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MAPPING THE HÆMAGGLUTININ AND NEURAMINIDASE FUNCTIONS
OF THE HUMAN PARAINFLUENZA VIRUS TYPE 3
HÆMAGGLUTININ-NEURAMINIDASE PROTEIN

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirements for the Degree
of
Master of Science
Department of Microbiology and Immunology
School of Medicine

By

Nicola Wheatley



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ABSTRACT

The haemagglutinin-neuraminidase protein (HN) of human parainfluenza virus type 3 is a bifunctional protein. To map the functions to the protein, three truncations of the gene were constructed by digesting the HN gene with the restriction endonucleases *Hind* III, *Bgl* II or *Xba* I and inserting a stop codon. An internal deletion using *Rsa* I was also constructed. The four mutants were expressed in the recombinant vaccinia virus system. The expression of the mutant proteins was analysed by both Western Blot and immunoprecipitation. The products of the three truncations migrated at the molecular weights predicted for the truncated proteins. The product of the internal deletion migrated more quickly than predicted and upon sequencing revealed a frame shift mutation and, therefore, a fourth truncation. The migration of the gene product was consistent with the molecular weight predicted for this truncation. The full length HN was functional in both haemagglutination and neuraminidase assays. The four mutants were active only in the neuraminidase assay, none of them haemagglutinated Guinea pig erythrocytes. This allows us to predict that the haemagglutination region is near the carboxy-terminus of the protein while the neuraminidase region is amino-terminal of amino acid 212.

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DEDICATION

This thesis is dedicated to my family--my husband, M.A.S. Aquino, who married me despite it, and to my parents, Drs. B. and M.A. Wheatley, who have supported me throughout my education.

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LIST OF ABBREVIATIONS

Amp	ampicillin
BBS	BES buffered saline (see Appendix 1)
bp	base pair
BUDR	5-bromodeoxyuridine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylene diamine tetraacetic acid
HPiV3	human parainfluenza virus type 3
IgG	immunoglobulin G
Krpm	kilo revolutions per minute
λ_{em}	wavelength of light emitted
λ_{ex}	wavelength of light used to excite fluorescence
MEM	minimal essential media
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MU	methylumbelliferone
NH ₄ Ac	ammonium acetate
nm	nanometer
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline (see Appendix 1)
PEG	polyethylene glycol
RIPA	radio-immunoprecipitation assay
SDS	sodium dodecyl sulphate
TBE	Tris-borate buffer (see Appendix 1)
TBS	Tris-buffered saline (see Appendix 1)
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

INTRODUCTION

BACKGROUND

Human Parainfluenza Virus Type 3 (HPIV3) is a member of the family Paramyxoviridae, genus paramyxovirus (Chanock and McIntosh, 1990). It causes recurrent respiratory infections in infants and young children (Pringle, 1987; Welliver *et al*, 1982). These infections can sometimes be severe enough to warrant hospitalization. In particular, HPIV3 is associated with bronchiolitis and pneumonia (Pringle, 1987), and is second only to Respiratory Syncytial Virus (RSV) as a cause of respiratory morbidity in infants (Welliver *et al*, 1982). HPIV3 can also cause recurrent upper respiratory tract infections in adults and older children (Welliver *et al*, 1982; Pringle, 1987). This ability to reinfect has confounded scientists for some time and will be discussed in detail below.

The classification of the viruses of family Paramyxoviridae is shown in Table 1. HPIV3, like other paramyxoviruses, is an enveloped pleomorphic virus that contains a single stranded negative sense RNA genome (Kingsbury, 1972). The genome of HPIV3 codes for 7 proteins that have been assigned functions based on location and homology to corresponding proteins of other paramyxoviruses (Galinski *et al*, 1987; Earl *et al*, 1987; Storey and Kang, 1984; Cowley and Barry, 1983; Elango *et al*, 1986; Jambou *et al*, 1985; Spriggs and Collins, 1986; Dimock *et al*, 1987; Sanchez and Banerjee, 1985; Storey *et al*, 1984). The L (for large) protein (MW 195 K) is believed to be an RNA

Table 1: Paramyxoviridae Classification¹

Genus	Principal Viruses
Paramyxovirus	Parainfluenza virus types 1-4 (HPIV1-4)
	Sendai virus
	Simian virus type 5 (SV5)
	Mumps virus
	Newcastle disease virus (NDV)
Morbillivirus	Measles virus
	Canine distemper virus
Pneumovirus	Respiratory syncytial virus (RSV)

¹ After Pringle, 1987

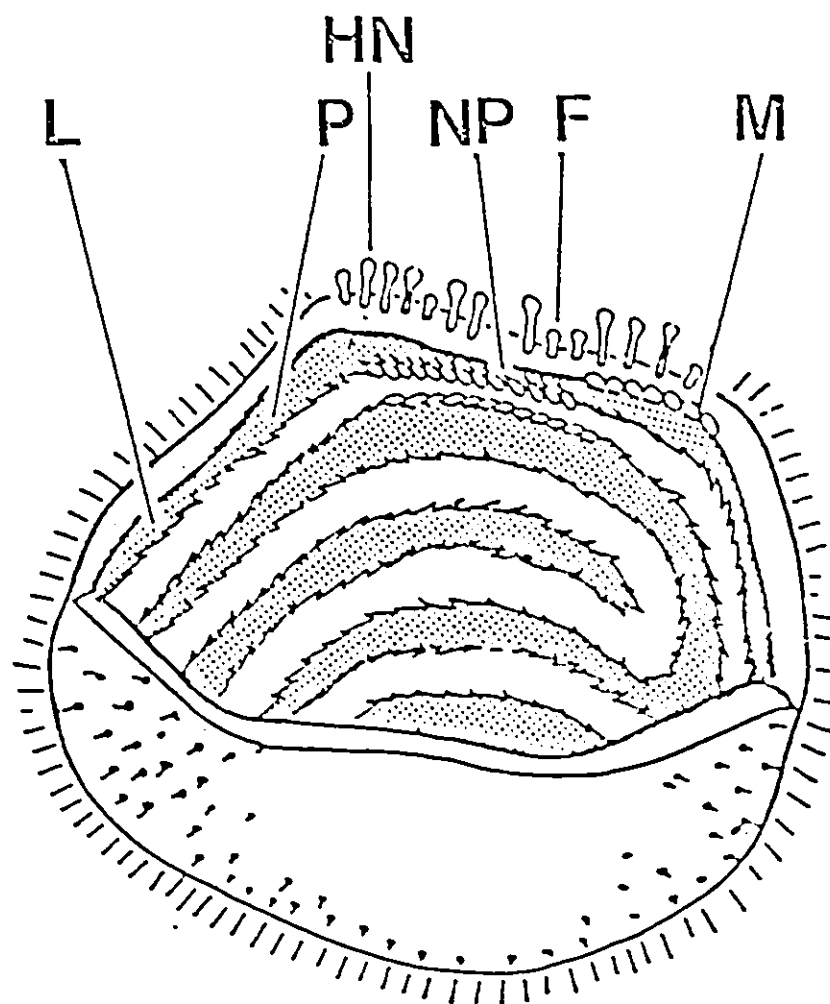
dependent RNA polymerase (Spriggs and Collins, 1986; Jambou *et al*, 1985; Elliott *et al*, 1989); the nucleocapsid protein (NP; MW 67 K) has a structural function, including encapsidation of the RNA, and a role in replication and transcription (Spriggs and Collins, 1986; Jambou *et al*, 1985; Storey *et al*, 1984); the polymerase associated protein (P; MW 87 K) functions during transcription and replication (Spriggs and Collins, 1986; Jambou *et al*, 1985; Storey *et al*, 1984; De *et al*, 1990) and C (MW 24 K) is non-structural (Spriggs and Collins, 1986) but may play a role in RNA synthesis (Vidal and Kolakofsky, 1989). The matrix protein (M; MW 35 K) is proposed to have a dual function (Jambou *et al*, 1985; Spriggs and Collins, 1986; Colman and Ward, 1985; Morrison, 1988): 1) to locate the nucleocapsid to the membrane and 2) to bind to the envelope glycoproteins—maintaining the overall structure of the virus. The two surface glycoproteins, fusion (F; MW 67K) and haemagglutinin-neuraminidase (HN; MW 69 K) (Galinski *et al*, 1987; Elango *et al*, 1986; Storey *et al*, 1987) will be discussed in detail below. The coding order is 3'-NP-P/C-M-F-HN-L-5' (Spriggs and Collins, 1986; Dimock *et al*, 1987) where the P/C region contains two alternate open reading frames, the C coding sequence overlapping the P coding region. The genomic RNA is closely associated with three proteins—NP, L and P which, along with a cellular protein component, make up the replication complex (De *et al*, 1990). Surrounding this nucleoprotein capsid is the M protein and a lipid bilayer, assimilated from the cell membrane, which contains

the two transmembrane glycoproteins (Lyles, 1979; Chanock and McIntosh, 1990). A schematic representation of these features is shown in Figure 1.

REPLICATION

Paramyxovirus replication occurs in the cytoplasm of the cell (Kingsbury, 1990). However, nuclear functions may be involved in maturation of the virus since Sendai virus infected enucleated cells do not release infectious particles (Pringle, 1987). The virus attaches to neuraminic acid residues on the cell membrane, probably through HN-receptor binding; then the fusion protein acts to fuse the viral envelope with the cell membrane, thereby releasing the nucleocapsid (NP, L, P and RNA) into the cytoplasm (Kingsbury, 1972). The L, P and NP proteins are required for RNA transcription, while replication requires ongoing NP synthesis as well (Vidal and Kolakofsky, 1989). De and co-workers have shown that a soluble cellular protein is also required for transcription (De *et al*, 1990). The RNA of the paramyxoviruses contains a 50 nucleotide leader RNA at the 3' end of the genome (Dimock *et al*, 1987) which is transcribed and is essential for transcriptase entry and function (Kingsbury, 1990). Seven subgenomic messenger RNAs are transcribed (Wechsler *et al*, 1985; Spriggs and Collins, 1986) from the genomic RNA at a frequency which decreases with distance from the 3' end (De *et al*, 1990). The ratio of the mRNAs reflects the final ratio of the proteins in the virion (Kingsbury, 1990). The mRNAs are capped, methylated and polyadenylated (De *et al*, 1990; Spriggs

Figure 1: Schematic representation of a paramyxovirus.
(After Kingsbury, 1972) NP-nucleocapsid protein, L-polymerase, P-polymerase associated protein, M-matrix protein, F-fusion protein, HN-hæmagglutinin-neuraminidase protein.



and Collins, 1986). Transcription occurs sequentially along the genome, each gene having its own initiation and termination signals. The transcriptional regulatory regions of the paramyxoviruses show considerable homology (Côté *et al*, 1987; Dimock *et al*, 1987; Spriggs and Collins, 1986; Storey *et al*, 1987), although refinement seems to have occurred within individual viruses (Chanock and McIntosh, 1990). For HPIV3, each gene ends with a stop sequence, "E", 11 nucleotides long and starting with a U. The "E" sequence is found at the end of all mRNAs immediately before the poly A tail. The poly A tail is made by polymerase "stuttering" on an oligo U region. Following the "E" region is the "T" or intergenic region, which in HPIV3, is a GAA triplet. This region is not transcribed and seems to be the true termination signal. The third region is the "S", for start region, a 10 nucleotide pyrimidine rich region which is found at the 5' end of all mRNAs, preceded by a capped G. The "T" region is the least variable of the three sequences, suggesting a dominant role in stop and start signalling. The RNAs are translated into their protein products using cellular protein synthesis systems (Kingsbury, 1990).

The switch from transcription to replication is poorly understood, but requires that the replicase read through the termination regions of the genome to form a full length anti-genome. Vidal and Kolakofsky (1989) suggest that in a minimal model the formation of a full length plus sense RNA probably requires a critical concentration of NP to catalyse the anti-termination mode. They modify this model to suggest that concurrent assembly of replicating RNA

into virion particles is required, not only for read-through but also for continuous reading, since read-through products synthesized in the absence of concurrent assembly terminate heterogeneously within a few hundred nucleotides of the start of the second gene. The antigenome can then be transcribed into genomic RNA. Both the genomic and antigenomic RNA require NP encapsidation for replication. The RNA and NP form a nucleocapsid to which is added the auxiliary proteins (L, P, and probably C) (Vidal and Kolakofsky, 1989; De *et al*, 1990). The RNP moves to the cell membrane which is lined by the M protein and contains integrated F and HN. The M protein may interact with both NP and the glycoproteins (Chanock and McIntosh, 1990). The mature virion is formed by budding through the membrane. The neuraminidase cleaves neuraminic acid residues to reduce aggregation of the virus. This may occur before budding. If F present on the cell surface has been cleaved and activated, it may cause cell to cell fusion.

GLYCOPROTEINS

Because of the roles the two surface glycoproteins, HN and F, play during initiation of viral infection and viral spread, they have been studied extensively (Blumberg *et al*, 1985; Coelingh *et al*, 1987; Jorgensen *et al*, 1987; Sakai and Shibuta, 1989; Scheid and Choppin, 1974; Shibuta *et al*, 1981; Spriggs *et al*, 1987; Thompson and Portner, 1987; Tuffereau *et al*, 1985; Ray *et al*, 1988a; Ray *et al*, 1988b; Paterson *et al*, 1987) as summarized by Morrison (1988) (also,

Schneider *et al*, 1989; Roux, 1990). The fusion protein is responsible not only for fusion of the viral envelope and the cell membrane during infection, with the subsequent release of the nucleocapsid into the cytoplasm, but also for cell to cell spread of the virus by formation of syncytia (Chanock and McIntosh, 1990). The F protein of HPIV3 is synthesized as an inactive precursor, F_0 , of 539 amino acids which is cleaved between residues 109 and 110 by a cellular protease, with trypsin-like specificity, to yield two subunits (F_1 and F_2) linked by a disulphide bond (Chanock and McIntosh, 1990). F has four potential glycosylation sites, although only three are likely used (the fourth is in a trans-membrane region). The positions of glycosylation sites, as well as 9 of 11 cysteines, are well conserved among the paramyxovirus F proteins (Morrison, 1988). Also well conserved are 3 hydrophobic domains. The C-terminal amino acid residues 404-516 constitute a transmembrane region and are part of F_1 after cleavage. The second hydrophobic region becomes the new N-terminus of F_1 (amino acids 110-135) after cleavage and is believed to be the fusion domain. Fifteen out of twenty six amino acids in the fusion peptide are conserved among the Paramyxoviridae, with the exception of the pneumoviruses. The final hydrophobic domain in the HPIV3 F protein is the N terminal 18 amino acid signal sequence, which is cleaved during translocation. The F_2 subunit has a predicted MW of 13 K and contains the original N-terminus minus the signal sequence, it remains bound to F_1 by a disulphide bond and may play a role in stabilizing the three dimensional

structure of the fusion protein. Cleavage is necessary for function and occurs following an arginine residue in all paramyxovirus F proteins. The region preceding this basic residue has a varying number of basic amino acids (from 1 in Sendai to 6 in canine distemper virus) (Morrison, 1988). The sequence in HPIV3 is Asp-Pro-Arg-Thr-Lys-Arg. The time of cleavage appears to vary among the paramyxoviruses, the NDV F protein is cleaved in the trans Golgi while the Sendai virus F protein is not cleaved until after it reaches the cell surface (Chanock and McIntosh, 1990). Morrison (1988) suggests that cleavage time may be affected by the number of basic residues preceding the cleavage site. Also, during transport from the rough endoplasmic reticulum to the cell surface, the protein undergoes N-linked glycosylation and conformational changes, possibly due to disulphide bond organization (Mottet *et al*, 1986; Vidal *et al*, 1989). F does not form covalently linked oligomers, however, the Sendai F protein has been shown to form non-covalently linked tetramers (Sechoy *et al*, 1987; Welling *et al*, 1987).

The other surface glycoprotein, HN, is bifunctional having both haemagglutination (HA) ability and neuraminidase (NA) or sialic acid cleavage activity. In addition, HN may play a role in the functioning of the F protein (as discussed below). The binding of sialic acid residues by the HA portion is presumed to be responsible for primary binding of the virus to the cell surface prior to fusion and infection. The neuraminidase activity may function during viral release from the cell by budding, cleaving the sialic acid residues to reduce

viral aggregation. The exact location of the active sites and studies determining them will be discussed below. It should be noted that not all genera of the family Paramyxoviridae have a bifunctional attachment protein. The viruses of the genus paramyxovirus have HN, the viruses of the genus morbillivirus have a protein exhibiting only HA activity and viruses of the genus pneumovirus have an attachment protein which has neither HA nor NA activity. Also, viruses from the family Orthomyxoviridae (eg. Influenza) have both HA and NA but on separate proteins. Sakai and Shibuta (1989) have shown that a functional HN is required for Fusion protein activity in Bovine PIV3, a result confirmed in our laboratory for HPIV3 (Ebata *et al*, 1991).

IMMUNE RESPONSE TO GLYCOPROTEINS

The surface glycoproteins, F and HN, not only play a significant role during infection and replication, they are also responsible for eliciting a neutralizing antibody response in individuals infected by HPIV3 or other paramyxoviruses. Several groups have tested the role that antibodies to the glycoproteins play in protection against paramyxovirus infection (Ray *et al*, 1988b; Coelingh *et al*, 1987; Paterson *et al*, 1987; Rydbeck *et al*, 1988; Spriggs *et al*, 1987). Similar results were obtained with passive immunization (Rydbeck *et al*, 1988) or if purified protein (Ray *et al*, 1988a; Ray *et al*, 1988b; Ray *et al*, 1985), recombinant vaccinia virus (Spriggs *et al*, 1987; Paterson *et al*, 1987), or cells infected with recombinant baculovirus (Coelingh *et al*, 1987) were used

to stimulate an immune response. Ray *et al* (Ray *et al*, 1985; Ray *et al*, 1988b; Ray *et al*, 1988a) used purified glycoproteins from HPIV3 as a subunit vaccine and showed that only when both glycoproteins were present in the vaccination of hamsters was there complete protection from subsequent challenge with HPIV3. The individual glycoproteins were only partially protective (Ray *et al*, 1988a). They also showed (Ray *et al*, 1988b) that intranasal immunization was more efficacious than subcutaneous inoculation, resulting in elevated IgA levels in bronchial lavage fluid. The IgA response to HN was greater than to F.

Spriggs *et al* (1987) used recombinant vaccinia virus expressing F or HN to vaccinate cotton rats intradermally and showed vaccination with vHN to be more protective than vaccination with vF. Vaccination with vF resulted in 3-fold less serum neutralizing antibody than vHN and 500-fold less reduction of HPIV3 in the upper respiratory tract upon subsequent challenge. Both recombinant vaccinia viruses were equally, almost completely, protective for the lower respiratory tract. Spriggs suggests immunity to HPIV3 is complex and that other immune mechanisms may well be involved (Spriggs *et al*, 1987). Use of recombinant vaccinia virus encoding the F and HN glycoproteins of SV5 produced essentially the same results (Paterson *et al*, 1987).

HPIV3 HN in recombinant baculovirus results in a protective immune response, decreasing HPIV3 replication upon subsequent challenge 100-fold in the upper respiratory tract and almost completely in the lower respiratory tract. Serum antibodies produced by vaccination with cells infected with recombinant

baculovirus were neutralizing but less so than antibodies raised to live HPIV3 intranasal immunization (Coelingh *et al*, 1987). Baculovirus producing F of HPIV3 has also been shown to be immunogenic and protective upon challenge of immunized hamsters with HPIV3 (Ray *et al*, 1989).

Passive immunization of newborn hamsters with monoclonal antibodies (MAb) against F or HN was partially protective against HPIV3 induced meningitis. The amount of protection was directly related to ability of the MAb to neutralize HPIV3 *in vitro*, with MAbs showing the greatest neutralization titre giving the greatest protection and non-neutralizing MAbs being non-protective (Rydbeck *et al*, 1988). Combined treatment with anti-F and anti-HN MAbs was not tested.

To summarize, antibodies to both surface glycoproteins can be protective *in vivo* (Ray *et al*, 1988b; Coelingh *et al*, 1987; Paterson *et al*, 1987; Rydbeck *et al*, 1988; Spriggs *et al*, 1987) and neutralizing *in vitro* (Rydbeck *et al*, 1988; Spriggs *et al*, 1987; Coelingh *et al*, 1987). However, *in vivo* the immune response to parainfluenza or the pneumoviruses does not appear to be sufficient to protect from subsequent infections (Coelingh *et al*, 1990). This is evidenced by the occurrence of reinfection and infection at a time when maternal antibodies are present in infants. It is also in direct contrast to some other paramyxoviruses (measles, mumps) where a self-limiting disease usually results in life long immunity (Chanock and McIntosh, 1990). Coelingh and co-workers (1990) have shown that there are several reasons for the poor

immunoprotection. First, it appears that the titre of secreted IgA in the respiratory tract is more important for protection than serum antibody titre (Coelingh *et al*, 1990; Ray *et al*, 1988b). However, serum antibodies are partially protective since infants with high maternal circulating antibodies show less illness than those without; also, the severity of illness is decreased upon reinfection. Secondly, more than one exposure to HPIV3 appears to be required for protection from significant morbidity. Antibodies to HN appear on initial infection with HPIV3, but they can protect solely against initiation of infection—viral adsorption; anti-F antibodies are required to protect from cell to cell spread by fusion mediated cell fusion and syncytium formation. Unfortunately, anti-F antibody titres reach protective levels only after repeated exposure to HPIV3 (Coelingh *et al*, 1990).

HÆMAGGLUTININ-NEURAMINIDASE

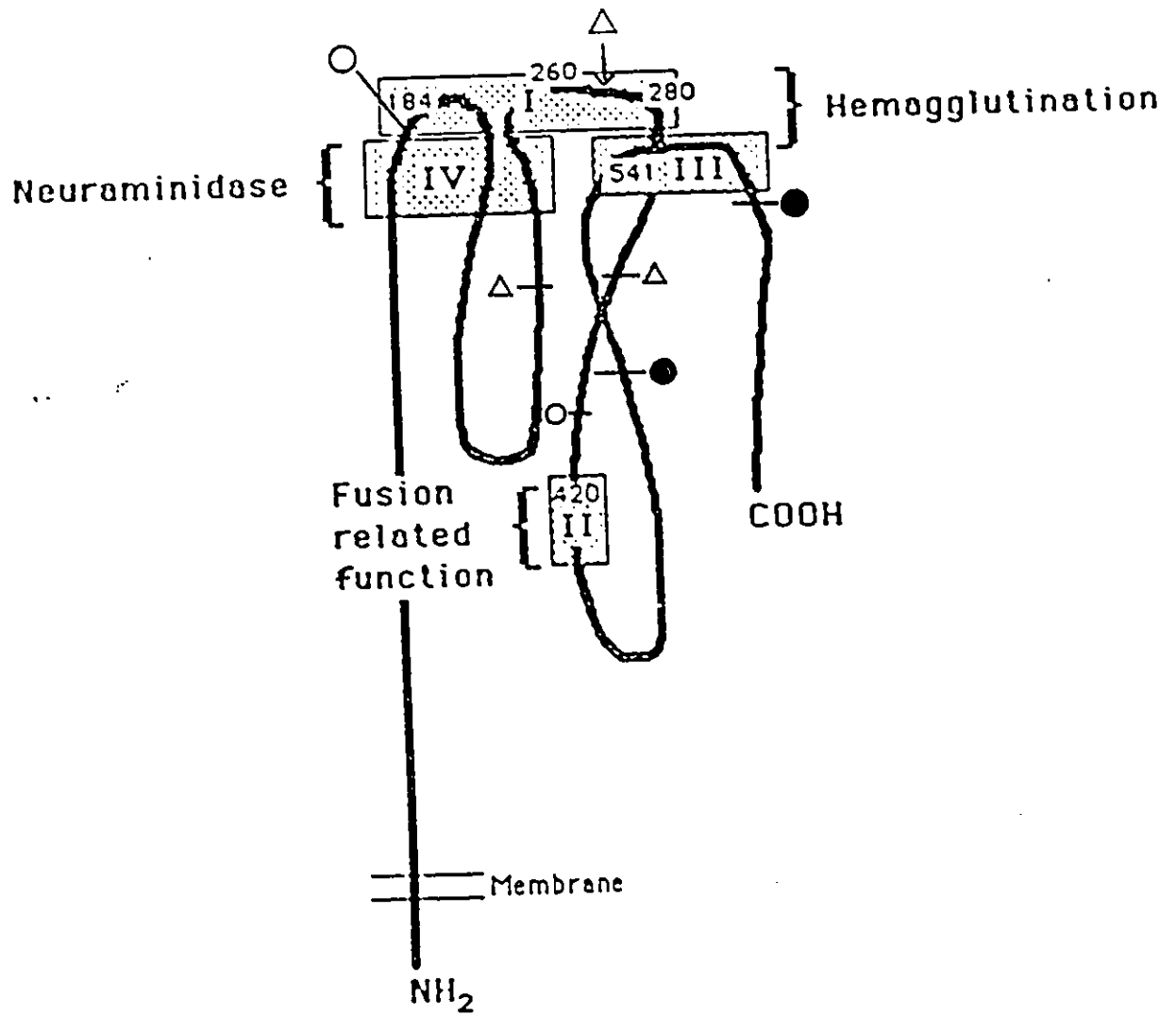
As noted above, the hæmagglutinin-neuraminidase protein is bi- and possibly tri-functional and has a MW of 69 K. Coelingh *et al* (1988) have shown that the HN protein consists of 3 domains. The N terminus, consisting of amino acid residues 1-90 has a hydrophilic cytoplasmic portion, and a hydrophobic transmembrane region (aa 32-53) leading into a helical stalk region. The second domain (residues 91-509) is well conserved among the paramyxoviruses and contains all the glycosylation sites and putative functional regions (Coelingh *et al*, 1988). Jorgensen (1987) has predicted that part of this domain (aa 234-323)

has a β -sheet-loop structure. The C terminal 63 amino acids (residues 510-572) make up the third domain which has a helical structure (Coelingh *et al*, 1988). The question is: How many active sites are present on an HN molecule, and where are they?

The attempts to map the functional regions to the protein have taken 2 routes: sequence homology comparisons and monoclonal antibody (MAb) mapping. A composite diagram of the resulting locations of the functional regions is shown in Figure 2. Jorgensen (1987) sequenced the HN gene of NDV and compared it to the HN genes of Sendai virus, SV5 and HPIV3 in an attempt to identify conserved structural features. She then compared these regions to neuraminidase (NA) sequences of influenza A. Jorgensen found that 10 of 12 cysteines are invariant among the four paramyxovirus HN proteins, suggesting their importance for protein integrity, but that glycosylation sites were not conserved. The region corresponding to HPIV3 amino acids 234-323 has limited sequence homology among the four viruses, although one stretch of 6 amino acids (265-270) is perfectly conserved. However, all four proteins show similar hydrophobicity and structure in this region, that is β -sheet characteristics (by Garnier analysis) joined by loops. Both the HA and NA of influenza have been studied by X-ray crystallography [(Weis *et al*, 1988; Wilson *et al*, 1981) and (Varghese *et al*, 1983; Colman *et al*, 1983) respectively] and the structures of the proteins predicted. Colman (1983) has shown NA to consist of β -sheet-loop structures surrounding a highly conserved catalytic pocket; the surface

Figure 2: Predicted locations of functional sites on the hæmagglutinin-neuraminidase protein of Sendai virus.

The diagram is modified from Thompson and Portner (1987), who identified conformational regions on the Sendai virus protein corresponding to hemagglutination (I and III) and neuraminidase (IV) activity. Although the exact location of region IV was not confirmed it was shown to be close to region I. Region II was identified as being essential for the function of the fusion protein. The results of Blumberg (1985) and Jorgensen (1987) are superimposed on the diagram and represent linear regions predicted from sequence comparisons, as discussed in the text. Blumberg predicts amino acids 163-382, bounded by open circles (○), to be involved in neuraminidase activity and amino acids 458-547 (equivalent to 477-567 of the HPIV3 HN protein), bounded by closed circles (●) to be involved in hæmagglutinin activity. Jorgensen predicts amino acids 234-323, bounded by open triangles (△) to be involved in neuraminidase activity and specifically identifies amino acids 265-270 as a putative sialic acid binding region, indicated by the arrow and open triangle.



loops are antigenically variable. Weis (1988) predicted a similar structure for HA, with antigenically variable regions surrounding a conserved active pocket. When HA was crystallized in the presence of sialyllactose, a trisaccharide receptor analog, the HA pocket was filled by the monosaccharide sialic acid—proving that this is the receptor for influenza. When the HN of HPIV3 region aa 234-323 is compared to influenza NA amino acids 208-287 (catalytic site) the structural similarities are striking (Jorgensen *et al*, 1987). Jorgensen concludes that this region is important for HN function and probably represents the sialic acid binding region of the enzymatic site of NA. She found no significant similarities to the haemagglutinin of influenza. Blumberg (1985) had previously attempted to compare Sendai HN to both the HA and NA of influenza types A, B and C. He found "weak but probably significant homology" between region 458-547 of Sendai HN and the influenza haemagglutinins, with the greatest similarity in regions where the influenza sequences were conserved. His comparison to influenza neuraminidases was "statistically indecisive" but he identified a region (aa 163-382) of HN which he felt, on other criteria showed similarities to influenza NA. These "other criteria" included cysteine location, potential for β -sheet structure and correlation of charged residues. Taken together, these two studies suggest that the NA region is at the approximate centre of the protein and HA may be towards the carboxy-terminus. The localization of the functions to these two regions is further supported by Kōvamees (1989) and McGinnes (1987) who have sequenced mumps and NDV

HN genes, respectively, and found similar clusters of conserved regions when compared to the other paramyxoviruses.

Monoclonal antibody mapping of the functional domains has yielded conflicting results. Thompson and Portner (1987) have found four antigenic regions on Sendai HN. Antibodies to sites I and III inhibit HA, to site II inhibit haemolysis by F and to site IV inhibit NA. That is, HA and NA can be selectively inhibited. Coelingh (Coelingh *et al*, 1987), with HPIV3, found 6 antigenic regions. Antibodies to three of which (A,B,C) had HA inhibiting ability and antibodies to three others (D,E,F) had no effect. However, several of the antibodies to region A which inhibited HA also inhibited NA. Neuraminidase could not be selectively inhibited. In both cases, when these MAbs were used to select escape mutants in culture, the resulting viruses were viable and had functional HN proteins. The predicted sequences of the escape mutant HN proteins show single amino acid changes which are spread through out the protein (Coelingh *et al*, 1986; Coelingh *et al*, 1987). Coelingh (1987) did find some clustering of changes around amino acids 350-400 but she found no correlation between a specific change and function. In all likelihood HN is designed like NA and HA of influenza, in which the conserved residues of the active site are surrounded by hypervariable regions (Colman and Ward, 1985; Thompson and Portner, 1987). One problem with monoclonal mapping experiments is that HN is only 67 Kd while the average IgG molecule is 200 Kd.

This raises a question of steric interference rather than true selective inhibition of activity.

The only mutant work on HN comes from Portner (1981), who has a temperature sensitive (ts) mutant of Sendai virus that is ts for HA but not NA. Sequencing has identified three amino acid changes at 262, 264 and 461 (Thompson and Portner, 1987). The fact that the mutation only affects one of the two functions demonstrated that there are distinct sites on the protein for HA and NA. In addition, he tested an inhibitory analog of neuraminic acid (2-deoxy,2,3-dehydro-N-acetyl neuraminic acid) against NDV and Sendai virus. The analog inhibited NA at 10^{-4} M but did not inhibit HA even at 100 fold greater concentration. While this may only indicate one site with different affinities, he was able to demonstrate that the analog selectively inhibited virus elution from erythrocytes but did not inhibit adsorption. Unfortunately, no one has repeated these results with other paramyxoviruses.

OBJECTIVES

When this project was initiated, no one had tried to mutate the HN gene of any paramyxovirus and look at protein function. Air *et al* (1987) have used site directed mutagenesis on influenza NA (type 2) and shown that any change to the active site resulted in total loss of activity. The information presented in the last section, while suggestive, does not provide a clear picture of HN structure-function relationship. It was decided, therefore, to study the functional

domains of HN by more direct means, through the use of deletion mutants. Since the literature has not pinpointed amino acids important in function, three truncations and one internal deletion were chosen for initial study. The internal deletion removes amino acids 236-290, which is a large proportion of the putative sialic acid binding region identified by Jorgensen (1987).

The objectives of the project were:

- 1) To express a functional HPIV3 HN using vaccinia virus as a vector
- 2) To optimize functional assays for neuraminidase and haemagglutinin
- 3) To construct truncation/deletion mutants of the HN gene
- 4) To express the mutant HN proteins using vaccinia virus as a vector
- 5) To analyse mutant HN genes for changes in activity.

Although Ballart and co-workers (1990) have recently created infectious measles from cDNA, studies of negative sense RNA viruses still require indirect study. Several expression systems are available and have been used to express HN, including baculovirus (Coelingh *et al*, 1987), transfection, under SV40 control (Paterson *et al*, 1985), SV40 recombinant virus (Spriggs and Collins, 1990) and vaccinia virus (Sakai and Shibuta, 1989; Spriggs *et al*, 1987). A vaccinia virus vector was chosen because it replicates in the cytoplasm (Moss

and Flexner, 1987; Mackett and Smith, 1986), avoiding potential problems of RNA splicing that might occur if the mRNA was produced in the nucleus. Since the project was initiated other researchers have shown that this is not a problem for paramyxoviruses (Paterson *et al*, 1985; Spriggs and Collins, 1990; Coelingh *et al*, 1987). Vaccinia virus also carries its own replication system. The expression system has been well characterized (Mackett *et al*, 1985; Moss and Flexner, 1987; Chakrabarti *et al*, 1985; Mackett and Smith, 1986) and the shuttle vector (Chakrabarti *et al*, 1985) and wild type virus are readily available. The vector we chose allowed for easy screening because insertion of foreign DNA into the non-essential thymidine kinase gene resulted in virus with a TK phenotype. It also contained the β -galactosidase gene, allowing for double selection/ detection (Chakrabarti *et al*, 1985). After this project was initiated, 2 papers were published using the recombinant vaccinia virus system to express HN and F of HPIV3 (Spriggs *et al*, 1987) and SV5 (Paterson *et al*, 1987). The proteins were appropriately glycosylated and co-migrated with native HN and F under both reducing and non-reducing conditions. The proteins were expressed at the cell surface and HN was functional in an assay for HA (Spriggs *et al*, 1987). F of SV5 was also functional in a Fusion assay (Paterson *et al*, 1987). M.J. Côté, in our laboratory, also successfully expressed F and HN of HPIV3 in recombinant vaccinia virus, but was unable to show function for F and did not assay HN activity (Côté, 1989).

MATERIALS AND METHODS

BUFFERS AND SOLUTIONS

The recipes for buffers and solutions named in the text can be found in Appendix 1.

1-1 RECOMBINANT DNA TECHNIQUES

i) RESTRICTION ENDONUCLEASE DIGESTION

Enzymes were usually obtained from Pharmacia or New England Biolabs. An aliquot of DNA, usually 1 μ g, was digested in a total volume of 20 μ l following manufacturers' recommendations for buffer. A five-fold excess of enzyme was routinely used in a 1 hour incubation. Digests of mini-prep DNA also included 5 μ g of DNase-free RNase A to digest contaminating RNA. Greater quantities of DNA were digested in appropriately larger volumes.

ii) FILL IN OF 5' OVERHANGS

After restriction endonuclease digestion, the restriction enzyme was heat inactivated at 65°C and the DNA was precipitated with ethanol by adding 1/2 volume of 7.5 M NH_4Ac and 2 volumes of ethanol at -20°C. The pellet was resuspended in water, at 1 μ g/20 μ l, and 10X Nick Translation buffer (1/10 volume), dNTPs (2 nmol of each nucleotide per μ g DNA) and the Klenow fragment of DNA Polymerase I (1 U/ μ g) were added. The mixture was

incubated for 30 minutes at room temperature. Agarose gel electrophoresis was used to separate the DNA from the unincorporated nucleotides.

iii) DEPHOSPHORYLATION OF VECTORS

After restriction endonuclease digestion, vector DNA was dephosphorylated using Calf Intestinal Phosphatase (CIP). The restriction enzyme was heat inactivated and the DNA precipitated with ethanol. The pellet was resuspended in water and 10X CIP buffer (1/10 volume) and 1 U CIP per μg DNA were added. The mixture was incubated for 30 minutes at 37°C. Samples were then electrophoresed on agarose gels.

iv) AGAROSE GEL ELECTROPHORESIS

Agarose gels (0.8% or 1.0%) were run in the Hoefer Scientific Instruments MINNIE or MAX Submarine Gel Units using TBE as the running buffer. Gels were electrophoresed at 50 volts for 2 hours (MINNIE) and 100 volts for 2-3 hours or at 35 volts overnight (MAX).

v) ELECTROELUTION

After electrophoresis and staining with ethidium bromide (5 $\mu\text{g}/\text{ml}$ for 15 minutes) DNA bands were cut out of gels under long wave UV light (to avoid thymidine-dimerization). The agarose fragment was placed in the cup of an IBI Unidirectional Electroelutor and the apparatus was filled with 450-500 ml of

buffer. The V channel was filled from the angled side with 125 μ l of 7.5 M NH_4Ac , 0.01% Bromophenol Blue. Electroelution was carried out at 100 V for 1 hour.

The salt cushion was removed from the straight channel in approximately 300 μ l, using a small piece of Intermedic tubing attached to the end of a yellow micropipette tip. The sample was extracted twice with water-saturated butanol and 2 volumes of ethanol were added (with or without 2 μ g of glycogen as a carrier). The DNA was allowed to precipitate for 10-15 minutes on ice or overnight at -20°C then collected by centrifugation. The DNA was precipitated once more before use.

vi) PHOSPHORYLATION OF OLIGONUCLEOTIDES

Prior to ligation, oligonucleotides were phosphorylated using polynucleotide kinase (PNK). A mixture of 1 μ g of oligonucleotide, PNK buffer and 20 U PNK were incubated in a total volume of 10 μ l at 37°C for 1 hour. The samples were heated at 65°C for 10 minutes to inactivate the kinase, then allowed to cool to room temperature to anneal. Since the oligonucleotides were palindromic, they could anneal to each other.

vii) LIGATION

Sticky- and blunt-end ligations were carried out in 10-20 μ l containing 1X ligase buffer. Sticky end ligations were performed using a DNA concentration

of 5-20 $\mu\text{g}/\text{ml}$ and 0.5 Weiss U/ ml of DNA ligase at 16°C for 16-20 hours. Blunt end ligations used 50 $\mu\text{g}/\text{ml}$ DNA and 100 Weiss U/ ml of DNA ligase and were incubated at 16°C for 16-20 hours.

I-2 TRANSFORMATION OF BACTERIAL CELLS

i) COMPETENT CELLS

A single colony of E. coli RR1 was used to inoculate 10 ml of 2YT and the culture was grown overnight, without shaking, at 37°C . The entire overnight culture was then used to inoculate 100 ml of P-Medium and incubated at 37°C , with shaking at 200 rpm, until an A_{600} of 0.3-0.4 was reached. The culture was cooled on ice then centrifuged at 4 Krpm, 4°C for 10 minutes in a Beckman JA20 rotor. The cells were washed with 100 ml of 10 mM NaCl at 4°C , centrifuged as above, then resuspended in 50 ml of 50 mM CaCl_2 and incubated on ice for 15 minutes. The cells were centrifuged again and the pellet was resuspended in 10 ml of 50 mM CaCl_2 , 16% glycerol. The cells were aliquoted (200 μl /tube) and flash frozen in a dry ice/ethanol bath then stored at -80°C .

ii) TRANSFORMATION PROCEDURE

For transformation of E. coli, an aliquot of competent cells was thawed on ice for 15-20 minutes and 10 μl of DNA (20-200 ng) was added. The DNA-cell mixture was incubated on ice for 30 minutes then heat shocked for 3 minutes at 42°C . The cells were then cooled on ice and 800 μl of fresh Luria

Broth (LB) was added. The cells were allowed to recover by incubating at 37°C for one hour with shaking (200 rpm). The cells were then plated, by spreading with a sterile glass rod, on LB plates containing 50 µg/ml ampicillin. Typically, 1, 10 and 100 µl of the cells were plated, then the remaining cells were pelleted and the pellet was resuspended in 100 µl and plated. The plates were inverted and incubated overnight at 37°C. Efficiency of transformation was calculated using 20 ng of uncut pIBI plasmid as the control DNA.

iii) SMALL SCALE PLASMID PREPARATION (MINI-PREP)

The small scale plasmid preparation ("mini-prep") was an alkaline lysis method as modified by Morelles (1989). Single colonies of transformed E. coli were inoculated into 5 ml LB + Amp (50 µg/ml) in 50 ml Erlenmeyer flasks and grown with agitation (200 rpm) at 37°C overnight. Bacteria from 1.5 ml were pelleted at 10 Krpm for 30 seconds in a microfuge and the medium was aspirated. The pellet was resuspended in 200 µl of Solution I containing 4 mg/ml lysozyme. After incubation for 5 minutes at room temperature, 400 µl of Solution II was added, the samples were mixed and incubated for a further 5 minutes. Then 300 µl of 7.5 M NH₄Ac was added and the mixture was incubated on ice for 10 minutes. The proteinaceous material and debris were removed by centrifugation at 10 Krpm for 3 minutes. The supernatant was removed to a fresh tube and 500 µl of isopropanol was added. After incubation at room temperature for 10 minutes the DNA was recovered by centrifugation

at 14 Krpm for 10 minutes at 4°C. The supernatant was aspirated and the pellet washed with 70% ethanol. After drying, the pellet was resuspended in 100 μ l TE. Any particulate matter was removed by centrifugation at 10 Krpm for 2 minutes. Restriction endonuclease digests were then performed using 5 μ l aliquots of the DNA.

iv) SEQUENCING PREPARATION

For sequencing, the mini-prep DNA was further purified as follows. RNase A was added to a final concentration of 20 μ g/ml and each sample was incubated at 37°C for 30 minutes. Each sample was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated by the addition of 50 μ l of 7.5 M NH_4Ac and 300 μ l of ethanol at -20°C. After 20 minutes on ice DNA was recovered by centrifugation and the pellet was resuspended in 16 μ l water to which 4 μ l 4 M NaCl and 20 μ l 13% PEG were added. The mixture was incubated on ice for 20 minutes then the precipitate was collected by centrifugation at 14 Krpm for 15 minutes. The pellet was resuspended in 20 μ l of water and reprecipitated with NH_4Ac and ethanol. The pellet was resuspended in 35 μ l of water and 7 μ l was used for sequencing reactions. Before sequencing the DNA was denatured by adding NaOH to a final concentration of 0.2 M and incubating at room temperature for 5 minutes. The solution was neutralized by the addition of 5

M NH_4Ac . Ethanol was then added and the DNA was precipitated overnight at -20°C.

v) SEQUENCING

The Sequenase Kit from United States Biochemical Corporation was used according to the manufacturer's directions. Some sequences were also confirmed using the Dupont Genesis 2000 DNA Analysis System (kindly performed by D. McLean).

Samples were heated to 95°C before loading onto an 8% acrylamide, 8 M urea gel prepared in 1.2X TBE. A running buffer gradient, 1X TBE in the upper chamber and 1.5X TBE in the lower, was used to optimize reading. The gel was electrophoresed at 50 W until the bromophenol blue marker was at the bottom of the gel.

vi) LARGE SCALE DNA PREPARATION (Maxi-prep)

The Maxi-prep was essentially a scaled up version of the mini-prep. A 200 μl aliquot of an overnight culture was used to inoculate 25 ml of LB + Amp. The culture was grown for 2-3 hours and was then used to inoculate 1 litre of LB + Amp. After 3-4 hours, 170 $\mu\text{g}/\text{ml}$ Chloramphenicol was added to amplify the plasmid DNA and the incubation was continued overnight. Cells were harvested by centrifugation in the Beckman JA10 rotor, at 6 Krpm, for 10 minutes at 4°C. Lysis was carried out as described in the mini-prep protocol

using 6 ml of Solution I, 12 ml of Solution II and 9 ml of 7.5 M NH_4Ac . Debris was removed by centrifugation either in the Beckman SW28 rotor for 25 min at 20 Krpm or in the JA20 rotor at 19 Krpm for 30 min. DNA was precipitated from supernatants using 10 ml of isopropanol. Pellets were resuspended in 4.2 ml of TE then 1.1 g/ml CsCl and 0.8 mg/ml ethidium bromide were added. A clearing spin of 10 Krpm, 10 minutes, 4°C in the JA20 was performed and the supernatants were transferred to 5 ml quick sealing tubes. Centrifugation was done overnight in a Beckman VTi 65 rotor at 55 Krpm. Plasmid bands were collected using 22 gauge needles and 1 or 3 ml syringes and transferred to polystyrene tubes. The ethidium bromide was removed by repeated extraction with water-saturated butanol. The aqueous phase was made up to 5 ml, transferred to cold corex tubes and DNA was precipitated by the addition of 10 ml of ethanol at -20°C . After centrifugation, pellets were resuspended in TE and precipitated twice from NH_4Ac with ethanol. The final precipitates were resuspended in water and the A_{260} of each sample was determined. The concentration of DNA was adjusted to 1 mg/ml, then aliquots were stored at -20°C .

II TISSUE CULTURE

The following cell lines were used: CV1 (African green monkey kidney; from the American Type Culture Collection, Rockville, MD); 143B (human osteosarcoma thymidine kinase negative cell line; from Dr. J. Campione-Piccardo

at the Laboratory Centre for Disease Control (LCDC), Ottawa). Cells were grown in Gibco Autoclavable MEM (catalogue #410-1700ED) supplemented with 2 mM Glutamine, 0.25% Sodium Bicarbonate, 50 µg/ml Gentamycin and either 5% (CV1 cells) or 10% (143B cells) foetal bovine serum (FBS). Cell cultures were incubated at 37° in a 5% CO₂ atmosphere in a Shell-Lab incubator.

When a plate reached confluency the cells were passaged as follows. The medium was aspirated and the cells were washed twice with TBS warmed to 37°C. Trypsin-EDTA (0.05%, 0.53 mM) was then added and the plate was incubated at 37°C for 3-4 minutes. The trypsin was inactivated by the addition of 9 ml of fresh medium, warmed to 37°C, which was used to resuspend the cells. The appropriate volume (eg. 1 ml per plate for a 1:10 split) of resuspended cells was then added to plates containing fresh medium and the cells were returned to the incubator for growth.

i) FREEZING

For freezing, cells were removed from plates with trypsin, as described above, then pelleted at 1500 rpm in a Sorvall GLC-2B bench top centrifuge. The cells were resuspended in 1 ml of freezing medium per plate. The cells were frozen progressively at -20°C for 1 hour, -80°C overnight then stored in liquid nitrogen.

ii) RESURRECTION

An aliquot of frozen cells was warmed in a 37°C water bath then separated onto 2 plates containing fresh medium. After the cells were allowed to settle (several hours to overnight at 37°C) the medium was changed to remove the cryoprotectant. The cells were grown to confluency and passaged as described above.

iii) INFECTION

For viral infection, medium was aspirated and replaced with 1 ml of serum free medium containing an appropriate quantity of virus. If a large number of plates were being infected with the same virus, the virus was diluted in an appropriate volume of medium and 1 ml was added to each plate. The plates were then incubated at 37°C, for 1 hour with occasional rocking. The medium was then removed and replaced with 10 ml of fresh medium and the plates were incubated at 37°C for 12-48 hours.

Cells were infected with HPiV3 at a multiplicity of infection (MOI) of 3-5 PFU per cell and incubated for 24 or 48 hours. Infection with vaccinia virus was at a MOI of 1 to 10 PFU per cell and infection was allowed to proceed for 8 to 16 hours. Vaccinia virus was incubated at 37°C with 0.05% trypsin for 15 minutes prior to infection to reduce viral aggregation.

iv) TRANSFECTION FOR PRODUCTION OF RECOMBINANT VIRUS

After infection of CV1 cells at a MOI of 0.1 PFU/cell with the WR wild type strain of vaccinia virus, cells were transfected with pSC11-HN recombinant plasmids. DNA (20 µg) was mixed with 0.25 M CaCl in 500 µl then added to an equal volume of 2X BBS and incubated at room temperature for 10-20 minutes. The mixture was added, dropwise, to a plate containing 10 ml of MEM. After an 8 hour incubation at 37°C, the plates were washed with MEM and the medium replaced. The plates were then incubated until CPE was observed, usually 48 hours. Virus was harvested by scraping the infected cells from the plates using a rubber policeman. The cells were pelleted, washed and resuspended in serum free medium. Virus was released from the cells by three rounds of freezing and thawing.

v) PLAQUE ASSAY and PLAQUE PURIFICATION

Serial dilutions of virus were done in serum free medium and infections were performed as above except that the plates were overlaid with 0.8% Agarose, 1X MEM and incubated for 2 (vaccinia) to 4 (HPIV3) days. The overlay for 143B cells included 25 µg/ml bromo-deoxy uridine (BUdR) and 10% FBS. Vaccinia plaques were picked for purification following a second overlay with agarose containing 300 µg/ml X-gal. Blue colour was allowed to develop for 6-8 hours. Blue plaques could then easily be identified and were picked using a sterile Pasteur pipette. Vaccinia plaques picked in this manner were

dissociated in 500 μ l of serum free medium by 3 rounds of freezing, thawing and vortexing.

When titrating virus, cells were fixed with 10% formal saline for 1 hour and stained with 0.01% crystal violet. After washing and drying the plates, plaques could easily be counted.

vi) CELL LYSIS

Cells were grown and infected as required, scraped from plates using a rubber policeman and transferred into a centrifuge tube. The cells were pelleted, washed once with TBS and transferred into microfuge tubes. The washed pellets were resuspended in ice cold RIPA buffer. An equal volume of RIPA containing 2X detergent was added, the contents were immediately mixed and nuclei and debris were removed by centrifugation. The supernatant was quickly removed to a fresh tube and maintained on ice.

vii) SDS-PAGE

Polyacrylamide gels were run in the Bio-Rad Mini-Protean or Protean II systems which were used according to the manufacturer's directions. A resolving gel (10 or 12.5% polyacrylamide in 0.75 M Tris-HCl, pH 8.8; 1% SDS) was poured, overlayed with water-saturated butanol and allowed to polymerize. The butanol was then removed and a 5% stacking gel (67 mM Tris-HCl, pH 6.8; 1% SDS) poured and allowed to polymerize.

Samples were prepared by mixing equal volumes of cell lysate and 2X sample buffer and heating at 95°C for 5 minutes. Rainbow markers (Amersham) were similarly prepared using 7-10 µl of marker. Samples were loaded onto the gel and electrophoresed for 1-2 hours at 150 V (Mini-Protean) or 5 hours at 100 V (Protean II) in SDS PAGE running buffer.

viii) WESTERN BLOT

After electrophoresis the proteins were transferred to Immobilon-P Transfer Membrane (Millipore) using the BioRad Mini-Electro or Trans-blot system. Transfer buffer was cooled to 4°C and the ice block (Mini) was frozen prior to starting the transfer. The membrane and 6 mm paper were cut to size and equilibrated with transfer buffer. The gel was removed from the glass plates and soaked for 15 minutes in transfer buffer to remove SDS. The blotting sandwich was prepared and placed in the apparatus. Electroblotting was at 100V for 1 hour (Mini) or overnight at 25 V (Trans blot). The membrane was removed and either air dried for storage or used immediately.

Immunostaining was accomplished using the Bio-Rad Protoblot system, which uses an anti-rabbit IgG conjugated to alkaline phosphatase as the secondary antibody and alkaline phosphatase hydrolysis of substrate for a colour reaction, following manufacturer's directions. Polyclonal rabbit anti-HPIV3 antiserum was used at a 1:500 dilution. The diluted antiserum was stored at 4°C and reused 5 or more times over 3 months. The colour reaction

was allowed to develop for 3-4 minutes, which maximized the signal to background ratio.

ix) IMMUNOPRECIPITATION

Cells for immunoprecipitation were labelled as follows. Cells were grown to approximately 80% confluency, infected as described above, washed twice with TBS and then placed in 3 ml of leucine free, serum free medium for 1 hour. The cells were then radioactively labelled by adding 60 μ Ci of [3,4,5- 3 H] leucine (Dupont) to the medium and continuing the incubation for a further 3 hours. Cells were harvested and lysed as described above.

For each precipitation 10 mg of Protein A Sepharose CL 4B beads (Sigma) were weighed out and allowed to swell in RIPA buffer containing 1X detergent. The beads were pelleted by centrifugation for 30 seconds, washed with RIPA 1X detergent and pelleted again. The pellet was resuspended at 60 μ g/ml and 170 μ l was mixed with 300 μ l of 3 H-cell lysate and 30 μ l of serum. The mixture was incubated at 4°C overnight with continuous end-over-end mixing. The beads were washed five times with RIPA 1X detergent and transferred into a 500 μ l microfuge tube. The final pellet was resuspended in 25 μ l of 2X protein sample buffer (for SDS-PAGE) and heated to 95°C for 5 minutes. The sample was separated from the beads by puncturing the bottom of the tube with a 22 gauge needle and spinning the liquid into a 1.5 ml microfuge tube. The samples were loaded onto a polyacrylamide gel and electrophoresed as described above.

After electrophoresis the gel was fixed in 30% Methanol 10% glacial Acetic Acid for 1 hour or longer, then transferred into En³Hance (Dupont) and incubated at room temperature for 40 minutes. The gel was then washed under cold running water for 20 minutes, and transferred onto 3mm paper. The gel was dried in a BioRad gel drier (cycle 1) at 60°C for 45-60 minutes, placed in a cassette with Cronex 4 X-ray film (Dupont) and exposed at -80°C overnight. If longer exposures were required, fresh X-ray film was added and the cassette replaced at -80°C for the desired length of time.

x) HEMAGGLUTINATION ASSAY

Cells were infected at approximately 90% confluency using a MOI of 1 (vaccinia) or 5 (HPV3) as described above, and incubated for 16 hours. The cells were harvested as described for cell lysis but were resuspended in PBS at 10^6 cells/ml and then sonicated for 30 seconds (Sonifier cell disruptor 350, Branson Sonic Power Co.; microtip, setting 7), on ice. Serial two-fold dilutions of 100 μ l aliquots were performed in a 96 well round bottom plate. Guinea pig erythrocytes were washed in PBS then diluted to 0.5% and 100 μ l were added to each well. The plates were incubated at room temperature for 1 hour, then scored on the basis that the titre was the inverse of the last dilution not showing a "tear drop" when the plate was tipped.

xi) NEURAMINIDASE ASSAY

Cells were infected, harvested and sonicated as described for the haemagglutination assay. The sonicate (equivalent to 10^5 cells, in 100 μ l) was mixed with 100 μ l PBS, 100 μ l NaAc buffer, pH 5.0 and 100 μ l (0.2 mg/ml) 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (Sigma) as described by Shibata *et al* (1983) and incubated for 4 hours at 37°C with constant end-over-end mixing. The mixtures were centrifuged to pellet any particulate debris then transferred into 3.6 ml of 0.25 M Glycine, 0.193 N NaOH. Fluorescence of the product methylumbelliferone (MU) was determined with a Perkin-Elmer Fluorometer using λ_{ex} 360 nm and λ_{em} 440 nm and 5 nm slit widths. A standard curve using the sodium salt of methylumbelliferone was performed in conjunction with every assay so that fluorescence units could be converted into nmol.

RESULTS

CONSTRUCTION OF ALTERED HN GENES

The HN gene of HPIV3 was initially constructed from cloned cDNA fragments by T. Binder and the construction is summarized in Appendix 3. The HN gene was then transferred into pIBI-31, to create pIBI-HN, a map of which is shown in Figure 3. During the first attempt to truncate HN, a mutant, pHN-H9, was created as summarized in Figure 4a. This construct resulted from the digestion of pIBI-HN with *Hind* III, which cut once in HN (at base 1755) and once in pIBI (in the multiple cloning region, 3' to HN), followed by ligation of these two sites to one another. This recreated a *Hind* III site and resulted in a useful vector for making truncations, since most of the multiple cloning region had been removed. The HN gene was truncated at 3 unique restriction endonuclease sites: *Hind* III, *Bgl* II and *Xbo* I. An internal sequence from base 780 to base 940 was also deleted using 2 *Rsa* I sites. The constructs were named after the enzyme used for the mutation: Hind, Bgl, Xho and Rsa, while a single letter designation (H, B, X, R) was used to identify the plasmids and viruses produced (eg. pHN-H, pSCFN-H and vHN-H). The truncations of HN were made by digesting 10 µg of pHN-H9 with *Hind* III, with *Hind* III and *Xbo* I, or with *Hind* III and *Bgl* II, as summarized in Figure 4b. The 5' overhangs were filled in using the Klenow fragment of DNA polymerase I and dephosphorylated using calf intestinal phosphatase, then the samples were

Figure 3: Restriction maps of pIBI-HN and HN constructs.

a) pIBI-HN, which contains the entire coding region of the HPIV3 HN gene; b) HN constructs, numbers indicate nucleotide position in the HPIV3 HN gene. Amp-Ampicillin resistance gene, MCR-multiple cloning region. Restriction sites: B-*Bam* H1, Bg-*Bgl* II, E-*Eco* R1, H-*Hind* III, R-*Rsa* I, X-*Xho* I, Xb-*Xba* I.

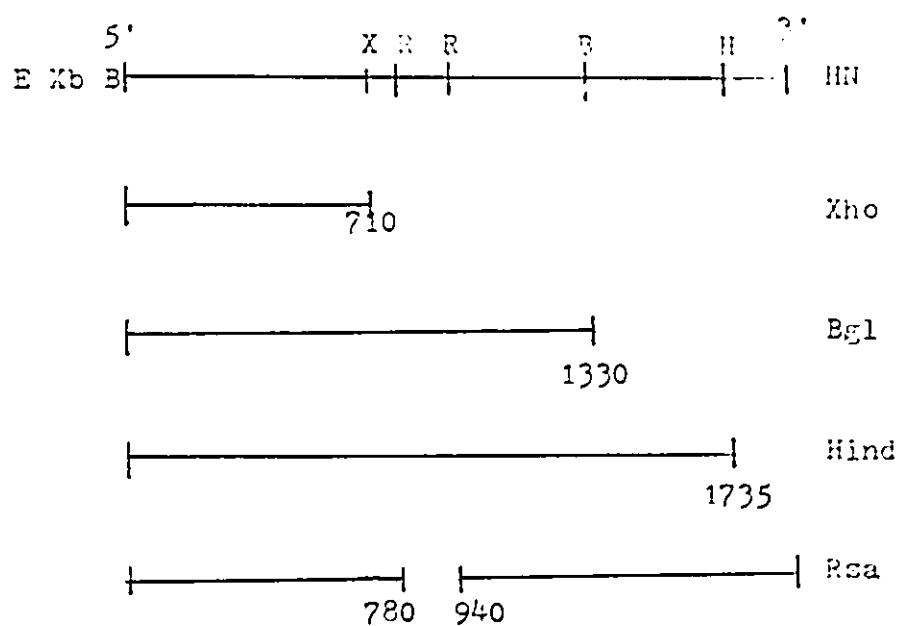
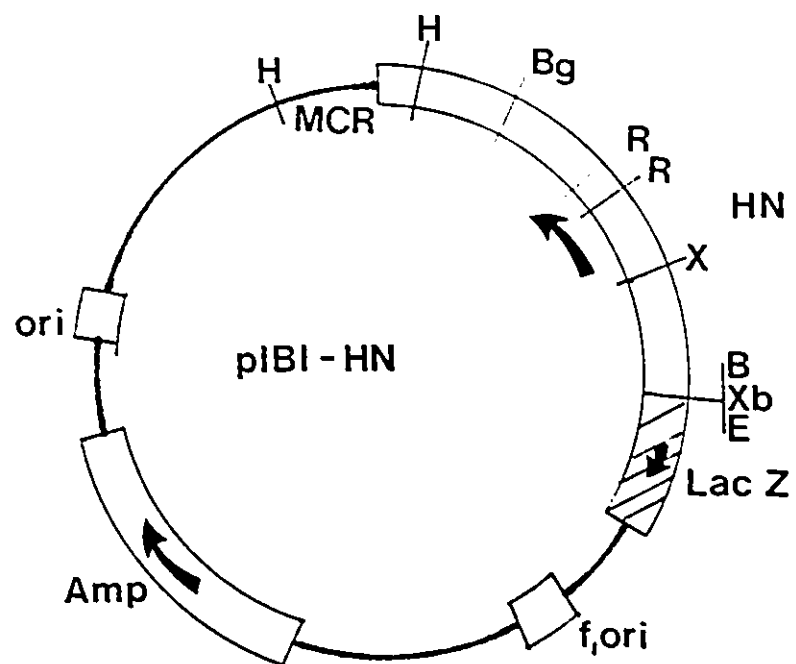
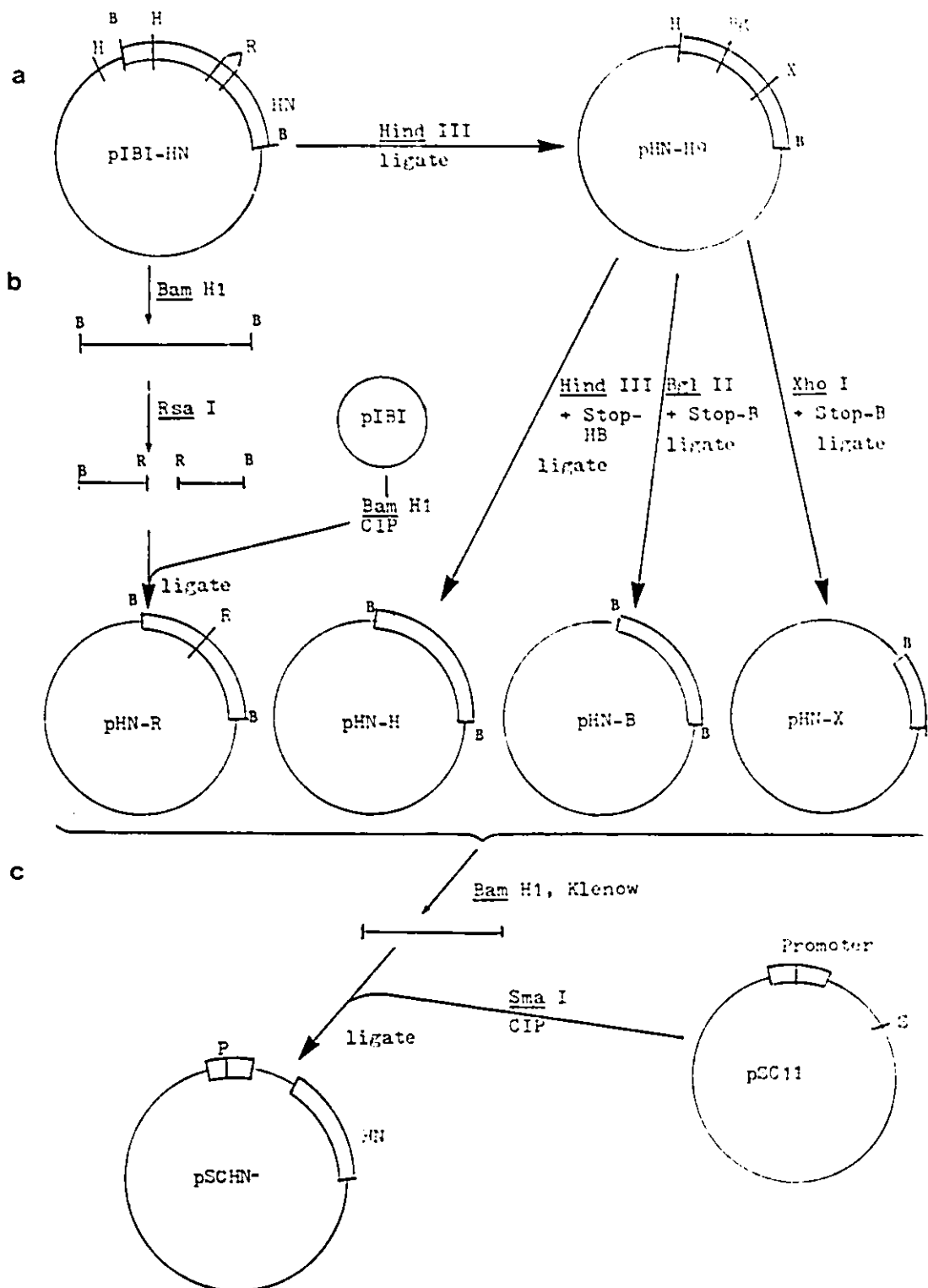


Figure 4: Construction of pSC11 clones containing mutant HN genes.

a) Truncation of pIBI-HN by *Hind* III (H) digestion and religation to produce pHN-H9

b) Truncations of pHN-H9 to produce: pHN-H, by digestion with *Hind* III and insertion of Stop-HB oligonucleotide; pHN-B, by digestion with *Hind* III and *Bgl* II (Bg) and insertion of Stop-B oligonucleotide; pHN-X, by digestion with *Hind* III and *Xbo* I (X) and insertion of Stop-B oligonucleotide. Construction of pHN-R by digestion with *Bam* H1 (B); the *Bam* H1 fragment was then digested with *Rsa* I (R) and the two large fragments were ligated into *Bam* H1- digested pIBI-30.

c) Transfer of mutant HN genes into pSC11 by digestion of pHN- constructs with *Bam* H1, fill in with the Klenow fragment of DNA polymerase I and ligation into pSC11 that had been prepared by *Sma* I (S) digestion and dephosphorylation (CIP). The pSC11 constructs were designated pSCHN-B, pSCHN-H, pSCHN-R, and pSCHN-X for Bgl, Hind, Rsa and Xho respectively.

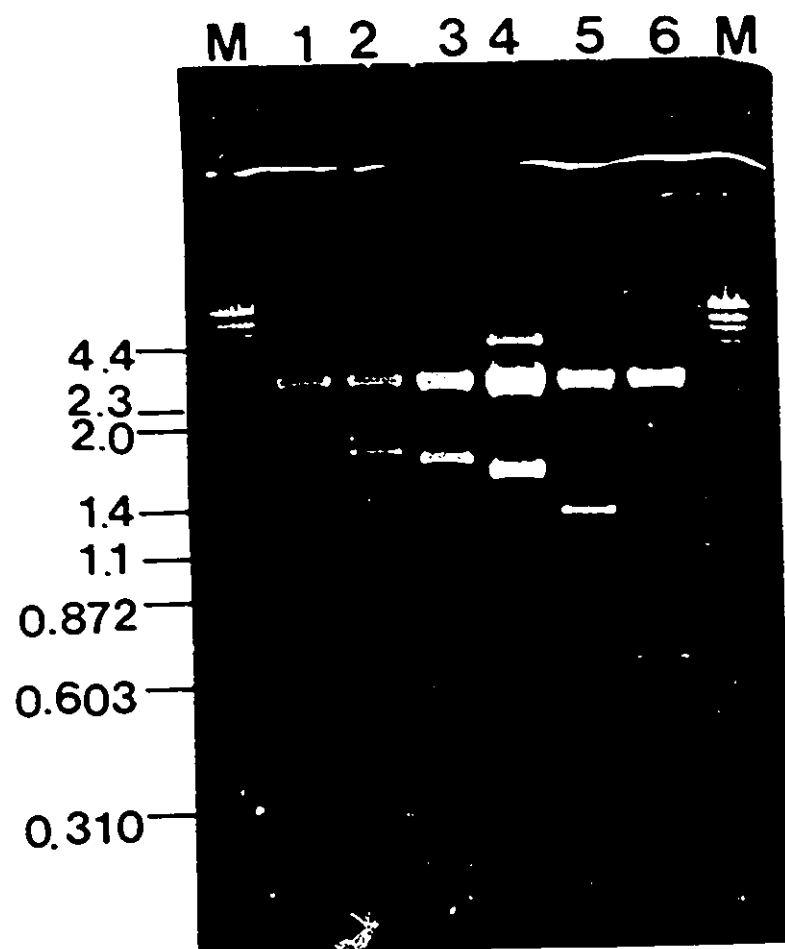


electrophoresed overnight in a 1% agarose gel, and the appropriate fragments were electroeluted. Oligonucleotides, containing a stop codon and a *Bam* HI recognition site (Stop-B and Stop-HB, sequences in Appendix 2) were phosphorylated with polynucleotide kinase, heated to 65°C and allowed to cool to room temperature to anneal. The "stop" oligonucleotides were ligated to the dephosphorylated, truncated DNA in a 5 fold molar excess. After transformation, mini-preps were screened by *Bam* HI digest, which should release a single HN fragment for each construct. The results of such a digest are shown in Figure 5, lanes 3, 5 and 6.

Since pIBI-31 contains 3 *Rsa* I sites, the full length HN fragment was released from pIBI-HN using *Bam* HI, then digested with *Rsa* I as diagrammed in Figure 4a. The two appropriate *Rsa* I fragments (950 and 750 bp) were electroeluted after agarose gel electrophoresis, then combined with *Bam* HI digested and dephosphorylated pIBI-30. A vector to insert-1 to insert-2 ratio of 2:1:1 was used in the trimolecular ligation. Screening of transformants was by digestion with *Sty* I and identifying the loss of one *Sty* I site. Figure 5, lane 4 shows the *Bam* HI digest of this construct.

The three truncations were sequenced at the junction regions to confirm that the stop codon was in frame (sequences of oligonucleotides used as primers are in Appendix 2). Due to an oversight, the sequence of the *Rsa* deletion was not confirmed. This will be discussed below.

Figure 5: Agarose gel electrophoresis of pHN-clones. Plasmid DNA was digested with *Bam* H1, electrophoresed on a 1% agarose gel and stained with ethidium bromide. lane 1: pIBI, lane 2: pIBI-HN, lane 3: pHN-H, lane 4: pHN-R, lane 5: pHN-B, lane 6: pHN-X. The upper band in lanes 1-6 is pIBI; the lower band is the corresponding HN fragment in each construct. M indicates lanes loaded with a mixture of a *Hind* III digest of λ DNA and a *Hae* III digest of ϕ X174 DNA, used as markers.



ISOLATION OF RECOMBINANT VACCINA VIRUS

As diagrammed in Figure 4c, each of the pHN constructs was digested with *Bam* HI to release the HN fragment and 5' overhangs were filled in with the Klenow fragment of DNA polymerase I. The vector, pSC11, was digested with *Sma* I and dephosphorylated. DNA was electrophoresed on agarose gels and the appropriate fragments were electroeluted. Blunt-end ligations of the HN fragments to pSC11 were done with the HN fragments in 5 fold molar excess. Screening of transformants was done by digestion of mini-prep DNA with *Eco* RV to determine orientation of the Hind, Bgl and Rsa constructs, while but orientation of the Xho construct was determined by digestion with *Sty* I and *Eco* RV/*Sty* I. Since only a limited map of pSC11 has been published (Chakrabarti *et al*, 1985), it was necessary to map several restriction sites before determining what restriction enzymes to use for screening. A partial map of pSC11 is shown in Appendix 4. An *Eco* R1 digest of the pSCHN constructs, which shows their relative sizes, is shown in Figure 6.

A schematic representation of the strategy used to produce recombinant vaccinia virus is shown in Figure 7. The pSCHN constructs were prepared in large scale and resuspended in water. Recombinant vaccinia viruses were produced by infecting monolayers of CV1 cells with the WR strain of vaccinia at a MOI of 0.1 then transfecting each monolayer with 20 μ g of a pSCHN construct. The cells were harvested 48 hours after infection, frozen and thawed three times, then 5 μ l (1/100 of a plate) was treated with trypsin (0.05%, 15 min) and

Figure 6: Agarose gel electrophoresis of pSCHN-clones. Plasmid was digested with *Eco* R1, electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. lane 1: pIBI-HN, lane 2: pSCHN-B, lane 3: pSCHN-H, lane 4: pSCHN-R, lane 5: pSCHN-X. The upper two bands in each lane are pSC11 fragments; the lower band is the HN fragment in each construct plus approximately 200 bp of pSC11 (see Appendix 4 for a restriction map of pSC11). M indicates a lane loaded with a mixture of a *Hind* III digest of λ DNA and a *Hae* III digest of ϕ X174 DNA, used as markers.

M 12345

4.4 —

2.3 —

2.0 —

1.4 —

1.1 —

0.872 —

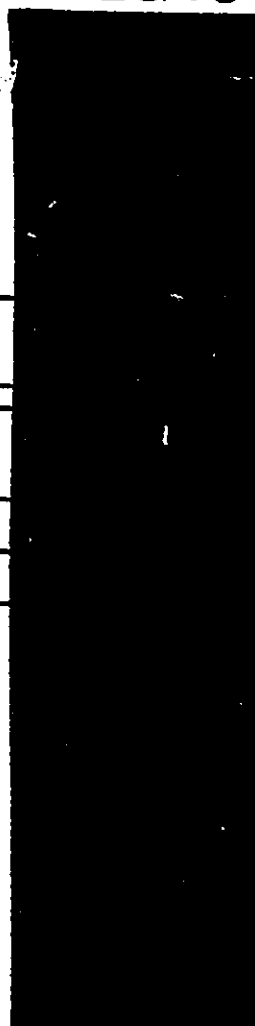
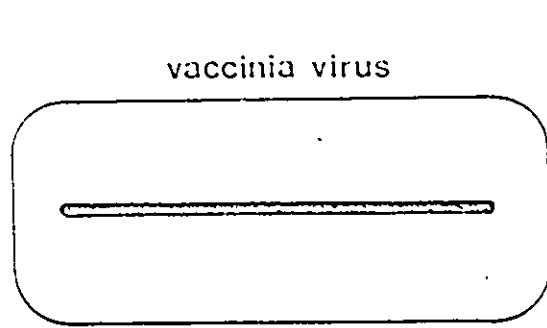
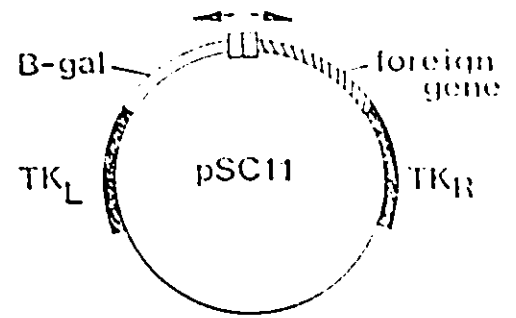


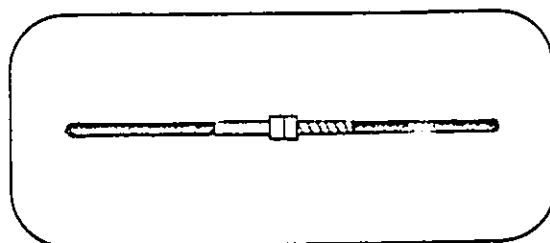
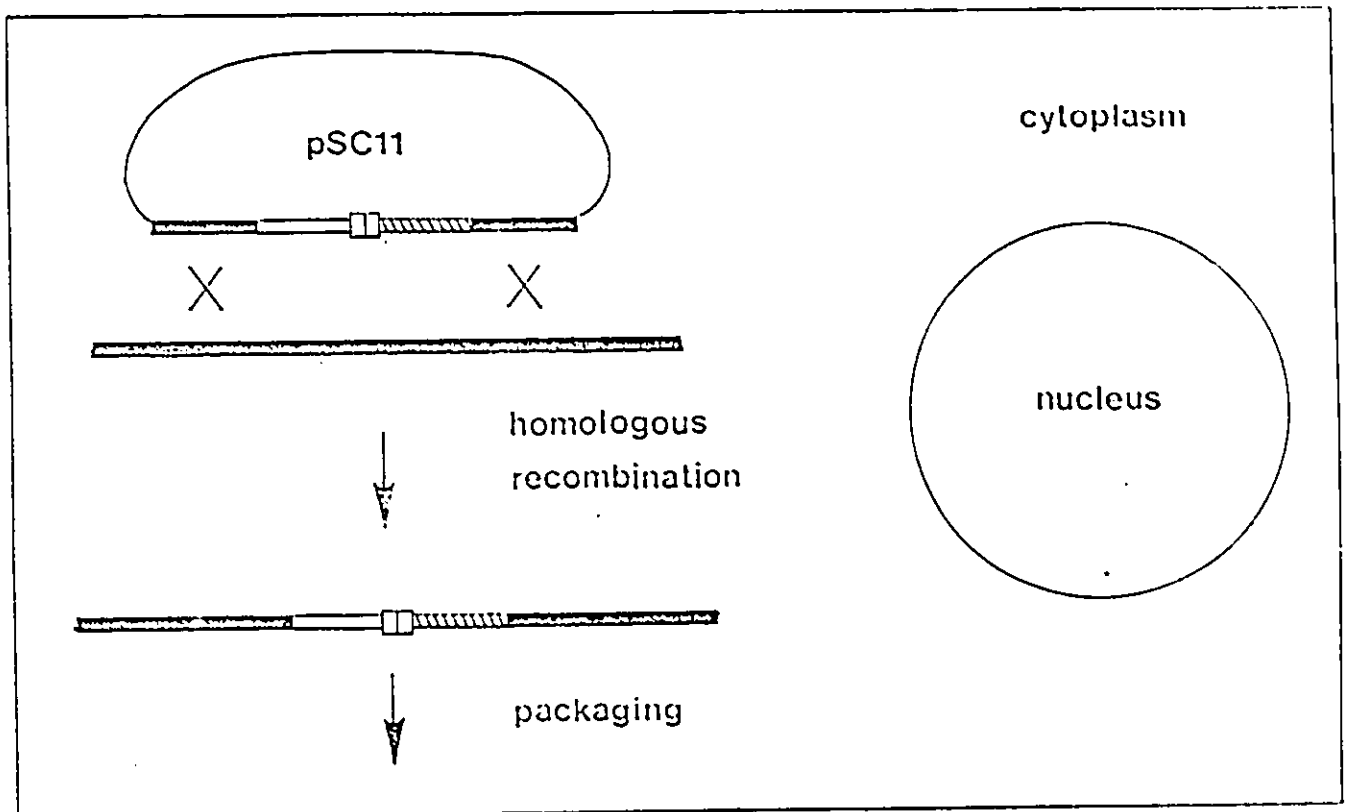
Figure 7: Schematic representation of recombinant vaccinia virus production. Cells were infected with vaccinia virus WR at a MOI of 0.1 PFU/cell and then transfected with 20 μ g of plasmid DNA (a pSCHN construct). Recombination between vaccinia virus DNA and the thymidine kinase sequences in the plasmid generates recombinant vaccinia virus which was selected with BUdR and X-gal.



infection



transfection



recombinant vaccinia virus

used to infect 143B cells. The 143B cells were overlayed with 0.8% agarose containing 1X MEM and 25 $\mu\text{g/ml}$ BUdR. Plaques were allowed to develop for 60 hours then the plates were overlayed again with 0.8% agarose containing 1X MEM and 300 $\mu\text{g/ml}$ X-gal. Plaques expressing β -galactosidase turned blue 6-8 hours after the second overlay. Blue plaques were picked and purified twice more on CV1 cells using X-gal to detect recombinant plaques.

The final plaques were used to prepare mini-stocks of the recombinants. One half a plaque was used to infect a 60 mm plate of CV1 cells and cells were incubated until extensive CPE was observed (approximately 72 hours). The cells were harvested, the virus released by freezing and thawing in 500 μl and 25 μl of each preparation was used to infect CV1 cells on 100 mm plates. These were again incubated until significant CPE was observed (approximately 48 hours), then harvested. The resulting virus stocks were titrated on CV1 cells then used for the expression assays. The titres were all in the $3\text{-}5 \times 10^8$ PFU/ml range.

EXPRESSION OF MUTANT HN GENES

A time course of expression of the HN gene product from recombinant vaccinia virus was carried out. Cells were infected with vHN at MOI's of 1, 5 or 10 PFU/cell and analysed by Western blot at 4 or 6 (MOI 10 only), 8, 16 or 24 hours after infection. Cells were harvested as described in the Materials and Methods and 10^4 cell equivalents were electrophoresed in a 10% polyacrylamide gel. Proteins were transferred to Immobilon membrane by electroblotting and

probed with rabbit anti-HPIV3 serum. The results of the Western analysis of the time course are shown in Figure 8. The level of expression of the HN product was as high 16 hours after infection at a MOI of 1 PFU/cell as at any other MOI or time. Therefore, these conditions were chosen for the functional assays.

Expression of the modified HN proteins from the recombinant viruses was analysed by Western blot and immuno-precipitation. CV1 cells were infected at a MOI of 1 PFU/cell and harvested 16 hours after infection. Cell lysate equivalent to 10^4 cells (HN, Hind, Bgl) or 10^5 cells (Rsa, Xho) was loaded onto either a 10% (HN, Hind, Bgl) or 12.5% (Rsa, Xho) polyacrylamide gel containing SDS. After electrophoresis, the proteins were transferred to Immobilon membrane by electroblotting and probed with rabbit anti-HPIV3 serum. Figure 9 shows the results of the Western blot analysis of the mutant protein products. The products from the Hind and Bgl constructs are shown in part a, while the products from the Rsa and Xho constructs are shown in part b. The mutant HN gene products were also immunoprecipitated from infected cell lysates labelled with ^3H -leucine. The results from an immunoprecipitation are shown in Figure 10. Only the Bgl and Rsa products are clearly identifiable in the immunoprecipitation. The product of the Hind construct was masked by cellular proteins, while the product of the Xho construct migrated at the bottom of the gel among other viral proteins.

The predicted molecular weights for the mutant proteins are: Hind 62.2 K, Rsa 58.2 K, Bgl 46.9 K and Xho 24.0 K. The products of the three

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Figure 8: Time course of the expression of the HN gene in vHN infected CV1 cells.

CV1 cells were infected with vHN at different MOI's and incubated for different lengths of time. Cells were harvested, lysed and 10^4 cell equivalents were loaded onto 10% polyacrylamide gels containing SDS. Gels were electrophoresed for 2 hours in the Mini-Protean system. Following electrophoresis, proteins were transferred to Immobilon-P membrane. HPIV3 proteins were identified using HPIV3 specific rabbit serum and alkaline phosphatase-conjugated anti-rabbit IgG. HN indicates the position of the HN protein, lane M indicates distances migrated by molecular weight markers (Rainbow markers, Amersham)

a) lane 1: HPIV3 infected cell lysate, lane 2: uninfected cell lysate, lanes 3, 4, 5: cell lysate from cells infected with vHN at a MOI of 1 and harvested at 8 (3), 16 (4) or 24 (5) hours post infection. Lanes 6, 7, 8: cell lysate from cells infected with vHN at a MOI of 5 and harvested at 8 (6), 16 (7) or 24 (8) hours post infection.

b) lane 6: HPIV3 infected cell lysate. Lanes 1-5: lysate from cells infected with vHN at a MOI of 10 and harvested at 4 (1), 6 (2), 8 (3), 16 (4) or 24 (5) hours post infection.

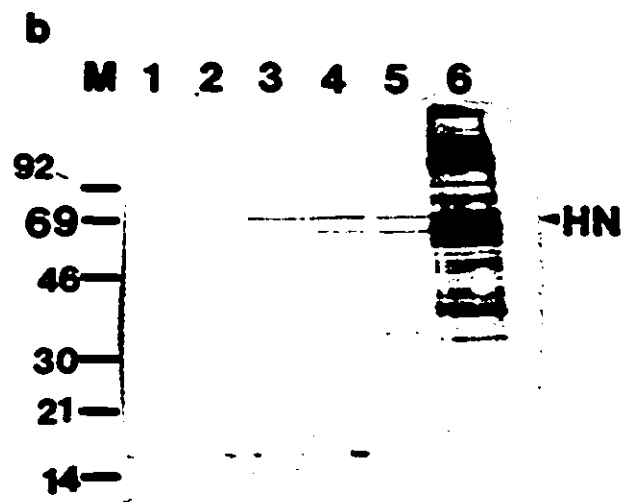
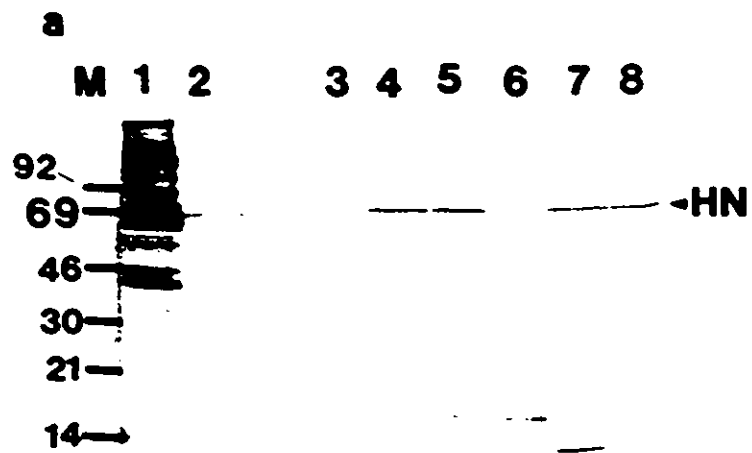


Figure 9: Western Blot analysis of proteins expressed from mutant HN genes. CV1 cells were infected at a MOI of 1 PFU/cell with vaccinia virus containing different HN constructs and incubated for 16 hours. Cells were harvested and lysed and cell lysate equivalent to 10^4 cells (a) or 10^5 (b) cells was loaded onto 10% (a) or 12.5% (b) polyacrylamide gels containing SDS and electrophoresed in the Protean II system at 100 V for 5 hours. The proteins were transferred to Immobilon-P membrane by electroblotting overnight at 30 V. Blots were probed using the Proto-blot system and polyclonal rabbit serum, as described in Figure 7. lane M as in Figure 7.

a) lane 1: HPIV3 infected cell lysate, lane 2: uninfected cell lysate, lane 3: vSC8 infected cell lysate, lane 4: vHN infected cell lysate, lane 5: vHN-B infected cell lysate and lane 6: vHN-H infected cell lysate. HN, H, and R indicate the positions of the proteins from the corresponding virus.

b) lane 1: uninfected cell lysate, lane 2: vSC8 infected cell lysate, lane 3: vHN infected cell lysate, lane 4: vHN-R infected cell lysate and lane 5: vHN-X infected cell lysate. HN, R and X indicate the positions for the proteins from the corresponding construct.

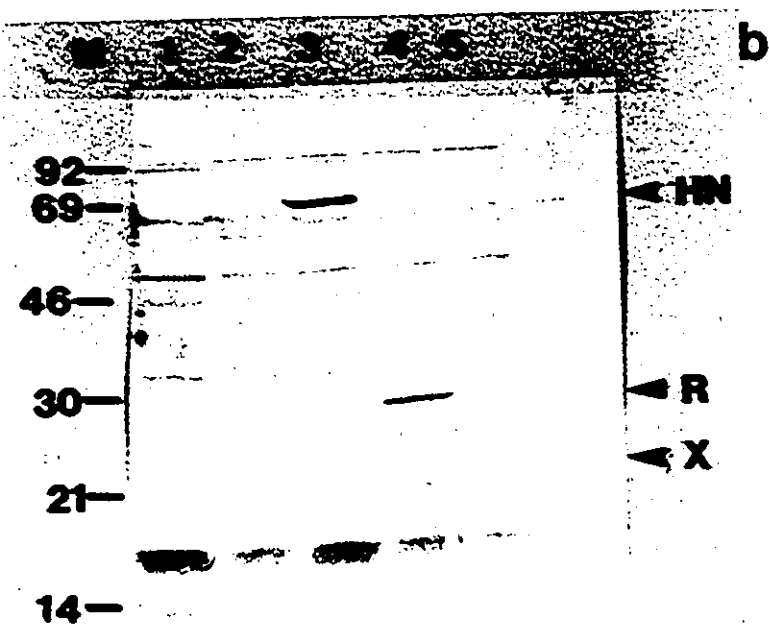
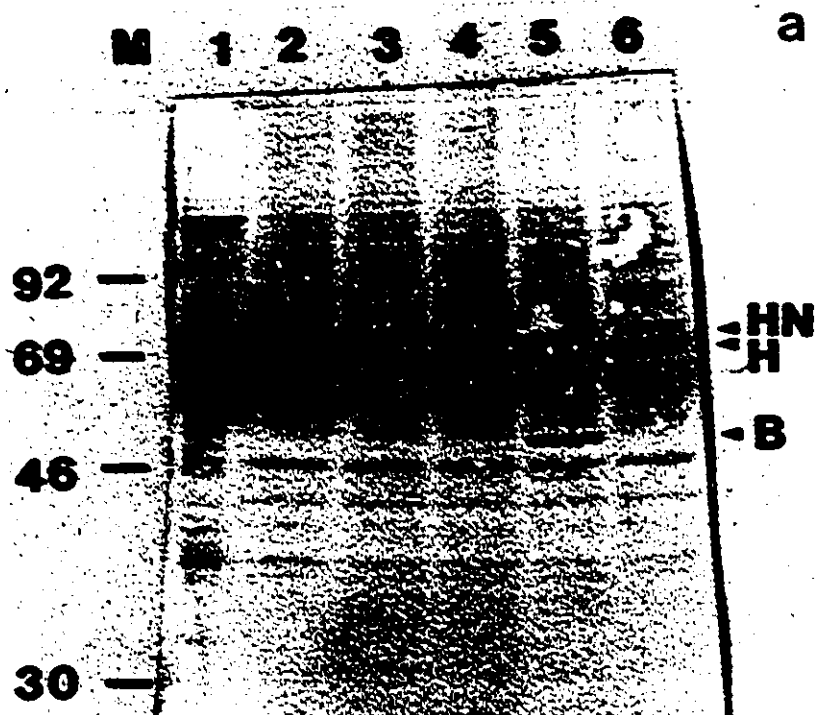
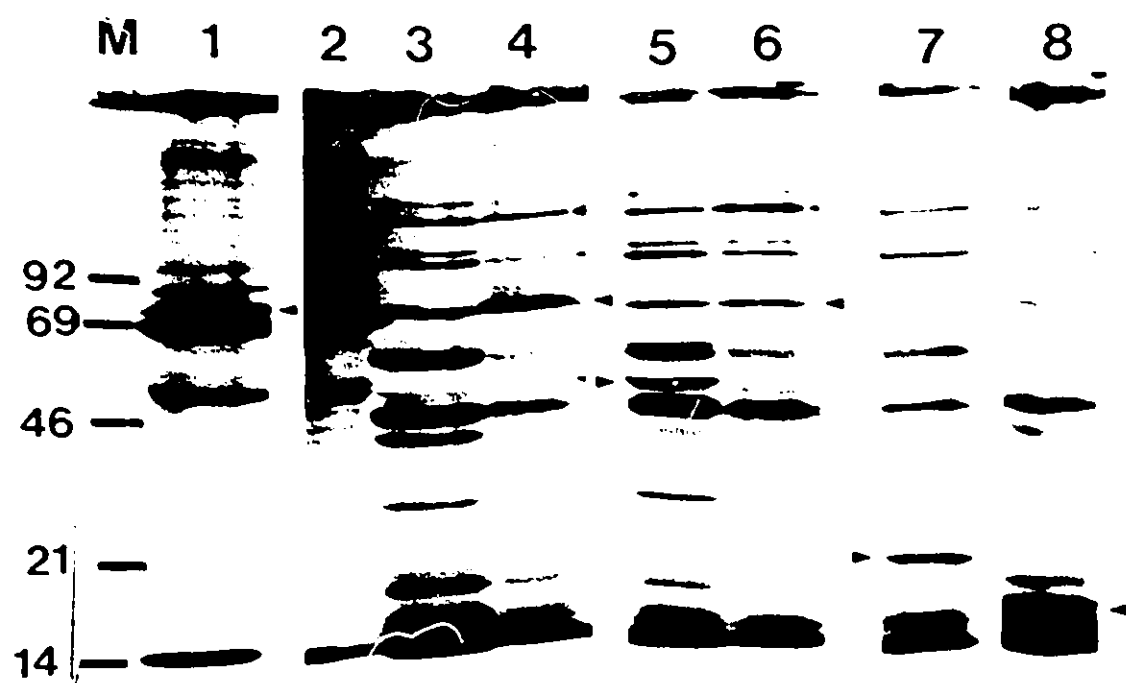


Figure 10: Immunoprecipitation of mutant HN proteins.

Cells were infected at a MOI of 10 PFU/cell with the vHN constructs and incubated for 4 hours, starved for 1 hour then labelled for 3 hours with 20 μ Ci/ml of 3 H-leucine. Cells were harvested, lysed in RIPA buffer, and incubated overnight with polyclonal rabbit serum and sepharose CL-4B beads. Beads were washed and the immunoprecipitated proteins were released by heating in 2X sample buffer. Samples were loaded onto a 10% polyacrylamide gel and the gel was electrophoresed for 2 hours at 150 V in the Mini-Protean system, fixed, treated with Enhance, dried and exposed to X-ray film for 3 weeks. lane 1: HPIV3 infected cell lysate, lane 2: uninfected cell lysate, lane 3: vSC8 infected cell lysate, lane 4: vHN infected cell lysate, lane 5: vHN-B infected cell lysate, lane 6: vHN-H infected cell lysate, lane 7: vHN-R infected cell lysate, lane 8: vHN-X infected cell lysate. Arrowheads indicated the expected location of the protein products as determined by Western analysis.



truncations, Hind, Bgl and Xho, all migrate to approximately the predicted position on gels, however, the product of the Rsa deletion appeared to be smaller than expected (approximately 30 K). Because of this, the sequences of the four HN constructs in pSC11 were all confirmed using the Genesis 2000 DNA analyser. The Rsa construct had a single nucleotide pair missing at the 5' end of the deletion, resulting in a frame-shift mutation. The protein product encoded by this mutant HN gene would terminate 15 amino acids downstream and is predicted to have a molecular weight of approximately 29 K.

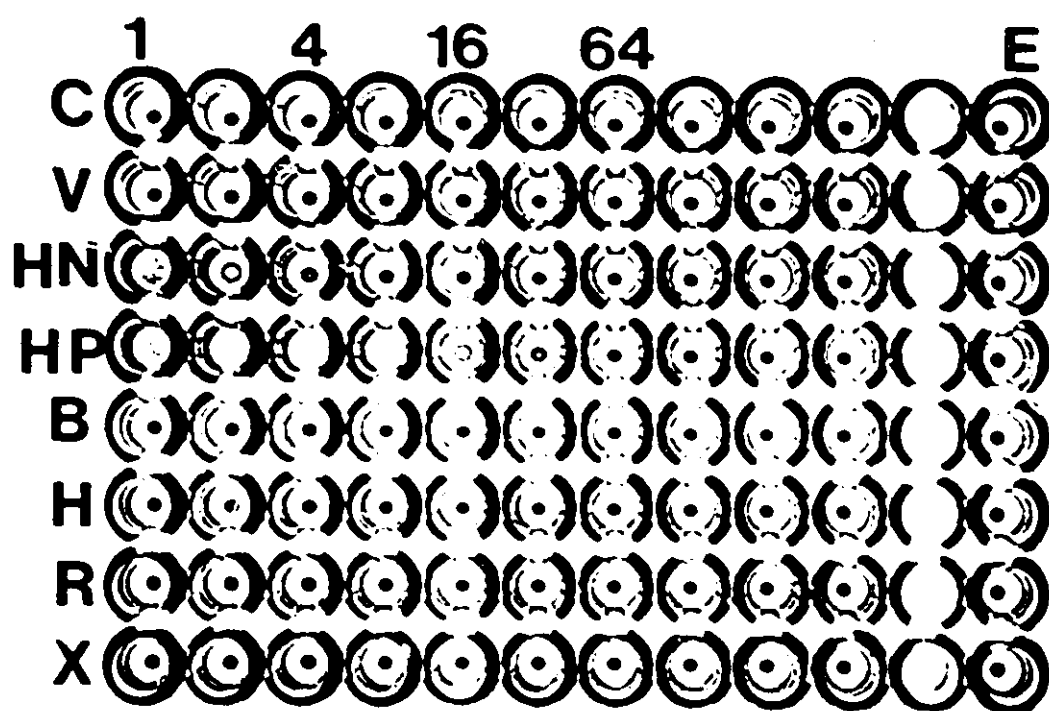
HEMAGGLUTINATION

Preliminary experiments established optimal conditions for the haemagglutination (HA) assay with the HN product expressed from vHN were observed when 10^5 cell equivalents of lysate from CV1 cells infected with vHN at a MOI of 1 PFU/cell for 16 hours and Guinea pig erythrocytes were used. The titre for vHN was 8-16, while the titre for HPIV3 (MOI 5 PFU/cell, 16 hr post infection, 10^5 cell equivalents), which was used as a positive control for HA, was found to be 128-256, based on "tear drop" appearance. The titre was defined as the reciprocal of the last well not showing a "tear drop" shape when the plate was tilted. The uninfected cells and vSC8 infected cell controls were both negative. All four mutants were also negative in the HA test, both with Guinea pig and human erythrocytes. Figure 11 is a photograph of a completed

Figure 11: Hemagglutination assay of mutant HN proteins.

CV1 cells were infected with each of the vaccinia viruses carrying the mutant HN genes, at a MOI of 1 PFU/cell and incubated for 16 hours. Cells were harvested and lysed, then serial twofold dilutions of lysate were done in 100 μ l of PBS. An equal volume of Guinea pig erythrocytes was added and the plate was incubated for 1 hour.

C-uninfected cell lysate, V-vSC8 infected cell lysate, HN-vHN infected cell lysate, HP-HPIV3 infected cell lysate, B-vHN-B infected cell lysate, H-vHN-H infected cell lysate, R-vHN-R infected cell lysate, X-vHN-H infected cell lysate, E-erythrocyte control (100 μ l PBS, 100 μ l Guinea pig erythrocytes).



HA. Tear dropping could not be successfully photographed but in Figure 11 occurred at 32 for vHN and 256 for HPIV3.

NEURAMINIDASE

The assay procedure was initially established using HPIV3. The results of an infection time course, at a MOI of 5 PFU/cell, and reaction time course are shown in Figure 12. Using a 36 hour infection, a 1 hour reaction with substrate produces approximately 1 nmol of the fluorescent product methylumbelliferone (MU). The neuraminidase activity (NA) of the products from the mutant HN proteins expressed by vaccinia virus was determined for samples prepared in parallel with those used for the HA test. The standard reaction time was 4 hours, at 37°C, having been determined previously by time course assays. vSC8 reproducibly gave lower background fluorescence than uninfected cells and was used as the control for background fluorescence in experiments done with products expressed in vaccinia (vHN and mutants). The results of the neuraminidase assay are shown in Table 1. With the exception of Rsa, the mutants have consistently demonstrated levels of neuraminidase activity between 50-90% of vHN activity. vHN-R gave inconsistent results in the NA assay, being negative in one run, positive in the second.

Figure 12: Neuraminidase activity in HPIV3 infected cells.

a) CV1 cells were infected with HPIV3 at a MOI of 5 PFU/cell for 24 or 36 hours. Cells were harvested and incubated with 20 μ g substrate, in NaAc buffer at pH 5.0, for 1, 2, 3 or 4 hours. Results are shown as nanomoles of methylumbelliferone produced after each reaction time for 24 (■) or 36 (●) hours infection. Cell controls are shown with open symbols □ (24) and ○ (36)

b) CV1 cells were infected with HPIV3 at a MOI of 5 PFU/cell and incubated for 12-48 hours. Cells were harvested and incubated for 1 hour with 20 μ g of substrate in NaAc buffer at pH 5.0. The background fluorescence, as determined from the uninfected cell control, was subtracted from each time point before plotting.

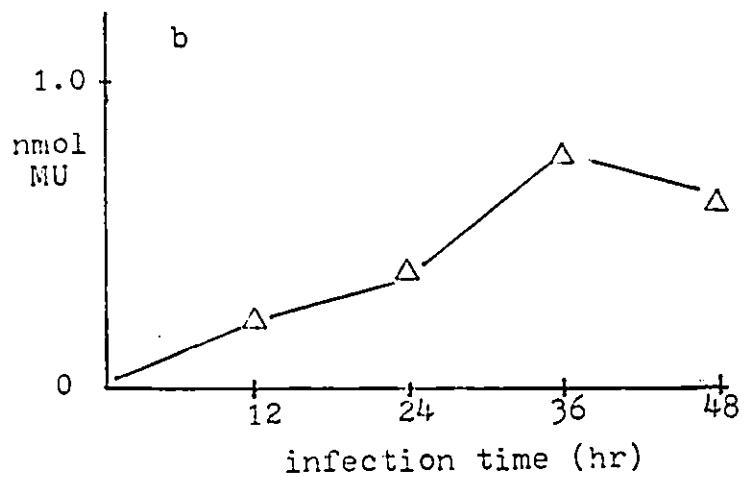
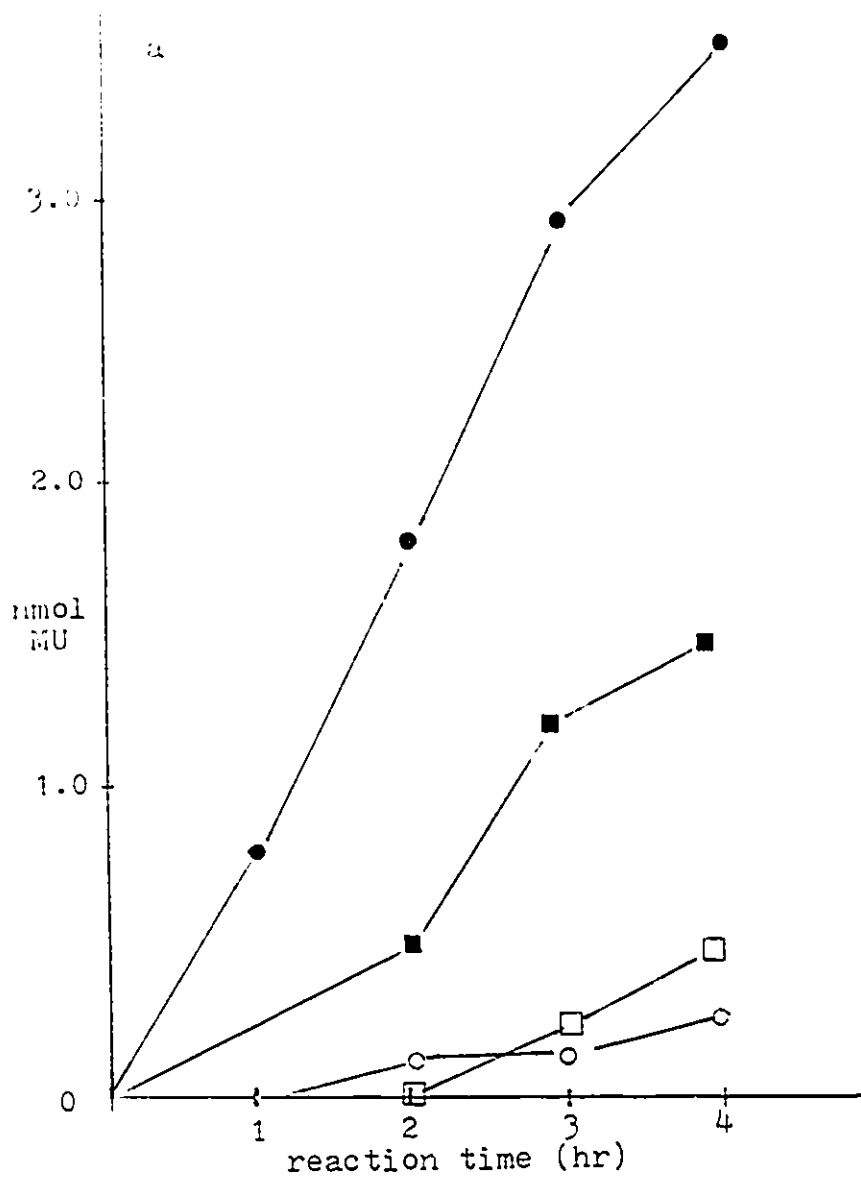


Table 2: Neuraminidase activity of HN gene products.

Sample ¹	pmol MU $\pm$ SD ²	Significance ³
vSC8	53.8 $\pm$ 59.1	
vHN	329.9 $\pm$ 20.8	p < 0.0001
vHN-B	233.5 $\pm$ 2.1	p = 0.0006
vHN-H	204.0 $\pm$ 53.2	p = 0.0139
vHN-R	111.9 $\pm$ 114.2	p = 0.1133
vHN-X	236.1 $\pm$ 98.0	p = 0.0181
Cells	204.9 $\pm$ 204.9	
HPIV3	910.0 $\pm$ 1.7	p < 0.0001

¹CV1 cells were infected with recombinant vaccinia virus at a MOI of 1 PFU/cell and incubated for 16 hours. Cells were harvested, lysed and assayed for neuraminidase activity as described in the text.

²Fluorescence was determined using a Perkin-Elmer fluorometer with λ_{ex} of 360 nm and λ_{em} of 440 nm, slit widths were set at 5 nm. Amount of methylumbelliferone product (MU) was determined by comparison with known standards. Numbers shown are the mean of 2 assays performed in triplicate. Background from spontaneous hydrolysis of substrate has been subtracted.

³Significance was determined using an unpaired 1 tail T-test from the T-EASE program. p $\leq$ 0.05 was considered significant. vHN samples were compared to vSC8, while HPIV3 was compared to uninfected cells (Cells).

DISCUSSION

The work in this thesis was designed to answer the question: Where do the hæmagglutinin and neuraminidase activities of the HN protein of HPIV3 map on the protein? To this end the immediate objectives of the work described herein were:

- 1) To express a functional HPIV3 HN using vaccinia virus as a vector
- 2) To optimize functional assays for neuraminidase and hæmagglutinin
- 3) To construct truncation/deletion mutants of the HN gene
- 4) To express the mutant HN proteins using vaccinia virus as a vector
- 5) To analyse mutant HN genes for changes in activity

All five of these goals have been accomplished. A recombinant vaccinia virus, vHN, containing the full length HPIV3 HN gene was constructed. The HN gene product expressed by vHN was found at the surface of vHN infected cells (Ebata *et al*, 1991) and was functional in a hæmadsorption assay (data not shown), and in assays for hæmagglutination and neuraminidase activity. The HN product of vHN was also shown to be necessary for the fusion activity of the F protein expressed from a recombinant vaccinia virus expressing the HPIV3 F gene, vF (Ebata *et al*, 1991). The HN product expressed from vHN was

indistinguishable from HN expressed by HPIV3 in both Western and immunoprecipitation analyses.

The assays for both haemagglutinin and neuraminidase activity have been established. Haemagglutination was shown to be more sensitive when Guinea pig, rather than human, erythrocytes were used (data not shown). Lysates from vHN infected cells gave 8-fold lower titres than HPIV3 infected cell lysates. Since cell numbers were constant, this is most likely because less HN protein is synthesized by vHN infected cells than by HPIV3 infected cells. This can be seen in the Western blots in Figures 8 and 9. The lower activity could also indicate a requirement for other HPIV3 proteins for optimal activity, as has been established for the F protein of HPIV3 (Ebata *et al*, 1991). Other HPIV3 proteins could be involved directly in HN activity or indirectly by affecting assembly of HN into correct conformation for activity. The HN protein of HPIV3 has recently been shown to form tetramers on the virus surface (Collins and Mottet, 1991) and this may require the functions of other proteins. Assays for haemagglutinin or neuraminidase activity following expression of HN together with other HPIV3 proteins using vaccinia virus vectors has not yet been attempted.

The neuraminidase assay, while producing consistent, statistically significant results for vHN activity, should be interpreted with caution. In fluorometric units, the signal was only slightly above background levels. One observes pmol increases in MU production over a 1.5 nmol background. The

majority of the background is from spontaneous hydrolysis of the substrate. The background fluorescence observed with uninfected cells and cells infected with vSC8 were variable, being almost non-existent during one assay and at approximately 200 pmol the next. The fluctuation does not seem to correlate with any changes in protocol, although freezing at -80°C might be responsible for the lower values. This variability leads to the high standard deviations shown for these controls. However, the NA activity was approximately the same amount above background whether background was high or low. Attempts to increase sensitivity or decrease background were unsuccessful. Increasing the quantity of cell lysate resulted in increased background and decreasing substrate concentration resulted in a concomitant decrease in activity (MU produced). The lower neuraminidase activity in cells infected with vHN compared to cells infected with HPIV3 can probably be explained as for the HA assay.

The fluorometric neuraminidase assay may well need further manipulation to optimize conditions for vHN. In one of the two original papers published (Myers *et al*, 1980; Potier *et al*, 1979), Potier and co-workers (Potier *et al*, 1979) show that sodium, calcium and EDTA concentrations, as well as pH, can all affect neuraminidase activity and can affect different neuraminidases differently. For example, 40 mM EDTA inhibits NA from Vibrio cholera by 98% but does not affect NA of fibroblasts or leukocytes. Under the conditions used in this work, even HPIV3 shows relatively low levels of activity. Potier (Potier *et al*, 1979) reports activity in nmol of methylumbelliferone product liberated

per minute while Myers (Myers *et al*, 1980) reports activity of fibroblast neuraminidase at 40 nmol of MU per hour per mg of protein. HPIV3 HN produces 1 nmol of MU per hour using approximately 10^5 cells after 36 hours of infection. Shibuta *et al* (1983) demonstrated activity of HN from wild type BPIV3 to be 1,000 nmol of MU per minute per mg of viral protein, using purified virus. This level of activity is approximately 10,000-fold greater than seen for HPIV3 infected cells but the amount of HN protein in 1 mg of virus may be as much as 10,000 times greater than in 10^5 infected cells. Shibuta (Shibuta *et al*, 1983) also demonstrated that BPIV3 expressing mutant HN proteins had a different pH optimum than wild type.

The HN gene was truncated using three unique restriction endonuclease sites to form the Hind, Bgl, and Xho constructs. After transfer into pSC11 and recombination with vaccinia virus, the truncated genes were found to be expressed by the recombinant vaccinia viruses vHN-H, vHN-B and vHN-X, respectively. The products of the truncated HN genes migrated with the expected electrophoretic mobilities. The migration of the gene product of the internal deletion mutant of HN (construct Rsa) expressed by vHN-R indicated that a smaller protein than expected had been produced. Confirmatory sequencing showed that this was due to a frameshift mutation which would result in early translation termination and a product of approximately 29 Kd. While the frameshift explains the size of the gene product, there is no easy explanation for the frame shift. *Rsa* I recognizes the sequence 5'GTAC3' and

makes a blunt end cut between the T and A to leave GT and AC at the DNA termini. Ligation of the *Rsa* I fragments should re-form 5'GTAC3', recreating the *Rsa* I site. In HN this should have produced an in-frame deletion. However, sequence analysis showed that upon ligation, the sequence 5'GAC3' was formed, indicating that the T-A base pair from the 5' side of the junction had been lost. Two possible explanations for this are: 1) the presence of exonuclease in the *Rsa* I enzyme (contamination of buffers and other solutions can be ruled out since they were also being used for other digests which did not exhibit this problem) or 2) the specificity of the *Rsa* I is not consistent. While exonuclease is the simplest answer, why did it only affect one base pair at the 5' end of the deletion when an identical site was available at the other free end of the DNA? All type II restriction endonucleases recognize palindromic DNA sequences and leave symmetrical cut sites, so even if *Rsa* I was not a blunt end cutter, but left a sticky end, ligation should re-form the *Rsa* I site. It seems unlikely that the enzyme behaved differently at different *Rsa* I sites.

With the limitations of the NA assay in mind, the mutant HN proteins consistently (except for the *Rsa* product) gave results above background. The *Rsa* construct was negative (essentially background) in the first run, but gave significant activity during the second run ($n=3$, 284.8 ± 35 pmol, $p = 0.0005$). The simplest explanation for this discrepancy is experimental error; possibly no lysate was added during the first run (substrate must have been added, since levels of activity were equivalent to background). However, there was one

difference between the two runs—the samples from the first run were frozen at -80°C between sonication and assay time. This had previously been shown to have no effect on the activity of HN expressed from either HPIV3 or vHN. The data for the truncated products (Hind, Bgl and Xho) indicate that freezing does not affect their activity either. The Rsa construct, however, has 15 non-native amino acid residues at its carboxy end and this might have affected stability upon freezing.

Several other issues warrant comment. In initial attempts to truncate HN, hexanucleotides containing two stop codons (TAA TAA) were used. These 6mers were never found to be inserted into the plasmid, but when larger oligonucleotides (10mers, Stop-B and Stop-HB) were used, insertion was successful. This may be explained by a lack of thermal stability of duplexes that must be formed by the 6mer for ligation.

During the isolation and analysis of recombinant vaccinia virus several issues came to light. First was the effect of using trypsin to decrease aggregation of vaccinia virus prior to infection. The use of trypsin increased the vaccinia virus WR titre 10 fold and increased the amount of recombinant virus retrieved from infected and transfected CV1 cells 100 fold over the amount of virus obtained without trypsin treatment (Côté, 1989).

Initial attempts to analyse expression of mutant HN proteins by Western Blot of proteins separated in polyacrylamide gels electrophoresed in the Mini-Protean unit were successful in identifying only 2 (Rsa and Bgl) of the 4 mutant

HN proteins. The same result was found when immunoprecipitated proteins were electrophoresed in the Mini-Protean system. With longer 10% polyacrylamide gels (Protean II system), 3 constructs (Hind, Rsa and Bgl) could be identified, while the final mutant HN protein (Xho) required long 12.5% polyacrylamide gels for conclusive identification. In the short gels the Hind product co-migrated with a 63 Kd cellular protein that was recognized by the rabbit serum, and the Xho product migrated near the dye front among several vaccinia virus proteins. Finally, for constructs in which a significant portion (>50%) of the HN protein was removed (Xho and Rsa) it was necessary to load more lysate from infected cells onto gels to readily detect the protein product by Western blot. This is probably due to the loss of many of the antigenic sites, in the carboxy half of HN, identified by Coelingh (Coelingh *et al*, 1986 as summarized in Chanock and McIntosh, 1990). Because less antibody would be bound to these truncated HN proteins, a decreased signal would be expected.

The overall aim of this project was to determine if one or two sites are responsible for the different functions of the HN protein of HPIV3. The results obtained in this study indicate that there are distinct haemagglutinin and neuraminidase sites on the HPIV3 HN molecule. These results support the conclusions of other groups. Portner (Portner, 1981; Portner *et al*, 1987; Thompson and Portner, 1987), Jorgensen (1987) and Blumberg (1985) all suggest that HA and NA activity can be mapped to distinct regions of the paramyxovirus HN protein. Even the shortest truncation of the HPIV3 protein

(Hind) shows no HA activity, suggesting that this activity maps to the carboxy terminus of the HN protein. All the truncations, however, show NA activity indicating that NA activity maps nearer the amino-terminus of HN. The data presented here suggest that the catalytic site for neuraminidase is not the middle of the protein (aa 224-323) suggested by Jorgensen (1987) but in fact lies amino terminal to amino acid 212.

Since immunoreactivity changes during glycoprotein processing (Roux, 1990; Mottet *et al*, 1986; Waxham *et al*, 1986; Morrison, 1988; Morrison *et al*, 1987; Vidal *et al*, 1989), it has been suggested that conformational changes to proteins because of truncations or drastic mutations might arrest protein processing and result in inactive molecules, whether this be by direct inactivation or by preventing the mutated protein from reaching the compartment required for activation. Preliminary screening of the mutants by h madsorption, which would have identified surface expression of active molecules was negative (data not shown) so we chose to do h magglutination and neuraminidase assays on cell lysates to avoid the possibility that the mutant proteins did not reach the cell surface. A monoclonal antibody that appears to recognize a conformational epitope of HN will not immunoprecipitate the mutant HN proteins, while polyclonal serum immunoprecipitates and recognizes the mutants on Western blots. These observations suggest that there are conformational changes in the mutants compared to the wild type protein. It is also possible that the epitope the MAb recognizes is at the carboxy terminus

of the HN protein and was removed by the truncation with *Hind* III. This would have been confirmed if the *Rsa* deletion construct had been formed as planned, since the 3' end of the gene (carboxy terminus of protein) would have been intact. The *Hind* truncation removes 18 amino acids including a single cysteine in the second last codon. If the HA activity lies between amino acids 458 and 547 of Sendai (equivalent to aa 477-567 of HPIV3) as suggested by Blumberg (1985), then the loss of 1 cysteine might alter conformation sufficiently to affect HA function. Activity would not be seen with the *Bgl* construct since it truncates at amino acid 418, removing 6 cysteines and the entire "HA domain". The cysteine at position 571 could possibly be involved in the formation of a loop that functions in haemagglutination.

Thompson *et al* (1988) have isolated a biologically active, soluble HN from Sendai by enzymatic cleavage of detergent solubilized virus particles. The cleavage was at amino acid 131 (equivalent to aa 130, HPIV3) in the stalk region, and the globular head retained full neuraminidase activity. With the results presented here, this isolates the catalytic site to between amino acids 130 and 212. Thompson (1988) was hoping that the soluble protein could be crystallised and used for X-ray crystallography. To date, no such information has been published, but it would be useful to conclusively solve the structure-function relationship debate about HN.

The conclusions drawn from the data presented in this thesis suggest that sites involved in HA and NA activity of the HN protein of HPIV3 are distinct and

well separated. The HA activity maps to the carboxy terminus of the protein while the NA activity maps near the amino terminus, close to where the stalk region and globular region meet. In functional terms, this makes good sense. The attachment portion of the protein is well away from the membrane to which it is bound where it is most likely to be able to interact with a receptor molecule. The neuraminidase activity is kept well away from the attachment region so it does not cleave receptors. It is also situated close to the membrane to which the HN protein is bound so that it can cleave sialic residues on the viral membrane to stop aggregation of viruses which would reduce the efficiency of viral release and spread.

Obviously, further work is required to confirm the conclusions presented here. Finer mapping of the two putative active regions is required. The functional assays should be made more sensitive, including identifying any other HPIV3 proteins that may be required for optimum HN function. Specifically, the mutants should be retested, the effects of freezing explored more thoroughly and the cell background should be minimized. It would also be useful to know if the mutant proteins expressed from vaccinia virus are expressed at the surface of infected cells. This could be done using immunofluorescence or by doing the neuraminidase assay on whole cells, rather than lysate. If the problems with the *Rsa* I enzyme can be solved, it would be interesting to analyse the function of the mutant protein expressed by vaccinia virus carrying an HN gene with the internal *Rsa* deletion, since it could confirm that an intact carboxy terminus results in a functional haemagglutinin.

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APPENDIX 1: BUFFERS AND SOLUTIONS**2X BBS**

50 mM BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid)
280 mM NaCl
1.5 mM Na₂HPO₄
adjusted to pH 6.96 with HCl

CIP BUFFER (CALF INTESTINAL PHOSPHATASE)

50 mM Tris-HCl, pH 9.0
1 mM MgCl₂
0.1 mM ZnCl₂
1 mM spermidine

ELECTROELUTION BUFFER

20 mM Tris-HCl, pH 8.0
200 mM EDTA
5 mM NaCl

LIGASE BUFFER

50 mM Tris-HCl, pH 7.4
10 mM MgCl₂
10 mM dithiothreitol
1 mM spermidine
1 mM ATP
0.1 mg/ml BSA

LURIA BROTH (LB)

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl

NICK TRANSLATION BUFFER

50 mM Tris, pH 7.2
10 mM MgSO₄
0.1 mM Dithiothreitol
50 µg/ml Bovine Serum Albumin

POLYNUCLEOTIDE KINASE BUFFER

50 mM Tris-HCl, pH 7.6
 10 mM MgCl_2
 5 mM dithiothreitol
 0.1 mM spermidine
 0.1 mM EDTA

PBS (PHOSPHATE BUFFERED SALINE)

140 mM NaCl
 2.5 mM KCl
 8.0 mM Na_2HPO_4
 1.5 mM KH_2PO_4
 0.8 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
 0.5 mM MgCl_2

P-MEDIUM

20 mM KPO_4 , pH 7.0
 15 mM $(\text{NH}_4)_2\text{SO}_4$
 1.8 μM FeSO_4
 10 mM MgSO_4
 1.0% Casamino acids
 0.2% Glucose

RIPA BUFFER

50 mM Tris-HCl, pH 7.2
 150 mM NaCl

2X DETERGENT

0.2% SDS
 2% Na-deoxycholate
 2% Triton X-100

Just before use add phenylmethylsulphonyl fluoride and Benzamidine-HCl to 1 mM

2X SDS PAGE SAMPLE BUFFER

4% SDS
 20% Glycerol
 10% β -Mercaptoethanol
 66 mM Tris-HCl pH 6.8
 0.1% Bromophenol Blue

SDS-PAGE RUNNING BUFFER

25 mM Tris
 192 mM Glycine
 0.1% SDS

SOLUTION I (PLASMID PREPARATION)

50 mM Glucose
25 mM Tris-HCl, pH 8.0
10 mM EDTA

SOLUTION II (PLASMID PREPARATION)

0.2 N NaOH
1% SDS

TBE (Tris-Borate Electrophoresis Buffer)

89 mM Tris
89 mM Boric Acid
2 mM EDTA

TBS (TRIS-BUFFERED SALINE)

137 mM NaCl
5 mM KCl
5.6 mM Glucose
0.7 mM Na_2HPO_4
25 mM Tris-HCl, pH 7.2

TE

10 mM Tris, pH 8.0
1 mM EDTA

TRANSFER BUFFER FOR WESTERN BLOT

25 mM Tris
192 mM Glycine
20% Methanol

2YT

1.6% Tryptone
1.0% Yeast Extract
0.5% NaCl

APPENDIX 2: SEQUENCES OF OLIGONUCLEOTIDES**STOP LINKERS FOR TRUNCATIONS:**

STOP-B: TAGGATCCTA (Bgl, Xho)

STOP-HB: AGGGATCCCT (Hind)

OLIGONUCLEOTIDE PRIMERS FOR SEQUENCING:

HN180: TATGGACAATAATCCTGGTG

HN415: GACACAACAAATGTCGGATC (Xho)

HN700: AATTACTCGAGGTTGCCAGG (Rsa)

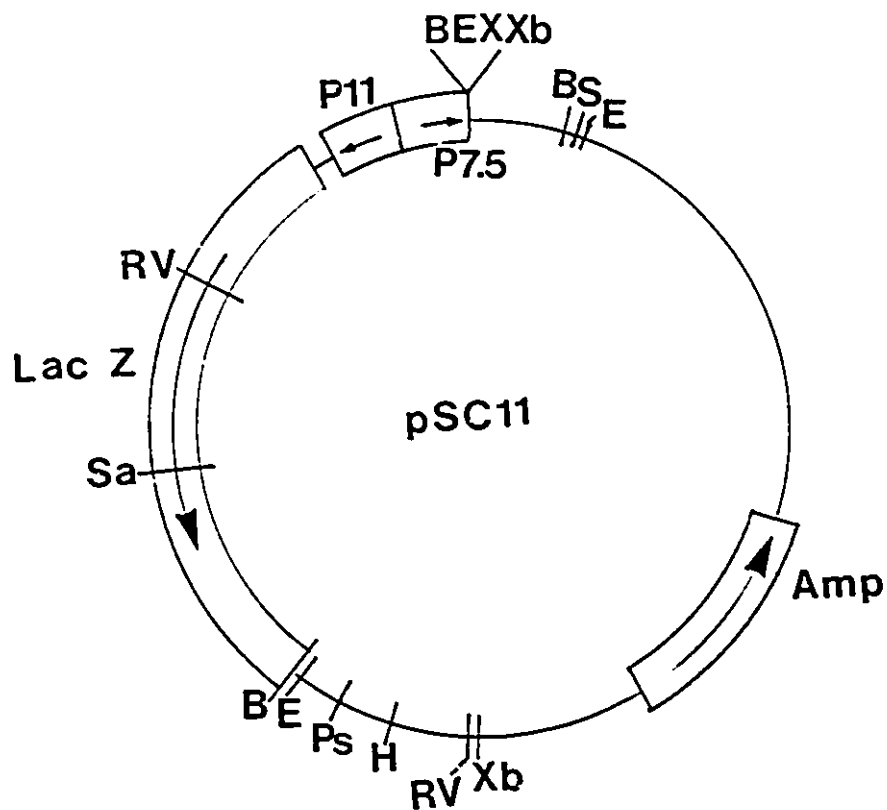
HN944: GATATTGTCAATCATGATGG

HN1191: CAGACAGAAGGATGGTCAAC (Bgl)

HN1430: AATGTGTTATCAAGACCAGG

HN1659: GATATACAACAACGAGCTGC (Hind)

APPENDIX 4: RESTRICTION MAP OF pSC11



LEGEND

P11--constitutive promoter,
vaccinia virus

P7.5--constitutive promoter,
vaccinia virus

Lac Z-- β -Galactosidase gene

Amp--ampicillin resistance gene

S-Sma I; B-Bam H1; E-Eco R1; X-Xho I
Xb-Xba I; RV-Eco RV; Sa-Sac I;
Ps-Pst I; H-Hind III