchez et al., 1986). Sanchez and Banerjee (1986) also compare the sequence to that of the more distantly related members of the Paramyxoviridae. These comparisons show that HPIV3 NP has no homology with RSV but limited homology with the NP sequence of two morbilliviruses, CDV and measles virus. The areas of greatest homology between the four viruses are located in the central portion of the molecule. The carboxy-terminal regions of the two morbillivirus NP proteins, like Sendai virus NP protein, show little homology to the NP protein of HPIV3. However, a common feature of this region is that it is negatively charged (Sanchez et al., 1986). Markwell and Fox (1980) show that a close association occurs between the NP and M proteins of Sendai virus and NDV during maturation of the virion. The association between the NP and M proteins of Sendai virus may be due to an interaction between the negatively charged C-terminal region of the NP protein and the positively charged M protein (Morgan et al., 1984). Therefore, it might be concluded that preservation of the biological function of the C-terminal region of the NP protein is due to a maintenance of charge rather than a conservation of sequence.

Three HPIV3 proteins were associated with the envelope of the virion, and were designated as the M,F and HN proteins (Figure 13). The HN and F proteins were glycosylated whereas the M protein was not (Figure

9). The envelopes of Sendai virus and Newcastle disease virus consist of a lipid bilayer, external glycoprotein spikes and an inner layer of carbohydrate-free M protein (Rott and Klenk, 1977). This envelope is acquired during the process of budding from the host cell surface (Rott and Klenk, 1977). The available evidence from selective extraction (McSharry et al., 1971) and chemical cross-linking studies (Markwell and Fox, 1980) suggests that the M protein is involved in transmembrane associations between the glycoprotein spikes and the nucleocapsid proteins.

The M gene of HPIV3 transcribed a mRNA of M.W. 4.6×10^5 (Figures 14; 15; 16). This mRNA directed the synthesis, in vitro, of a protein with a molecular weight of 35,000 (Figure 17a). The M protein in HPIV3 virions had a M.W. of 35,000, (Figure 9), aggregated in low salt buffer (Figure 13), was phosphorylated (Figure 10) and appeared to have intramolecular disulfide bonds (Figure 11). Intracellularly, a M protein was not easily detected (Figure 18). The lack of detection of the M protein intracellularly suggests that the M protein may undergo a post-translational modification. In Sendai virus infected cells, the M protein is found in two forms, phosphorylated and non-phosphorylated (Lamb et al., 1978). Phosphorylated M protein is also detected in the virions of NDV and Sendai virus (Smith and Hightower, 1981; Hsu and Kingsbury, 1982). Two forms of the M protein of morbilliviruses also occur (Graves, 1981). However, neither form of the M protein of morbilliviruses may be phosphorylated (Rima et al., 1981; Rima, 1983). In all cases the migration pattern, in SDS-polyacrylamide gels, of the phosphorylated M protein is different from that of the non-phosphorylated M protein. The implication is that phosphorylation of the HPIV3 M protein may account for the differences in the migration of the intracellular and virion M protein. However, a

protein with a M.W. of 35,000 is immunoprecipitated from HPIV3 infected cells using M-specific monoclonal antibodies (Song and Seligy, personal communication). If an intracellular M protein has the same size as the virion M protein then it is likely that the M protein is not undergoing a detectable post-translational modification.

An alternative explanation of the low levels of M protein in HPIV3-infected cells is that the M protein resides in infected cells for a brief period. A rapid assembly of M protein into virions is observed for other enveloped viruses. For example, the NDV M protein incorporates into the plasma membrane immediately after synthesis and has a short intracellular half life (Nagai et al., 1976). The M protein of the rhabdovirus VSV has the shortest intracellular half-life of the VSV proteins (Kang and Prevec, 1971; Figure 5). An explanation for the lack of intracellular HPIV3 M protein could be that the M protein is rapidly incorporated into the budding virus particles and thus transported swiftly out of the cell. Another possibility for the lack of M protein intracellularly is that the M protein is unstable and is degraded in infected cells. In BHK-21 and LLC-MK2 cells, persistently infected with Sendai virus, the M protein is unstable (Roux and Waldvogel, 1982). However, the rates of synthesis of M protein remain constant between acute and persistent infections (Roux and Waldvogel, 1982). Thus, M protein instability may be a consequence of a persistent infection rather than a universal property of the protein.

Emphasis has been placed on the role of the M protein in persistent infections because of the involvement of the protein in assembly and budding (Rima, 1983). Of particular interest is the role of the M protein in subacute sclerosing panencephalitis (SSPE), a persistent infection of human brain cells by measles virus (Rima, 1983). In cells persistently

infected with measles virus, the M protein is unstable, has an altered electrophoretic mobility and/or is synthesized in low amounts (Rima, 1983). Concomitant with the instability of the measles virus M protein is a drop in the number of viral particles released into the culture medium (Roux and Waldvogel, 1982; Rozenblatt et al., 1979). A reduced amount of M protein relative to the other proteins of measles virus is found in brain cells cultured from SSPE patients and in brain cells of SSPE patients (Hall and Choppin, 1979; 1981). Baczko et al, (1984) took these observations one step farther by showing that both the transcription and translation of M protein mRNA is impaired in infected brain tissue. The reasons for the absence or reduced level of M mRNA transcription and translation are not clear. However, a correlation exists between persistence, reduced maturation of virus, and a lack of M protein production.

HPIV3 can cause persistent infections both in vitro (Hodes, 1982), and in vivo (Gross et al., 1973; Muchmore et al., 1981; Parkinson et al., 1980). It is not yet established if the HPIV3 M protein has any role in the initiation or maintenance of these infections. The HPIV3 M protein exhibited features similar to other members of the Paramyxoviridae and so would be a prime candidate for study in HPIV3 persistent infections.

The paramyxovirus F protein is required for virus induced cell fusion, hemolysis and initiation of infection (Homma and Ohuchi, 1973; Scheid and Choppin, 1974; 1976; 1977). These biological activities imply that the F protein has a role in the pathogenicity of the viruses and the host immune response to these viruses (Choppin and Scheid, 1980). As a result, much interest has focussed on the F protein of HPIV3. The F glycoproteins of Sendai virus, SV5 and NDV consist of two disulfide-linked polypeptide chains (F_1 and F_2) which are derived from the precursor glyco-

protein (F_0) by proteolytic cleavage (Scheid and Choppin, 1977). When this study was initiated it immediately became apparent that HPIV3 could induce cell fusion in LLC-MK2 cells. Thus, there was little doubt that HPIV3 possessed a fusion protein analogous to the other paramyxoviruses. Subsequently, the virion of HPIV3 was shown to contain 3 glycoproteins (Figure 9). The largest of these glycoproteins (M.W. 69,000) has both hemagglutinin and neuraminidase activities (Ray et al., 1985; Ray and Compans, 1986; Van Wyke-Coelingh et al., 1985). The other two glycoproteins of HPIV3 represented different configurations of the F protein, presumably the F_0 uncleaved precursor (M.W. 60,000) and the cleaved F_1 protein (M.W. 46,000). The basis of this assignment was that the F_1 protein must be disulfide-linked to another polypeptide chain in order for it to migrate under non-reducing conditions with an apparent molecular weight of 60,000 (Figure 11, 12). The glycoprotein of M.W. 60,000 must be the uncleaved precursor of the $F_{1,2}$ protein otherwise it would dissociate into 2 polypeptide chains and would not be visualized on reducing gels. Further support for these assignments came with the identification of a 12-15,000 glycoprotein found associated with virions of HPIV3 (Wechsler et al., 1985). The presence of both F_0 and $F_{1,2}$ proteins on the surface of the virion was somewhat surprising as it has been well documented for Sendai virus that the F_0 protein was biologically inactive (Choppin and Scheid, 1980). It should be pointed out that the F_1 was the predominant form of F protein present in the HPIV3 virion with F_0 being almost non-existent in some virus preparations (Compare virus preparations in Figures 9 and 13). The presence of both F_0 and $F_{1,2}$ on the virion is not found for either Sendai virus or SV5. However, a small amount of F_0 is detected in purified NDV and measles virus, but again the cleaved forms predominate (Rima and Martin, 1979;

Stallcup et al., 1979; Morrison and Simpson, 1980).

The cDNA clone pPI 14 synthesized from F mRNA hybridized to two HPIV3 specific mRNA's of M.W. $1.3x10^6$ and $8.0x10^5$ (Figure 16). The mRNA of M.W. $8.0x10^5$ was the F mRNA. (Dimock et al., 1986a; Figures 14, 16, 17a). The mRNA of M.W. $1.3x10^6$ hybridized to clones representing both the F gene and the M gene (Figure 16). Presumably, this mRNA species was a bicistronic M/F mRNA. The M/F RNA species was quite abundant in HPIV3-infected LLC-MK2 cells and appeared to function as a mRNA in vitro for the synthesis of M protein as indicated by hybrid-select translation (Figure 17a). Translation of the M protein from the bicistronic mRNA is predictable because the M mRNA sequences would be located at the 5' end of this mRNA. However, the F protein likely would not be translated from this RNA species. Polycistronic mRNA's of this type are found for the other paramyxoviruses (Paterson et al., 1984a). However, this was the first paramyxovirus polycistronic RNA translated in vitro. The implication of this finding was that polycistronic mRNA's may not just be mistakes in transcription but may function in vivo to boost production of certain proteins.

The translation product from the F mRNA had a molecular weight of approximately 54,000 as determined by SDS-polyacrylamide gel electrophoresis (Figure 17a). Recently, the F gene of HPIV3 has been completely sequenced (Spriggs et al., 1986; Côté et al., manuscript submitted), confirming the designation of the F gene clones. The predicted M.W. of the unglycosylated F protein is approximately 60,000, somewhat higher than the in vitro translation product synthesized from F mRNA of M.W. 54,000. This discrepancy is hard to explain. However, it may be that the unglycosylated protein migrates anomalously in polyacrylamide gels. Support for this suggestion comes from analysis of viral protein

synthesized in the presence of the glycosylation inhibitor tunicamycin. HPIV3 infected LLC-MK2 cells grown in the presence of tunicamycin produced a protein of M.W. 54,000 (Figure 18) which may represent the unglycosylated F_0 protein. An analogous situation occurs for the F protein of SV5. The unglycosylated F protein of SV5, in vivo and in vitro, has a molecular weight of 48,000 (Paterson et al., 1984b). In contrast, the predicted M.W. of the peptide backbone of the F protein is approximately 58,000. Thus, the unglycosylated F protein of HPIV3 synthesized in vitro migrated faster than the actual molecular weight would suggest.

A comparison of the amino acid sequence of the F proteins of HPIV3, Sendai virus, SV5 and RSV reveals a number of interesting common features. First, HPIV3 F protein shares considerable amino acid identity with Sendai virus (41.1%), but less identity to either SV5 F protein (25.5%) or RSV F protein (19.4%) (Spriggs et al., 1986). The reported order of decreasing relatedness to HPIV3 was: Sendai virus, SV5, and RSV (Spriggs et al., 1986). More detailed comparisons between HPIV3, SV5 and Sendai virus F proteins revealed that: 1) the proteins are similar in length, 539 amino acids (HPIV3), 529 amino acids (SV5), and 565 amino acids (Sendai virus), 2) the locations of the cleavage sites are similar, amino acid 110 for HPIV3, 116 for Sendai virus, and 102 for SV5, and 3) the locations of the cysteine residues are highly conserved especially in the F_1 subunit (Spriggs et al., 1986; Côté et al., manuscript submitted). The similarities between the F proteins of HPIV3, Sendai virus and SV5 suggest a conservation in higher order structure of the F protein.

The sixth major structural protein of HPIV3 is the HN protein. This protein possesses both the hemagglutinin and neuraminidase activities of HPIV3 (Ray et al., 1985; Ray and Compans, 1986; Van Wyke-Coelingh et al., 1986). It is the first of the HPIV3 proteins to have a biological function

attributed to it. The HN protein had a molecular weight of 69,000, was glycosylated, had intramolecular disulfide bonds and was located in the envelope of the virion (Figures 9; 11; 13). The function and gross structure of this protein closely parallels that of the other paramyxoviruses (Scheid et al., 1972; Scheid and Choppin, 1973; 1974; 1975b).

In this study the HN gene of HPIV3 and the regions between the F and HN genes and the HN and L genes were sequenced. The single large open reading frame in the HN sequence coded for a protein consisting of 572 amino acids with a deduced M.W. of 64,178. Two clones (pPI 10 and 40) constructed from viral infected cell mRNA hybridized to a 2.4 Kb viral RNA species (M.W. 8.2×10^5)(Figure 16). When the HN mRNA was hybrid-selected and translated in vitro it directed synthesis of a protein of M.W. 64,000 (Figure 17a). This protein was also found in in vitro translation products of the mRNA extracted from HPIV3 infected cells (unpublished observations) and in intracellular proteins isolated from HPIV3 infected tunicamycin treated cells (Figure 18). Therefore, the protein of M.W. 64,000 represents the unglycosylated HN protein of HPIV3 and the cDNA clones which hybridize to the 2.4 Kb mRNA contain HN gene sequences. The similarities between the molecular weights of the HN proteins from in vitro translation products, from tunicamycin treated cells, and deduced from the amino acids sequence suggest that unlike the HN protein of NDV, HPIV3 HN does not appear to undergo a cleavage to form an active molecule (Schuy et al., 1984).

Recently Elango et al.(1986) published a sequence for the coding region of the HN gene of HPIV3. The results presented here confirmed this sequence. Indeed, only 14 base pair differences were found in the coding regions of the gene. Furthermore, these differences resulted in only two

amino acid changes: a glycine instead of a serine at amino acid position 489 and an alanine instead of a serine at position 512. The same strain of HPIV3 was used in the two studies. Thus, the 14 base differences suggested that the viral genome was very stable under different growth conditions and passage histories. These slight differences in growth patterns may be enough to account for the amino acid changes between the two sequences. In any case the amino acid differences were conservative. Differences of greater importance were found in the positioning of the 3' end of the HN mRNA. Elango et al.(1986) show that the gene ends with the sequence $CAAAG(A)_n$. In contrast, the HN mRNA sequence shown in Figure 20 ended further downstream with the sequence $TAT(A)_n$ as determined from the HN mRNA clone pPI 10. This position for the 3' end of the HN mRNA confirmed that found by Spriggs and Collins (1986). Using HPIV3 genomic clones (provided by M.J. Côté, University of Ottawa), the RNA polymerase recognition site for the L and HN genes, a potential polyadenylation signal for the HN gene and the CTT intergenic sequence between the HN and L gene were identified (Figure 20).

The accumulating evidence in recent years suggests that HPIV3 is closely related to Sendai virus. Van Wyke et al.(1985) show that HPIV3 HN is antigenically more closely related to Sendai virus HN than to either parainfluenza viruses 1,2 or 4, even though Sendai virus is a murine parainfluenza virus. HPIV3 also resembles Sendai virus in the translation patterns of its P protein (Sanchez and Banerjee, 1985a;b; Luk et al., 1986). The NP, P, F and HN genes of HPIV3 have extensive homologies to their counterpart Sendai virus genes. The F genes of HPIV3 and Sendai virus show 41% overall homology and regions of much higher homology (Spriggs et al. 1986; Côté et al., manuscript submitted). The amino-terminal portion of the NP protein showed 70% homology to the NP of Sendai virus (Galinski et

al. 1986; Sanchez et al. 1986). The largest deviation between the NP gene products of the two viruses occur in the C-terminal amino acids in which there was virtually no homology (Galinski et al., 1986; Sanchez et al., 1986). The P genes of HPIV3 and Sendai virus show an overall homology of 40% (Luk et al., 1986).

Comparison of the amino acid sequences of the HN proteins of Sendai virus and HPIV3 also revealed some striking similarities. There was 43% overall homology between the HN protein sequences of the two viruses. However, there were a number of highly conserved regions with homologies of up to 78%. These highly homologous regions tend to be located towards the C-terminus of the protein. The 30 amino acids of the C-terminus showed the highest homology between the two gene products. Indeed, the C-terminal amino acids of the HN gene products of NDV, SV5 and Sendai virus also have significant homologies (Chambers et al., 1986). The conserved sequence at the C-terminus of the HN genes of HPIV3, Sendai virus, Newcastle disease virus and SV5, indicate a biologically significant role for this region.

Blumberg et al.(1985) compare the HN amino acid sequence of Sendai virus to that of the influenza virus glycoproteins. They identified 2 areas of limited but potentially significant homology between the 2 sequences. One occurs between amino acids 163-382 of Sendai virus HN and is weakly homologous to influenza neuraminidase sequences. The other occurs at amino acids 458-547 and is weakly homologous to the deduced amino acid sequence of the influenza hemagglutinin. Two regions of high homology between Sendai virus and HPIV3 HN gene products also fell into these same regions (Figure 23, region B and C). However, the homologies between the influenza virus sequences and the HPIV3 sequences were even more limited than between Sendai virus and influenza virus. Three cysteine residues are found to be

invariant between the neuraminidase genes of 3 strains of influenza and the HN of Sendai virus (Blumberg et al. 1985). These cysteine residues were also found in the same relative positions locations in the HPIV3 HN at positions 214, 269, and 355.

The positions of cysteine residues were remarkably conserved between the Sendai virus HN protein and HPIV3 HN protein. All of the HPIV3 cysteine residues occurred within 2 amino acids of their counterparts in Sendai virus HN (Figure 22). The only difference was that Sendai virus had 3 more cysteine residues at the N terminus of the amino acid sequence. It should be pointed out that there was little homology between the 90 amino acids at the N-terminus of Sendai virus HN and HPIV3 HN. Similarities in the positions of cysteine residues are also found between Sendai virus HN and the HN of SV5 (Shioda et al., 1986). Disulfide bonding occurred in the HN protein of HPIV3 (Figures 10, 11). This result, coupled with the invariant positions of the cysteine residues among the paramyxoviruses indicates that these residues play an important role in the maintenance of the three dimensional configuration of the HN protein by the formation of disulfide bonds.

There was also a close relationship in the positions of the proline residues along the backbones of Sendai virus, SV5 and HPIV3 HN proteins (Figure 22; Shioda et al., 1986). As proline residues are usually found at folds along the polypeptide backbone this correlation also suggests a similar three dimensional configuration among all the paramyxovirus HN proteins.

The deduced amino acid sequence of the HN from Sendai virus and SV5 indicates only one hydrophobic region sufficiently long to anchor the protein in the membrane (Blumberg et al., 1985; Hiebert et al., 1985a; Shioda et al., 1986). This hydrophobic region is located near the N-

terminus of the protein. The HN protein of HPIV3 also had a single highly hydrophobic region located near the N-terminus of the protein at amino acids 45-90 (Figure 21). It has been speculated that these proteins are oriented with the N-terminus inserted in the membrane like the neuraminidase protein of influenza virus (Blumberg et al., 1985; Heibert et al., 1985a; Shioda et al., 1986). Evidence for N-terminal insertion of the HN of paramyxoviruses comes from the end group analysis of the HN protein of NDV carried out by Schuy et al.(1984). They reported that the amino termini of both HN_0 and HN are blocked, but that a free amino terminus was found on a small cleavage fragment of the HN_0 protein. They also reported that HN_0 and HN proteins have different C-termini and concluded that the C-terminal end of the HN protein is exposed at the surface of the viral envelope. Therefore, the N-terminus of the NDV HN protein must be inserted in the membrane.

Regions from all six genes of HPIV3 are cloned and the HN, F, NP and P genes are sequenced. There are striking similarities between the gene products of Sendai virus and HPIV3 (Elango et al., 1986; Galinski et al., 1986; Luk et al., 1986; Sanchez et al., 1986; Spriggs et al. 1986; Côté, manuscript submitted). In this thesis it was shown that the HN of HPIV3 followed this pattern and was remarkably similar to the HN of Sendai virus. These findings imply a close evolutionary relationship between the viruses and similarities in biological functions between corresponding protein domains of the paramyxoviruses.

There are a number of factors which indicate that viral glycoproteins of enveloped viruses play important roles in virus-host cell interactions. These include their external location on the surface of virions, their involvement in adsorption and penetration of the virus and their importance

as antigens against which neutralizing antibodies are produced (Choppin and Scheid, 1980). Other viral proteins may modulate the role of the glycoproteins in virus-host cell interactions. The evidence presented in this thesis suggested that deletion of functional VSV M protein influenced the fusion activity of the G protein. In Sendai virus an interaction between the M protein and the glycoproteins must occur for the latter to attain the proper configuration in the host membrane (Heggeness et al., 1982). How this interaction might modulate the cytopathic effects caused by Sendai virus is not clear. However a role for the M protein of measles virus in the maintenance of a persistent infection and in SSPE suggests that the paramyxovirus M protein is also involved in the virulence of the virus. The similarities between Sendai virus and HPIV3 genes as documented by this study and others (Elango et al., 1986, Galinski et al., 1986; Luk et al., 1986; Sanchez et al., 1986; Spriggs et al., 1986; Côté et al., manuscript submitted) suggest that M proteins of the two viruses would behave in a similar manner. The hydropathy plot of the HN protein of HPIV3 (Figure 21) reveals a hydrophilic stretch of amino acids preceding the hydrophobic anchor sequence. While this stretch may be located, after folding, on the external surface of the membrane it is more likely to be located on the internal surface, like that of the Sendai virus HN (Lyles, 1979). It is possible that this internal portion of the HN protein may have an electrostatic interaction with the M protein. Such an interaction could conceivably alter the configuration of the HN protein on the membrane. Therefore, the M protein of both enveloped viruses (VSV and HPIV3) may modulate the virus-host cell interaction by influencing the configuration and activity of the glycoproteins on the surface of either the virion and/or the infected cell.

CHAPTER 5:BIBLIOGRAPHY

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Appendix 1: Media Formulation

DULBECCO'S MODIFIED EAGLE MEDIUM

	mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	200.00
$Fe(NO_3)_3$-$9H_2O$	0.10
KCl	400.00
KNO_3	--
$MgSO_4$ (anhyd.)	97.67
$MgSO_4$-$7H_2O$	--
NaCl	6400.00
$NaHCO_3$	--
NaH_2PO_4-H_2O	125.00
Na_2SeO_3-$5H_2O$	--
VITAMINS:	
Biotin	--
D-Ca pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-Inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Vitamin B_{12}	--

DULBECCO'S MODIFIED EAGLE MEDIUM (Continued)

AMINO ACIDS:	
L-Alanine	--
L-Arginine-H_2O	--
L-Arginine-HCL	84.00
L-Aspartic acid	--
L-Cystine	--
L-Cystine-2HCl	62.57
L-Glutamic acid	--
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl-H_2O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Proline	--
L-Serine	42.00
L-Threonine	95.00
L-Tryptophane	16.00
L-Tyrosine	--
L-Tyrosine (Disodium salt)	103.79
L-Valine	94.00
OTHER COMPONENTS:	
D-Glucose	1000.00
Phenol Red	15.00
HEPES	--
Sodium pyruvate	110.00

EARLE'S BALANCED SALT SOLUTIONS (10X)

	gms/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	2.00
$CaCl_2$-$2H_2O$	--
KCl	4.00
KH_2PO_4	--
$MgCl_2$ (anhyd.)	--
$MgCl_2$-$6H_2O$	--
$MgSO_4$ (anhyd.)	--
$MgSO_4$-$7H_2O$	0.20
NaCl	6.80
$NaHCO_3$	2.20
Na_2HPO_4	--
Na_2HPO_4-$7H_2O$	--
$NaH_2PO_4H_2O$	1.40
OTHER COMPONENTS:	
D-Glucose	1.00
Phenol red	0.01

MEM VITAMIN SOLUTIONS (100X)

	mg/L
INORGANIC SALTS:	
NaCl	8500.00
Boitin	--
D-Ca pantothenate	100.00
Choline chloride	100.00
Folic acid	100.00
i-Inositol	100.00
Nicotinamide	100.00
Pyridoxal HCl	100.00
Riboflavin	10.00
Thiamine HCl	100.00

MEM AMINO ACID SOLUTIONS (50X)

AMINO ACIDS:	mg/L
L-Arginine-HCl	6320.00
L-Cystine	1200.00
L-Glutamine	14600.00
L-Histidine	--
L-Histidine HCl-H_2O	2100.00
L-Isoleucine	2625.00
L-Leucine	2620.00
L-Lysine HCl	3625.00
L-Methionine	755.00
L-Phenylalanine	1650.00
L-Threonine	2380.00
L-Tryptophane	510.00
L-Tyrosine	1800.00
L-Valine	2340.00

Appendix 2: Concentrations of deoxynucleotide triphosphates and dideoxynucleotide triphosphates used in sequencing reactions.

Nucleotide triphosphates	Concentration ($\times 10^{-3}$ M) dATP/ddATP	dCTP/ddCTP	dGTP/ddGTP	dTTP/ddTTP
dCTP	0.062	0.004	0.082	0.082
dGTP	0.062	0.082	0.004	0.082
dTTP	0.062	0.082	0.082	0.004
ddATP	0.07			
ddCTP		0.01		
ddGTP			0.025	
ddTTP				0.25

Chase solution: 0.5×10^{-3} M dATP, dCTP, dGTP, and dTTP

LIST OF PUBLICATIONS BY D.G.S. RELATED TO THIS THESIS

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Manuscripts in Preparation

Storey, D.G., M.J. Côté, K. Dimock, and C.Y. Kang. The nucleotide sequence of the HN gene and flanking regions of human parainfluenza virus 3: comparison to other paramyxoviruses.

Storey, D.G., M.J. Côté, A. Song, M. Seligy, R. Rybeck, E. Norrby, C.Y. Kang and K. Dimock. Genetic analysis of human parainfluenza virus 3.