A Mutational Analysis of Structural Determinants Within the Newcastle Disease Virus Fusion Protein: a Dissertation

Julie N. Reitter
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A MUTATIONAL ANALYSIS OF STRUCTURAL DETERMINANTS WITHIN THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN

A Dissertation Presented
By
Julie N. Reitter

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

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April 1994
Molecular Genetics and Microbiology
A MUTATIONAL ANALYSIS OF STRUCTURAL DETERMINANTS WITHIN THE
NEWCASTLE DISEASE VIRUS FUSION PROTEIN

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April 1994
This work is dedicated to my family, John and Bette, Anne, Becky and Trudy.
ACKNOWLEDGEMENTS

I would first like to thank my advisor, Trudy Morrison, for her support, guidance, and patience during the years that I have been a student in her lab. I also want to extend my appreciation to her for the expert editing of my numerous drafts and for the independence that allowed me to freely explore. I would next like to thank all the members of MGM whose advice and assistance were invaluable. I also would like to express my gratitude to members of my advisory committee, RG, DT, RW, MP, HR, and TP, for their efforts on my behalf. Also, Bob, Jane, and Pete, who made my entrance into graduate school possible. I also want to acknowledge my appreciation to the past and present people of the Morrison lab, Cheryl for taking the time to share her skills with me, and Lori, Cathy, Tia and Judy for sharing their lab benches and good humor with me. Finally, I would like to thank Mark, friends, and family who provided me with many enjoyable diversions.
The fusion protein of the Newcastle Disease Virus (NDV) contains three hydrophobic domains. To explore the topogenic signals of these domains, mutants were constructed in which each of the hydrophobic domains was deleted. The membrane insertion and topology of these proteins was characterized in a wheat germ cell-free translation system supplemented with canine microsomal membranes. The results indicated that the first 13 amino acids of the fusion protein are necessary to confer translation inhibition by SRP. Translocation of the nascent chains containing all or part of the first hydrophobic sequence resulted in the appearance of a species of higher molecular weight consistent with glycosylation of at least four of the five potential N-linked glycosylation sites. When glycosylation was inhibited with a glycosylation competitor peptide, signal sequence cleavage was detected. Protease digestion of mutants missing the C-terminal hydrophobic domain indicated that the C-terminus has stop transfer activity. A comparison of membrane insertion of the wild-type fusion protein to that of a mutant missing the second hydrophobic domain, the fusion sequence, indicated that the fusion domain has stop-transfer activity when synthesized in vitro. Furthermore, the fusion domain shows little signal sequence activity when positioned near the amino terminus of the fusion protein.

The fusion protein has a highly conserved leucine zipper motif immediately upstream from the transmembrane domain of the F1 subunit. In order to determine the role that the conserved leucines have for the oligomeric structure and biological activity of the NDV fusion protein, the heptadic leucines at positions 481, 488, and 495 were changed individually and in combination to an alanine residue. Whereas single amino acid changes had little effect on fusion, substitution of two or three leucine residues abolished the fusogenic activity of the protein although cell surface expression of the mutants and sedimentation in sucrose gradients was similar to that of the wild type. Furthermore, deletion of the C-terminal 91 amino acids, including the leucine zipper motif and transmembrane domain resulted in secretion of an oligomeric structure. These results indicate that the conserved leucines do not play a role in oligomer formation but are required for the fusogenic ability of the protein. When the polar face of the potential alpha helix was altered by nonconservative
substitutions of a serine-to-alanine (position 473), glutamic acid-to-lysine (position 482) or an asparagine-to-lysine (position 485), the fusogenic ability of the protein was not significantly disrupted.

A phenylalanine residue is at the amino terminus of the F1 protein of all paramyxovirus fusion proteins with the exception of the avirulent strains which have a leucine residue in this position. To explore the role of this phenylalanine in the fusion activity of the protein, this residue was changed to leucine (F117L) or to glycine (F117G) by site-specific mutagenesis while maintaining the cleavage site sequence of virulent strains of NDV. Whereas both the wild-type and the F117G proteins were proteolytically cleaved and F1 was detected, the leucine subsitution abolished cleavage. When co-expressed with the HN protein, the fusion protein with either a phenylalanine and glycine residue at position 117, but not a leucine, was shown to stimulate membrane fusion. However, incubation in trypsin activated the fusion activity of the F117L protein. Thus the presence of a leucine at position 117 of the precursor sequence blocks cleavage, but not fusion activity, and indicated that the phenylalanine at the amino terminus of the F1 subunit is not conserved for the fusion activity of the protein.
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CHAPTER I

INTRODUCTION

Newcastle disease was initially recorded near Batavia, Java, in Indonesia prior to 1926, where it caused a fatal respiratory and nervous system disease. The virus was next detected on a poultry farm near Newcastle-on-Tyne, England and then spread throughout the continent (reviewed in Shope, 1964). When the disease erupted, none of the infected birds survived and the disease would often temporarily disappear. In 1936 the same virus mysteriously appeared in the United States. The disease was much milder than had occurred in other countries and was not identified for many years. Whereas the original strains were very virulent with a mortality rate near 100%, the American version had attenuated virulence. During this time, poultry production was increasing in importance and economic pressures led to the study of the virus (Brandly, 1964).

The virus has now been noted in virtually every country in the world, possibly due to the wide variety of reservoir hosts (Roberston, 1964). The virus can infect virtually all of the 8,000 species of wild and domestic birds, making it a very important pathogen (Kaleta, 1988). The strain of Newcastle disease virus (NDV) most frequently identified throughout this country is the B₁ isolate. Due to ease in disseminating a live virus, this avirulent strain is used to vaccinate poultry flocks against virulent isolates. This continuing practice perpetuates the viral infection and may also allow for the possible emergence of more pathogenic isolates from the avirulent vaccine strains (Hitchner, 1964).
NDV is a member of the *Paramyxoviridae* family. This family of viruses is structurally similar to orthomyxovirus and rhabdovirus families. All have a negative strand RNA genome protected by a helical nucleocapsid and a lipid bilayer membrane. The single strand genome of the paramyxoviruses and rhabdoviruses is nonsegmented whereas that of the orthomyxoviruses is segmented.

The *Paramyxoviridae* family is divided up into three genera based on the possession of hemagglutination and neuraminidase activities (described in Kingsbury, 1990). Members of the *Morbillivirus* genus lacks neuraminidase activity and include the measles and canine distemper viruses. Members of the *Pneumovirus* genus lack both neuraminidase and hemagglutination activities and include the respiratory syncytial virus. Members of the *Paramyxovirus* genus possess both neuraminidase and hemagglutination activities and include Sendai, human parainfluenza, mumps, SV5 and Newcastle disease viruses.

The envelope of the NDV virion is studded with spike structures made up of the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. The hemagglutinin activity of the HN protein is believed to mediate attachment of the virion to the target cell (reviewed in Morrison and Portner, 1991). The protein binds to cellular sialic acid-containing receptors and can cause agglutination of erythrocytes. The neuraminidase activity allows the virion to cleave sialic acid. This activity presumably prevents the virions from aggregating with sialic acid-containing glycoproteins on neighboring virions. The other spike protein, F, mediates fusion of the viral membrane with that of the target cell membrane and fusion of an infected cell with an adjacent cell (Bratt and Gallagher, 1969).

After the virion attaches to its target cell, the nucleocapsid must enter the cell for replication to occur. This crucial step involves fusion of the viral membrane with the membrane of the target cell and release of the nucleocapsid
directly into the cytoplasm (Kingsbury, 1990). This fusion reaction is mediated by the virion surface proteins and occurs at a neutral pH at the plasma membrane (reviewed in White, 1990, and Kielian and Jungerwirth, 1990). Whereas HN is required for fusion, the F protein is thought to directly mediate the membrane fusion event (Scheid and Choppin, 1974). Once the nucleocapsid is inside the cytoplasm, it uses the biosynthetic machinery of the cell to begin generation of new virus particles (reviewed in Peeples, 1988). The NDV genome is replicated in the cytoplasm and serves as a template for synthesis of the viral mRNAs.

**Hydrophobic domains.** The amino acid sequence of a protein encodes all of the information required for proper folding and post-translational modifications necessary for biological activity. For membrane proteins, determinants within the sequence direct membrane targeting and integration and are often hydrophobic segments within the protein (reviewed in Lingappa, 1989, and von Heijne, 1988). The amino acid sequence of the two NDV spike proteins predicted hydrophobic segments near the amino-termini of both proteins. The single hydrophobic domain of the HN protein is located near the amino terminus. The F protein contains three hydrophobic domains. One domain is located near the amino terminus, another near the carboxy terminus, typical of type I integral membrane proteins, and a third hydrophobic domain is located at an internal position within the nascent protein.

The amino-terminal hydrophobic segment of integral membrane proteins is often a signal sequence. The function of this signal has been analyzed in cell-free systems that reproduce the steps required for translocation of nascent proteins across the ER membrane (Erickson and Blobel, 1983). These signals are recognized cotranslationally by a protein-RNA complex termed the signal recognition particle (SRP; Walter and Blobel, 1981a, reviewed in Walter and Lingappa, 1986, and Rapoport, 1992). This interaction has been shown to arrest
translation in a wheat germ cell-free system (Walter and Blobel, 1981c). The SRP-signal complex directs the ribosome to a SRP receptor in the ER membrane (Gilmore et al, 1982; Walter and Blobel, 1981b). SRP is released in a GTP-dependent manner, protein synthesis resumes, and the growing polypeptide is translocated across the ER membrane (Connolly and Gilmore, 1989; Walter and Blobel, 1981c). Protein domains on the luminal side of the ER are eventually exposed on the outside of the cell. The ultimate orientation of the protein in the plasma membrane is determined by the method of insertion into the ER membrane.

According to the loop model, amino terminal signal sequences are inserted into the ER membrane as a hairpin loop with the N-terminus of the signal remaining on the cytoplasmic side (Engelman and Steitz, 1981). The final topology of the protein is determined by the presence or absence of a cleavage site. Cleavage of the signal (also known as leader peptide) allows release of the N-terminus from the membrane. Proof for this loop model was provided by Shaw and coworkers who have shown that mutations of the VSV G protein cleavage site resulted in a protein that was anchored by the signal sequence (1988). An additional mutant with an extension of the amino-terminus indicated that the extreme amino-terminal residues were cytoplasmically exposed.

Cleavable signal sequences display a remarkable lack of primary sequence homology. A comparison of many signal sequences revealed no regions of strict homology, outside of the cleavage site (von Heijne, 1983). However, several characteristics have been identified. They usually have a tendency to take up an alpha-helical structure in nonpolar environments (Gierasch, 1989). Von Heijne (1983, 1985) has shown that the signal consists of three regions. The amino terminus is variable in length and composition but carries a net positive charge. The internal region of the signal is approximately
10 residues in length and is very hydrophobic. The third region is immediately upstream from the cleavage site, and usually five to seven mainly polar residues comprise this C-terminal side of the signal.

Noncleavable signal sequences are functionally similar to cleavable signals but, due to the absence of a cleavage site, the segment also serves to anchor the protein in the membrane (Spiess and Lodish, 1986; Wickner and Lodish, 1985). The hydrophobic nature of a type II signal, and amino terminal location, are often sufficient for both membrane targeting and anchoring functions (Zerial et al., 1987). However, the residues adjacent to this segment are likely to influence the membrane orientation. Wilson and coworkers have shown that removal of 28 residues upstream of the type II signal in the NDV HN protein did not abolish recognition by SRP but resulted in reversal of the membrane orientation of the protein (Wilson et al., 1987). Also, an analysis of the sequences of known transmembrane proteins led to the proposal of the "positive inside rule" in which the most positively charged end of the protein is retained in the cytoplasm (von Heijne, 1986).

For integral membrane proteins, translocation is terminated when a hydrophobic stop-transfer sequence, often located near the C-terminus, enters the translocation site (Yost et al., 1983). The mechanism by which translocation is terminated by these segments is unknown. Twenty-two amino acids are required to span a bilayer as an alpha-helix, and membrane-spanning segments are on average 24 residues long (Gierasch, 1989; Lodish, 1988). The transport channel could contain a receptor for stop-transfer sequences and may disassemble to allow integration of membrane proteins (Gilmore, 1993; Mize, 1986). Alternatively, termination may be nonspecific and only a consequence of hydrophobicity. Davis and Model (1985) have used artificial hydrophobic sequences to demonstrate that the hydrophobicity of a segment suffices to stop
transfer of the coliphage pIII membrane protein. The hydrophobic noncleavable signal sequence is structurally similar to the stop-transfer sequence. The most significant difference between the two segments appears to be the location within the protein.

Integral membrane proteins that span the lipid bilayer one time are classified as type I, type II or type III (von Heijne, 1988). Type I proteins contain the N-terminal cleavable signal sequence. The final topology is achieved by cleavage of this signal and membrane integration by a C-terminal hydrophobic stop-transfer sequence. Type II proteins also have an amino-terminal or internal signal sequence, but this hydrophobic segment is not cleaved and serves to both target and anchor the protein (Spiess and Lodish, 1986). Following insertion of type II signals, and in the absence of a C-terminal anchor sequence, continued elongation leaves the amino terminal residues exposed to the cytoplasm and the C-terminal portion of the protein completely translocated into the lumen of the ER. The unusual type III topology is achieved by an internal uncleaved signal-anchor segment that adopts an orientation opposite to that of type II proteins: the carboxy terminus remains on the cytoplasmic side of the membrane.

Multi-spanning proteins achieve their topology by a series of signal sequences and membrane-anchoring stop-transfer sequences (Rothman et al, 1988). Insertion is initiated by the first signal sequence in an SRP-dependent manner and only this first hydrophobic domain may be recognized by SRP (Wessels and Spiess, 1988). Additional translocation following membrane integration is facilitated by subsequent hydrophobic segments and may occur spontaneously (Wickner and Lodish, 1985) or due to the alternating action of uncleaved signal and stop transfer sequences (Rothman, 1988).

The NDV fusion protein, as well as many other viral fusogenic glycoproteins, contains a third hydrophobic domain denoted the fusion peptide
Like stop-transfer and type II signals, these fusion peptides contain over 20 uncharged residues and have the potential to form an alpha-helix in the presence of lipids (White 1990; Brasseur, 1990). However, this segment is completely translocated across the ER membrane as indicated by exposure on the surface of infected cells and secretion in the absence of the C-terminal anchor (personal observation, Chapter III).

The mechanism by which the fusion peptide fails to halt translocation has been explored by several laboratories. Paterson and Lamb (1987) examined the ability of the position to influence its membrane interactions. When the C-terminus of an influenza hemagglutinin (HA) molecule (a type I protein) was replaced with the SV5 fusion peptide and expressed in eukaryotic cells, immunoprecipitation indicated that this chimeric protein was retained within the cell as an integral membrane protein. In its natural position, the wild-type SV5 fusion domain was completely translocated. This result suggested that the fusion domain is sufficiently hydrophobic to function as a membrane anchor when at the C-terminus of a protein but not in an internal position.

In similar work, Davis and Hsu (1986) inserted the fusion peptide from the Sendai F protein into an internal position of a secreted version of the coliphage pIII protein and found by immunoprecipitation that the fusion peptide also failed to stop transfer in a bacterial expression system. This result led the authors to suggest that the fusion peptide in viral proteins has evolved to be compatible with translocation by keeping the overall hydrophobicity below an unknown threshold.

Although these studies indicated that the fusion peptide can be translocated, they may not have detected all populations of the protein. Fusion proteins that are anchored by the fusion peptide would likely be misfolded and underglycosylated and may not be detected by antisera generated against mature molecules. In both studies, protease digestion of proteins exposed on the
surface of isolated microsomal vesicle or bacterial spheroplast membranes was used for determination of membrane topology. Unless the antisera used for immunoprecipitation recognized the epitopes of the protein upstream of the fusion peptide, this potential population may not have been detected by the subsequent immunoprecipitation. Neither work addressed this issue.

An alternative system that overcomes these limitations when examining the topology of a protein is to utilize a cell-free translation system. Translation that employs cytosolic extracts from wheat germ allows the translation of a single species of mRNA to direct the synthesis of a single species of labeled protein. When this translation system is also supplemented with microsomal membranes derived from the ER, the proteins are translocated in a manner identical to that observed in vivo (Katz and Lodish, 1979). Protein translocation can be identified by post-translational processing reaction such as the addition of core oligosaccharides and by signal sequence cleavage. The topology with respect to the ER membrane can be determined by treatment with a protease to identify protected and exposed domains. With these tools, all resulting topological species can be examined.

Precursor processing. While the precursor fusion protein is in the ER, oligosaccharides are added, the molecule folds and intramolecular disulfide-linked bonds form (McGinnes et al, 1985). During transport through the Golgi apparatus, the molecule undergoes a disruption of intramolecular disulfide bonds and a conformational change (McGinnes et al, 1985; Morrison et al, 1987). The precursor is often cleaved by host cell proteases and generates two disulfide-linked polypeptides, F₁ and F₂ (see Figure 13; Scheid and Choppin, 1977; Gotoh, 1992). Detergent binding and spectroscopic studies have indicated that cleavage of the Sendai virus fusion precursor causes a conformational change, exposes previously solvent-inaccessible hydrophobic domains, and causes an
increase in the alpha-helical nature of the protein (Hsu et al, 1981). This cleavage positions the fusion peptide at the extreme amino terminus of the F₁ subunit and is essential for the fusogenic activity of the protein (Scheid and Choppin, 1974, 1977; Gorman et al, 1988).

The fusogenic proteins from the orthomyxovirus, retrovirus, and alphavirus groups also are made as precursors (reviewed in White, 1990). For the fusion proteins of orthomyxovirus, paramyxovirus and retrovirus, the transmembrane subunit retains the fusion peptide and associates with the amino-terminal subunit through disulfide bonds or non-covalent interactions. Susceptibility to cleavage is often a major determinant of the virulence of a virus (Webster and Rott, 1987). The readily cleaved fusion proteins from virulent paramyxo- and orthomyxoviruses contain two pairs of basic residues which are recognized by the ubiquitous Golgi-associated protease furin (Gotoh, 1992; Barr, 1991; Steineke-Grober, 1992). The avirulent strains have single basic residues at the cleavage site and require a trypsin-like protease for activation in culture (Nagai and Klenk, 1977; Gotoh, 1990).

**Fusion activity.** Eukaryotic expression of cloned viral glycoproteins has allowed the assignment of specific biological activities to the individual paramyxovirus glycoproteins. When the SV5 F cDNA was expressed in CV1 cells, the F protein caused syncytium formation (Horvath and Lamb, 1992). However, accumulating evidence suggests that the remaining paramyxoviruses require both an attachment and a fusion protein to induce cell-to-cell fusion. Expression of the F and HN proteins of the bovine parainfluenza 3 virus from a vaccinia virus vector indicated that the proteins must be coexpressed (Sakai and Shibuta, 1989). In addition, the measles virus, mumps virus, HPIV-3, HPIV-2 and NDV have shown a requirement for coexpression of F and HN (or H of the morbilliviruses) (Wild et al, 1991; Tanabayashi et al, 1992; Ebata et al, 1991; Hu
and Compans, 1992; Morrison et al, 1991). Furthermore, mixing experiments of cells expressing F with those expressing HN suggested that the two glycoproteins must be expressed in the same cell (Morrison et al, 1991).

The results from these studies led to a determination of the specificity of the HN fusion-promotion activity. Coexpression of the NDV F and the influenza hemagglutinin proteins did not induce fusion, indicating that more than an attachment function is required of the HN protein (Morrison et al, 1991). When expressed from a vaccinia virus vector, the human parainfluenza virus type 2 (PIV2) HN could not function as a substitute for the genetically similar parainfluenza type 3 (PIV3) HN protein (Hu et al, 1992). Similarly, coexpression of the NDV F and SV5 HN from an SV40 vector could not induce fusion (Horvath et al, 1992). These reports led to the suggestion that a specific interaction occurs between the F and HN proteins to mediate cell fusion (Hu et al, 1992; Sergel et al, 1993).

**Functional domains.** The question of which domains of the HN molecule participate in fusion promotion have been the subject of several investigations. A deletion mutant lacking nine amino acids at the base of the presumed stalk region was deficient in fusion promotion but not hemadsorption (Sergel et al., 1993a). Iorio and coworkers (1992) have shown that monoclonal antibodies against HN residues 263, 287, and 321 inhibit fusion but not attachment. An escape mutant of Sendai virus (SV) HN with a substitution at residue 420 was selected with a monoclonal antibody that inhibits fusion (Portner et al, 1987), while a change at position 539 severely diminished the ability of a PIV3 HN protein to promote fusion (Sakai and Shibuta, 1989). In contrast, early studies with the fusion protein have focused on one region that appears to be directly involved in the fusion event. Labeling with hydrophobic photoaffinity probes has shown that the Sendai virus and influenza fusion peptides directly interact with
target lipid bilayers (Novick and Hoekstra, 1988; Harter et al., 1989). Synthetic peptides that mimic the fusion peptide sequence inhibit both cell fusion and virus penetration (Richardson and Choppin, 1983; Richardson et al, 1980).

The significance of the fusion peptide in the fusion event is further implied by its presence within the transmembrane subunit of fusogenic proteins from diverse families of enveloped viruses (reviewed in White, 1992). In addition, a putative fusion peptide has been identified within the fusogenic sperm glycoprotein, PH-30 (Blobel et al, 1992). White (1992) has identified several general properties of fusion peptides. The segments average 28 residues for proteins with an amino-terminal fusion peptide and have an average hydrophobicity value between signal (low) and transmembrane (high) sequences. Most fusion peptides have a potential to form 'sided' helices with a segregation of polar and nonpolar residues. The amino acid sequences of fusion peptides are highly conserved within a virus family but not between families (White, 1992). This suggests that the particular amino acids may have a nonsequence specific function in the fusion event. When the requirement for sequence conservation was tested for a paramyxovirus, a functional assay indicated that conservative substitutions can be tolerated while nonconservative changes often disrupt cellular transport of the molecule (Horvath and Lamb, 1992). In addition, changes that were expected to increase the alpha helical nature of the peptide increased the fusion activity. Thus, hydrophobicity and potential to form an alpha helix appear to be the only structural constraints placed on this segment.

Evidence from several laboratories indicates that regions other than the fusion peptide also may have critical roles for the fusogenic activity of the F protein. Both Toyoda et al. (1988) and Portner et al. (1987) have shown that monoclonal antisera against a cysteine rich region of the NDV and SV fusion
proteins, respectively, can inhibit fusion. Viral membrane proteins, as well as most cellular glycoproteins, contain numerous disulfide bonds which are critical for folding and stability of the mature protein (Doms et al., 1993). Paramyxovirus fusion proteins contain a cluster of seven conserved cysteine residues approximately 100 residues upstream from the transmembrane domain that may form a series of cross-linked loop structures (Morrison and Portner, 1991). The importance of this conserved cysteine-rich region in fusion was indicated when synthetic peptides that inhibited fusion allowed the isolation of a mutant measles virus with changes only in this cysteine region of F (Hull et al. 1987).

An additional determinant of fusogenic activity for F proteins may be located downstream from the cysteine-rich region and adjacent to the transmembrane domain. This sequence consists of a series of leucines occurring every seven residues and another nonpolar residue in every fourth position (see Figure 13; Buckland and Wild, 1989; Lamb, 1993). A comparable repeat sequence is similarly positioned within the coronavirus fusogenic protein (Britton, 1991). If these regions are displayed on an alpha-helical wheel, the leucines and the fourth position amino acid align along one face of the helix, and an amphipathic nature of the helix is evident. This series of leucines is highly conserved within the family of Paramyxoviridae and resides against a background of apparently nonconserved residues. Mutational analysis by Buckland and coworkers (1992) has indicated that maintenance of the leucine residues is necessary for the biological activity, but not the oligomeric structure, of the measles virus fusion protein.

The NDV fusion proteins contain a second potential alpha-helical structure that may play a role in fusion (Wang et al., 1992; Chambers et al., 1990). The segment is made up of six heptad repeats and occurs immediately downstream from the fusion peptide. A potential helix is similarly placed within the fusion
proteins of coronaviruses, influenza viruses and retroviruses (Chambers et al, 1990). Mutations in this heptad repeat (a leucine zipper motif) of the HIV 1 env protein inhibited syncytium formation and infectivity (Chen et al, 1993). The repeat sequences within these proteins are predicted to form long amphipathic alpha-helices (Chambers et al, 1990; Delwart et al, 1990) and may obtain a coiled coil conformation through association of the nonpolar faces (Harbury et al, 1993). X-ray crystallography studies have indicated that this helix of the nascent influenza HA protein points toward the virion before acidic pH triggers a conformational change (reviewed in Wiley and Skehel, 1987). This action may relocate the fusion peptide toward the target membrane and allow formation of a three-stranded coiled coil (Carr and Kim, 1993). This inducible conformational change suggests that the native form of the HA molecule is a metastable structure (Lamb, 1993). The fusion proteins of the paramyxoviruses, as well as the coronaviruses and some retroviruses fuse membranes in a pH-independent manner and the trigger for this activation is unknown (White, 1990). However, by analogy with the HA protein, this potential helical structure within the NDV fusion protein may function to correctly position the fusion peptide.

Surface glycoproteins of virions are often subject to immune surveillance and usually evolve sequence variation to avoid this attack. The evolutionary conservation of these determinants within the fusion proteins of the Paramyxoviridae family suggests that the amino acids in these regions are dedicated to structural or functional roles of the mature protein and substitutions may be lethal (Kingsbury, 1990). A number of these conserved elements of the NDV fusion protein are examined in the following chapters of this thesis.
CHAPTER II

Materials and Experimental Methods

Viruses. The strain Australia-Victoria (AV), a virulent NDV strain, was propagated and purified as described previously (Hightower and Bratt, 1975).

Cells. Cos-7 cells were obtained from the American Type Culture Collection. They were maintained in dulbecco’s modification of Eagle’s medium (D-MEM) supplemented with 5% complement-inactivated fetal calf serum, 4.5 gm/l glucose, nonessential amino acids, vitamins, 2 mM glutamine, 0.17% NaHCO₃, penicillin/streptomycin, and 20 nM sodium selenite.

Antibodies. Fu1a is a monoclonal antibody that was generated against a disrupted virion preparation enriched in the matrix protein and was a generous gift of Dr. Mark Peeples. This antibody has been shown to react with mature F protein, but not the nascent molecule (Morrison et al., 1987). Anti-NDV antibody was raised in rabbits against UV-inactivated NDV virions (strain AV) and was also shown to react with only mature protein (Sergei et al., 1993). Anti-F was raised against a peptide consisting of the 29 C-terminal residues of the protein and was a gift from Dr. Mark Peeples (Wang et al., 1992) and was also generated in the University of Massachusetts Medical Center (UMMC) facility.

Site Directed Mutagenesis. To construct the single-stranded DNA template, the F gene was excised from pSP6Fst (clone 15) by using the XbaI and Sacl sites in the cloning cassette positioned at the 5' and 3' ends of the coding sequence,
respectively. This fragment was ligated into M13mp18 and used to transform TG1 (E. coli) cells. A single, white, phage plaque was picked and grown on TG1 cells in 2 x TY medium (16 g/l bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl). The resulting phage stock was precipitated with PEG 6000, the protein coat was removed by phenol extraction and the DNA was precipitated in sodium acetate and isopropanol. The resulting template positioned the F gene in the negative sense direction. Positive-sense oligonucleotides were synthesized by the DNA Facility of UMMC. The oligonucleotides used for mutagenesis were, starting with the 5-prime nucleotide: (L3A)GATAAGGCAGAGGAAAG; (L4A)CAGCAAAGCAGACAAAGTC; (L5A) CAATGTCAAAGCGAACAG; (dC49) CATTACCTAGATCGCTTT; (S473A) CAACAAACGCAGATAATGC; (N485K) GGAAAGCAACAGCAAAC; (Fg) CAACATAAACACCTCATCC; (E482K) GGATAAGTTAAGGAAAGC. The oligonucleotides were phosphorylated and annealed to the template. Oligonucleotide-directed in vitro mutagenesis of M13mp18 DNA was achieved by methods and reagents from Amersham. The L3A mutant template was mutated further to create the L34A and L35A mutants. Similarly, the L4A template was used to produce the L45A and, subsequently, the L345A mutants. Following synthesis of the mutant DNA strand and the complementary DNA strand, the extraneous single-stranded DNA was removed, and the resulting RF DNA was used to transform TG1 cells. The resulting phage DNAs were screened for the desired mutation by sequencing. A 564 bp restriction fragment (AvaI- Sacl) containing the desired leucine mutations was ligated in the remainder of the wild-type F gene and the entire fragment was sequenced. The AvaI-Sacl fragment from L345A was ligated into the remainder of A140K and K155L to produce the A140K/L345A and K155L/L345A mutants. Most cDNAs were shuttled between M13, pSVL and pSP6 via the Xbal and Sacl sites positioned in the cloning cassette at the 5' and 3' ends of the F gene, respectively.
Sequencing. The nucleotide sequences of mutants were verified by sequencing methods and reagents from United States Biochemical. The template DNA was denatured, an oligonucleotide primer was annealed upstream from the region of interest, and DNA synthesis was initiated off the primer by the bacteriophage T7 DNA polymerase (Sequenase, United States Biochemical). Extension of the complementary strand occurred in the presence of [α-35S]-dATP. The labelling reactions were then terminated with a dideoxynucleoside triphosphate of either GTP, CTP, ATP, or TTP, which lack the 3' hydroxyl group necessary for DNA chain elongation. The reactions were stopped by the addition of sample buffer, denatured and resolved on a 6% sequencing gel.

Plasmid Constructions. Full-length cDNA containing the entire coding region of the NDV fusion gene (strain Australia-Victoria or AV) was assembled from two overlapping clones, pTL831 and pTL438, using a common Xmal restriction site as shown in Figure 1A. These clones were derived and characterized as previously described (McGinnes et al., 1986). The resulting pTLF contained G-C tails at the 5' and 3' ends generated by the cloning protocols and were considered likely to be detrimental in cell-free transcription. The G-C tails were removed using a BamHI site and a MnII site located in the 5' and 3' noncoding regions, respectively. Figure 1B outlines the method used to remove the 5' and 3' G-C tails to produce the pSP6Fst cDNA. From pTLF, the BamHI-BamHI (52 nucleotides), the BamHI-Sphl (1545 nucleotides) and the Sphl-MnII (97 nucleotides) fragments were ligated into an pSP64 vector cut with BamHI and Smal. The mutant pSP6F13 cDNA resulted from the subsequent deletion of the 52 nucleotide BamHI restriction fragment.

The mutant pSP6SF (sans fusion domain) was generated by deleting the sequence between the sites at nucleotides 167 and 446 in pSP6Fst. From
pSP6Fst, the 1335 basepair Pvull (at nucleotide 446) to EcoRI fragment, the 1621 base pair EcoRI-Pvul vector fragment, and the 1508 basepair Pvul to Pvull (at nucleotide 167) fragment were ligated. The 93-codon fragment removed from the fusion protein sequence encodes amino acids 39 through 131 and includes the first 15 of the 26 amino acid hydrophobic fusion domain found at the amino terminus of F₁.

The plasmid pSP6FSS (sans signal sequence) was constructed by removal of a 162 nucleotide PstI-PstI restriction fragment from pSP6Fst. The construct deleted 68 codons from the 5' end of the wild-type fusion gene by forcing translation initiation off the third natural methionine codon.

The plasmid pSP6MF was constructed by placing the 5' region including the initiator methionine codon, from pSP6M (contains the NDV matrix protein cDNA; McGinnes et al., 1987) 42 nucleotides upstream from the coding region for the amino terminus of F₁. Construction involved cleaving at the Hinfl site at the fourth base of the coding sequence for the NDV matrix protein and ligating to the Hinfl site at nucleotide 362 of the NDV fusion protein cDNA. The 4-way ligation included the 1400 basepair pSP6 EcoRI-BglII piece, the BglII-Hinfl (at nucleotide 43) from pSP6M, Hinfl (nucleotide 362) to Haell (nucleotide 764) from pSP6Fst, and Haell (nucleotide 764) to EcoRI from pSP6Fst.

The pSP6dC91 plasmid (without leucine zipper motif) was constructed by deleting a 182 basepair fragment between the EcoRV and SphI restriction sites found at nucleotides 1438 and 1620, respectively. Plasmid SP6Fst was digested with SphI and the site was blunted with T4 DNA polymerase and further digested with BglII. Ligation of the 1478 nucleotide SphI-BglII fragment to the 3017 nucleotide pSP6Fst BglII-EcoRV fragment resulted in a frame-shift and a premature translation termination. This deletion substituted 91 codons from the carboxy
terminus of the parental plasmid, including the leucine zipper and the transmembrane cytoplasmic domains, with a new sequence (following the EcoRV site) coding for the amino acids LPNCQAKGATKDLVMAWE. The plasmid SP6SFdC91 was constructed similarly by ligating the BglII-EcoRV and SphI (blunted)-BglII fragments isolated from pSP6SF. This construct lacked the coding sequence for both the second and third hydrophobic domains.

For in vivo expression, the NDV genes were expressed in Cos cells using the SV-40 based expression vector, pSVL (Pharmacia). Most genes were ligated into the XbaI and ScaI sites of the cloning cassette. Ligations were incubated at room temperature for 4 hours in 50 mM Tris (Triis(hydroxymethyl)aminomethane), 10 mM MgCl₂, 0.1% BSA, 5 mM DTT, and 1 mM ATP and 1 unit of T4 DNA ligase (Boehringer Mannheim Corp.). Proper construction of all cDNAs was verified by sequencing as well as translation of mRNAs generated by transcription of the resulting DNA with SP6 polymerase (Krieg and Melton, 1987).

**DNA stocks.** HB101 bacterial stocks were transformed with plasmid DNA and grown from a single colony in a half liter of LB broth (10g/l bacto-tryptone, 5g/l yeast extract, 10g/l NaCl, 1mM NaOH) for 6 hours and amplified with chloramphenicol (85 mg) for an additional 12 hours. Bacteria were pelleted and the plasmid DNA was purified with the reagents and columns supplied with either the pZ523 (5 Prime - 3Prime, Inc.) or Qiagen maxi-prep purification kits.

**Cell Free Transcription and Protein Synthesis.** Full-length mRNA transcripts of each cDNA were generated by linearizing the plasmids downstream from the coding region with EcoRI. The purified DNA was transcribed with SP6 RNA polymerase (Promega) according to a modified procedure which included capping of the newly made transcripts (Krieg and Melton, 1987). Transcription reactions contained 0.5 mM each of ATP, CTP, and UTP; 0.1 mM GTP; and 0.4 mM
m7G(5')ppp(5')G. Translation reactions (25 µl) using 30% (vol/vol) nuclease-treated wheat germ extract and 18.75 µCi [35S]methionine followed previously described methods (Erickson and Blobel, 1983). Wheat germ extract was supplemented with 25 mM creatine phosphate, 1.8 mM ATP, 0.3 mM GTP, 0.18 mM of each amino acid excluding methionine, 25 mM HEPES (pH 7.8), 64 µg/ml creatine phosphokinase, 250 mM spermidine, 3.2 mM dithiothreitol (DTT), 0.0016% Nikkol, 7U/25 µl RNasin (Promega), 0.9mM MgOAc, and 20 mM KOAc. Translation reactions were incubated for 90 minutes at 24°C. Canine pancreatic membranes stripped of SRP (KRM) and SRP were isolated as described previously (Walter et al., 1981a) and were the generous gift of Dr. Reid Gilmore. One µl (1 pmol) of SRP or SRP buffer (50 mM Triethanolamine, TEA; 500 mM KOAc, 5 mM MgOAc, 0.01% Nikkol, 1 mm DTT) and 1.5 µl of KRM (1.5 equivalents) or KRM buffer (1 mM DTT, 250 mM sucrose and 50 mM TEA) were added to each 25 µl translation reaction. To quantitate SRP translation inhibition, the M protein mRNA, generated from M cDNA described previously, were added to a 50 µl reaction containing the second species of mRNA, and the mixture was split prior to the addition of SRP or SRP buffer. The tripeptide Asn-Tyr-Thr (NYT) is used to inhibit N-linked glycosylation and was synthesized and blocked as described previously (Welply et al., 1983). The acceptor tripeptide (gift from R. Gilmore) was added to membrane-supplemented reactions to a final concentration of 400 µM 10 minutes prior to the addition of the mRNA. Translation products (5 µl) were dissolved in 10 µl of a 6.25% SDS and 0.5 M Tris pH 8 solution. Prior to loading the gel, the samples were boiled in the presence of 0.1 M DTT for 5 minutes and alkylated with 56 mg/ml iodoacetamide. To determine whether transcription in the presence of translation affects protein translocation, a transcription-linked translation assay was developed. The transcription reactions using non-linearized plasmids were incubated at 41°C for 30 minutes. Two microliters from this reaction was added
directly to the translation mix and transcription-translation was allowed to occur for 90 minutes at 22°C. To compare results from the wheat-germ system to those of a mammalian system, a rabbit reticulocyte translation system was used following protocols supplied by the manufacturer (Promega).

**Protease Protection Assay.** Five microliter aliquots of each translation reaction were diluted to 25 μl with RSB (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris, pH 7.4) and proteinase K. Final enzyme concentrations ranged from 0.1-200 μg/ml and are indicated in figure legends. Digestions were for 60 minutes on ice and were stopped by adding phenylmethylsulfonyl fluoride (PMSF) to 10 mM. Samples were TCA-precipitated, resuspended in sample buffer, dissolved and boiled prior to loading half of each sample onto the gels. To test for leakiness of the vesicles, preprolactin mRNA isolated from bovine pituitary (gift from R. Gilmore) was added to the translation reaction.

**Transient Gene Expression.** Several systems were employed to express the cDNAs. For transfections requiring a high level of transfection efficiency, DEAE-Dextran was used following a modification of the method of Levesque, et al (1992). Cos cells were plated on 35 mm plates (Falcon Primaria) at 2.5 x 10⁵ cells 20 hours prior to transfection. The monolayers were 80% confluent at the time of transfection. Two micrograms of DNA were mixed with 0.5 ml of a solution of DEAE/Dextran (Pharmacia) suspended in Tris buffered saline (TBS; 25 mM Tris base, 137 mM NaCl, 5.1 mM KCl, 0.7 mM Na₂HPO₄; pH 7.4) at 0.5 mg/ml. The plates were rinsed once with TBS and the DNA was allowed to adsorb to the cells at room temperature for 20 minutes then incubated at 37°C for one hour. The DNA/Dextran mix was replaced with 1 ml of 100μM chloroquine in OptiMem (Gibco) and the cells were incubated at 37°C for an additional 5-6 hours. The chloroquine was removed, the plates were rinse with TBS, and 2 ml of Cos media
was applied. At 48 hours post-transfection, the cells were washed in TBS and cells were lysed in 0.4 ml of lysis buffer (1% Triton X-100, 2 mg/ml iodoacetamide, 1 mM PMSF in RSB). Plates were scraped with a rubber policeman, the extract was pulled through a 21-gauge needle 5 times, and centrifuged for 30 seconds to remove nuclei. Extracts were stored at 4°C until use.

For Lipofectin transformation, Cos cells were plated at 2 x 10^5 24 hours prior to transfection and were 50-60% confluent at the time of transfection. Ten µl of Lipofectin (Gibco) in 200 µl of Opti-MEM (Gibco) was incubated with the DNA for 15 minutes then 0.3 ml of Opti-MEM was added. The assay was optimized for fusion activity and required that 3 µg of F DNA and 1.5 µg of HN DNA be used. To account for background fusion, 4.5 µg of pSVL DNA was transfected. The plates were washed twice with TBS and the DNA/Lipofectin mixture was applied. The plates were incubated for 6 hours, and the DNA mixture was replaced with 2 ml of Cos media.

**Fusion Assay.** At 24, 48 and 72 hours post transfection, the plates were systematically scanned for areas of fusion. In each field of view, the number of nuclei in every syncytia with 3 or more nuclei were counted for a total of 20 syncytia. Two individuals scored the plates and the numbers were averaged. An index value was obtained by subtracting the average value obtained from pSVL from the value for each assay.

**Immunofluorescence.** Cos cells were plated on glass cover slips in 35 mm plates containing glass cover slips at 3.5 x 10^5 20 hr prior to transfection. To fix cells, at 48 hours post transfection, the cells were washed in PBZ buffer (1% fetal calf serum, 0.2% NaN₃ in PBS) and dried at room temperature. After 20 minutes, the cover slips were placed in 95% ethanol and stored at -20°C. After a minimum of one hour the slides were rinsed in PBS. Buffer PBZ was applied and plates
were stored a minimum of 16 hours at 4°C. For live cells, the plates were washed in PBS and incubated with cold PBZ for one hour. All subsequent incubations were performed at 4°C. One-half ml of primary antibody diluted 1:100 in PBZ was applied to the coverslips and incubation proceeded for 90 minutes at 4°C for live cells or 30 minutes at room temperature for fixed cells. The unbound antibody was removed by thorough washing in PBZ. Fluorescein isothiocyanate (FITC) conjugated to anti-rabbit or anti-mouse IgG (Southern Biotechnology Associates) was used at a 1:100 dilution. The coverslips were incubated on ice for 30 minutes, washed thoroughly in PBS and inverted onto slides treated with a drop of DABCO (25 mg/ml triethylenediamine, 90% glycerol, in PBS).

**Radiolabeling.** Labeling with [35S]methionine (100 μCi/ml, 1450 Ci/mmol; Amersham) was accomplished in methionine-free minimal essential medium supplemented with nonessential amino acids and 10% dialyzed fetal calf serum. For pulse-chase experiments, labeling was for times indicated in figure legends and followed by the addition of complete Cos media.

**Immunoprecipitations.** Cell lysates were incubated with a 1:100 dilution of antibody for one hour at room temperature. The immune complexes were precipitated with Immunobeads (Bio-Rad) to which goat anti-rabbit or anti-mouse immunoglobulin antibody was covalently coupled to the micron-sized hydrophilic (polystyrene) beads. Before use, the beads were prepared by washing once in 5 mg/ml BSA, twice in 1 mg/ml BSA/PBS/0.5% NP40 and resuspended in 1 mg/ml BSA at a concentration of 19 μg antibody/10 mg beads/ml. One hundred microliters of beads were added to each sample and incubated on a shaker for 30 minutes. The beads were pelleted by spinning for 30 seconds in a microfuge, and washed 3 times in wash buffer (0.5% NP-40, 0.4%SDS in PBS). Samples were resuspended in 80 μl of sample buffer and divided into two equal portions.
Immediately before loading onto a gel, the samples were denatured by boiling 5 minutes (with and without 1 µl of β-mercaptoethanol; βME), 1 mg of iodoacetamide was added, the beads were pelleted, and the supernatants were loaded onto a 10% SDS gel.

Detection of Cell Surface Molecules. After a pulse label and a nonradioactive chase, monolayers were washed in ice-cold PBS (0.15 M NaCl, 7.7 mM K$_2$HPO$_4$, 2.4 mM KH$_2$PO$_4$) and incubated on ice with a 0.5 ml of a 1:50 dilution of anti-NDV antiserum (heat inactivated at 56°C for 30 min) in PBZ. After 60 minutes, the unbound antiserum was removed by extensive washing in ice-cold PBS. The cells were lysed in 0.5 ml of lysis buffer containing 1% Triton-X 100 and 0.5% sodium deoxycholate. The monolayers were scraped with a rubber policeman, the lysate was pulled through a 21-gauge needle several times, and the nuclei were removed from the lysate by a 30 second centrifugation. The immune complexes were precipitated with Immunobeads as previously described.

Sucrose gradients. To examine the oligomeric structure of the glycoproteins, sucrose gradients were used. A linear gradient of 25% to 10% sucrose (w/v) dissolved in RSB and 0.1% Triton X-100 was created using a gradient maker to mix the 5 mls of each solution into a polyallomer 14 x 89 mm centrifuge tube (Sarstedt). A 200 µl cushion of 2 M sucrose was positioned at the bottom of the tube. Each 35 mm plate was lysed in 0.4 ml of RSB, 1% Triton X-100, 10 mM iodoacetamide, and 1mM PMSF. The lysate was homogenized by pulling through a 21-gauge needle several times. Nuclei were pelleted by spinning in a microfuge for 30 seconds. A total of 0.6 ml (1-1/2 plates) was carefully layered over the gradient. The lysates were fractionated by centrifugation at 38,000 rpm, at 17°C, for 18 hours in an SW41 rotor. One-half ml fractions were collected from the top of the gradient. Fractions were numbered beginning with fraction 1 at the
bottom and fraction 21 at the top. The pelleted material was dissolved in 90 μl SDS-Tris sample buffer (6.25% SDS, 0.5M Tris pH 8), and 30μl was loaded on the gels. Fractions were TCA-precipitated, and the pellets were dissolved by incubating in 30 μl of SDS-Tris sample buffer at 50°C for 15 minutes. Prior to loading, 15 μl of reduction mix (50% glycerol and 0.01% bromophenol blue) and 1 μl of βME were added, the samples were boiled for 5 minutes, and 0.2 mg iodoacetamide was added. A marker of 0.12 μl of NDV virions was loaded in the first lanes. In most cases, fractions 15, 17, and 19 were omitted, as any molecules that may sediment in those fractions were expected to be too small to be resolved. To calibrate the gradients, molecular weight protein markers (Boehringer Mannheim) were similarly processed, and the resulting gels were stained with Coomassie Blue stain.

**Polyacrylamide Gel Electrophoresis.** Electrophoresis on SDS-polyacrylamide slab gel was carried out by the procedure of Laemmli (Laemmli, 1970) with the modifications of Morrison et al (Morrison and Ward, 1984). For the cell-free translations, proteins were resolved on 12% polyacrylamide-SDS gels. For all other assays, samples were resolved on 10% gels. Following electrophoresis, the gels were subjected to western analysis or fixed, dried and subjected to autoradiography using Kodak X-Omat AR X-ray film. For immunoprecipitations, gels were treated with PPO. A BetaScope (Betagen) was used for quantitation of [35]S-methionine labelled products. Rainbow markers (Amersham) were used for determination of molecular weights.

**Western Analysis.** Following electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine and 5% methanol) for 20 minutes. The hydrophobic membrane Immobilon PVDF (Millipore) was pretreated with methanol and also equilibrated with transfer buffer. The proteins were transferred
from the gel onto the membrane while immersed in transfer buffer at 100 mA for at least 14 hours. To reduce nonspecific signals, the blot was soaked in 10% (w/v) non-fat powdered milk (dissolved in wash buffer: 0.4% Tween-20 in PBS) for 4 hours. The blot was washed 3 times and probed with the primary antibody (1:2500 in wash buffer) for 90 minutes at room temperature. Following 3 washes, the blot was treated with a 1:40,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Boehringer Mannheim Corp.) for one hour. The blot was allowed to wash overnight at 4°C. The blot was treated with the luminol-based ECL reagents (Amersham) for one minute, drained, wrapped in plastic wrap and immediately detected by placing against film (Kodak X-AR). To obtain adequate signals, exposures were varied from 1-120 seconds.

Construction, expression and purification of trpE fusion proteins. To generate specific antisera, segments of the fusion protein gene were fused to the amino terminus of the trpE gene under the control of the tryptophan operon of E.coli. The genes were expressed in HB101 cells and the resulting protein is insoluble and was purified from the bacterial lysate as previously described (Koerner et al., 1991). Constructs were made with four segments from the coding sequence for the fusion protein. Nucleotides 76-1715 (BamHI-Clal) and 76-445 (BamHI-PvulI) were ligated into the BamHI and Clal sites of pATH1 to generate the pATH-E (entire) and pATH-5' proteins, respectively. Nucleotides 750-1715 (Pvull-Clal) and nucleotides 202-801 (HindII-HindII) were inserted into the pATH 2 SmaI restriction site to create pATH-3' and pATH-M, respectively. Approximately 250 µg of semipurified protein was electrophoresed on eight percent polyacrylamide-SDS gels. The appropriate bands were excised and homogenized in an equal volume of PBS. Freunds complete adjuvant was added (25%v/v) and 6 subcutaneous injections were made along the back and hips of each rabbit. After 4 weeks, 150 µg of protein mixed with Freunds incomplete adjuvant (25%v/v ) was used to boost.
Two weeks after boosting, the rabbits were bled weekly.

**Chemical cross-linking.** To analyze protein-protein associations, the cell surface proteins were chemically crosslinked prior to cell lysis. Intact monolayers were washed twice in PBS. The crosslinker was applied at a final concentration of 10 mM in PBS for 30 minutes at 4°C. The reaction was stopped by washing once in TBS followed by 25 mM ethanolamine for 2 minutes. The noncleavable crosslinkers, DMS, DMA, and DMP were tested. The thiol-cleavable crosslinker DTSSP (all from Pierce) was also used. The plates were washed, lysed in Triton X-100 and deoxycholate, and immunoprecipitated or subjected to western analysis.
NEWCASTLE DISEASE VIRUS (NDV) is a paramyxovirus whose viral envelope is derived from the infected cell membrane and contains the virally-encoded fusion glycoprotein. The fusion protein mediates membrane fusion between virions and the target cell and also between infected and uninfected cells (Bratt and Gallagher, 1969; Scheid and Choppin, 1974). The complete nucleotide sequence for the NDV fusion protein gene predicts an amino acid sequence with hydrophobic domains at the amino terminus and near the carboxy terminus (McGinnes and Morrison, 1986). This arrangement is typical of type I membrane glycoproteins, proteins with the amino terminus outside the plasma membrane and the carboxy terminus, cytoplasmic.

The amino terminal hydrophobic sequence of type I membrane proteins corresponds to an endoplasmic reticulum-specific targeting sequence that is recognized by the signal recognition particle (SRP) upon emergence of the nascent chain from the large ribosomal subunit (for a review see Lingappa, 1989). Further elongation of the polypeptide is halted or markedly diminished until the SRP-ribosome-nascent chain complex is targeted to the endoplasmic reticulum.
membrane via interaction with the membrane bound SRP receptor (for review see Walter et al., 1984). SRP is displaced from the signal sequence by the SRP receptor in a GTP-dependent reaction that leads to the subsequent insertion of the nascent chain into the membrane (Connolly and Gilmore, 1989). Polypeptides undergoing translocation are in direct contact with a 39 kD membrane glycoprotein termed the signal sequence receptor (Wiedman et al., 1987; Krieg et al., 1989). Translation resumes, and the polypeptide is translocated across the membrane until the hydrophobic stop transfer sequence of the transmembrane protein emerges from the ribosome (Yost et al., 1983). Further translocation of the polypeptide is abolished when the stop transfer sequence is inserted into the membrane. Unlike the amino terminal signal sequence that is removed by the lumenal enzyme signal peptidase (Evans et al., 1986), the hydrophobic stop transfer sequence is retained to function as the transmembrane spanning segment of the mature type I integral membrane protein.

Stop transfer sequences derived from type I membrane proteins have been shown to function as signal sequences in certain contexts. A stop transfer sequence can be recognized by SRP and act as a noncleavable signal sequence when it is placed at the extreme amino terminus of a polypeptide (Mize et al., 1986; Zerial et al., 1987). A cleavable signal sequence, however, cannot replace a stop transfer sequence as a transmembrane span when it is placed near the carboxy terminus of a type I membrane protein (Rothman et al., 1988). N-terminal signal sequences are less hydrophobic than stop transfer sequences, and this may account for the failure of a signal sequence to halt translocation of subsequent portions of the polypeptide when it is positioned at the end of a membrane protein.

The amino acid sequence of the fusion protein contains a third hydrophobic domain of 26 uncharged amino acids beginning 117 residues from the amino
terminus (McGinnes et al., 1986). This additional hydrophobic region, often called the fusion peptide (Morrison, 1988), is located at the amino terminus of the F₁ portion of the mature protein. The paramyxovirus fusion protein is synthesized as a biologically inactive precursor, F₀, which is subsequently cleaved by a host cell protease into two disulfide-linked polypeptides, F₁ and F₂ (Scheid and Choppin, 1974, 1977). This cleavage is necessary to activate the fusion activity of the protein required for infection (Scheid and Choppin, 1974, 1977). A comparison of the amino acid sequence of the amino terminus of the NDV F₁ subunit with that of other paramyxoviruses indicates that the sequence of this hydrophobic region is highly conserved and is, therefore, likely to be functionally important (Richardson et al., 1986; Morrison and Portner, 1991).

The hydrophobic fusion peptides in viral fusogenic glycoproteins are intermediate in hydrophobicity relative to signal sequences and stop transfer sequences (White, 1990). Also, the fusion peptides of the paramyxovirus fusion proteins are preceded by positively charged amino acid residues, typical of stop transfer sequences of type I membrane proteins and signal/anchor sequences of type 2 membrane proteins (Figure 24; von Heijne, 1988). However, previous reports have indicated that the fusion peptides are completely translocated across the ER membrane during cellular biosynthesis (Paterson and Lamb, 1987; Davis and Model, 1985; Davis and Hsu, 1986). In addition, antigenic determinants on both sides of this hydrophobic domain are exposed on the external surface of the virion (Toyoda et al., 1988). However, when the fusion sequence of the simian virus 5 (SV 5) fusion protein was moved to the extreme carboxy terminus of the influenza virus hemagglutinin and expressed in vivo, it served to anchor the protein in membranes (Paterson and Lamb, 1987). The SV 5 fusion sequence can also act as a signal sequence in vivo when placed near the N-terminus of a hybrid protein.
(Paterson and Lamb, 1990) Given these observations, it was of interest to examine the behavior of the NDV fusion sequence using a cell free system.

This work characterized the membrane insertion of the fusion protein in a cell-free system. To identify the topogenic signals of the protein, deletion mutants were constructed in which each hydrophobic segment was deleted. Expression of these constructs in wheat germ extracts containing ER-derived membranes indicated that the fusion protein behaves like a type I integral membrane glycoprotein. Both the amino terminal 13 amino acids and SRP were necessary for efficient targeting to membranes and translocation. The carboxy terminal region remains untranslocated, consistent with the notion that the third hydrophobic domain is a stop transfer/membrane anchor sequence. Further, the fusion domain by itself shows little signal sequence activity but does have stop-transfer activity in its normal position.
RESULTS

Plasmid Constructions. The strategy used for construction of the full-length coding region of the fusion gene in pSP64 is shown in Figures 1A and 1B. The full-length fusion gene was assembled in pBR322 by using a common Xma I restriction site in an overlapping region of two fusion protein gene specific clones to produce pTLF. The 5' and 3' G-C tails generated by the cloning protocols were removed as shown in Figure 1B to generate pSP6Fst. The coding region for the 553 amino acids of the fusion protein lies within the 5' Bam HI site and the 3' Mnl I sites, sites used to eliminate the G-C tails.

Mutants which eliminated each of the hydrophobic domains of the fusion protein were constructed as described in Materials and Methods and Figure 2. Three mutants deleted part or all of the amino terminal signal sequence. The pSP6F13 mutant deletes nucleotides 24-75 removing the methionine codon from the amino terminal signal sequence. Translation is forced to initiate at the second methionine codon in the coding sequence resulting in the deletion of the first 13 amino acids of the protein. The pSP6FSS mutant deletes the amino terminal signal sequence including the first two methionine codons of the coding region of the fusion protein gene. This construct forces translation to initiate at an internal methionine 68 codons downstream from the original translation start site. The pSP6MF mutant deletes the first 103 codons and initiates translation from a methionine codon placed 42 nucleotides upstream from the sequence encoding the hydrophobic fusion peptide. The pSP6SF cDNA deletes much of the second hydrophobic domain of the fusion protein by removing nucleotides 168-446, which encode amino acids 39 to 131, including the first 15 amino acids of the fusion peptide. The coding region for the third hydrophobic domain was deleted from the
F and SF cDNAs by introducing a frame shift mutation 91 codons upstream from the carboxy terminus to produce the dC91 and SFdC91 cDNAs, respectively.

**Cell Free Translations.** In order to characterize the proteins encoded by the pSP6Fst, pSP6F13, pSP6FSS, pSP6MF, and pSP6SF DNAs, the linearized DNAs were transcribed using SP6 polymerase, and the mRNAs synthesized were used to direct protein synthesis in a cell-free wheat germ extract. The resulting [35S]methionine-labelled products are shown in Figure 3 (largest molecular weight species in each lane). The pSP6Fst, pSP6F13, pSP6FSS, pSP6MF, and pSP6SF-derived transcripts encode full-length products with calculated molecular weights of 59, 57.6, 52, 48, and 49 kD, sizes expected for molecules of 553 (wild type F protein), 540 (F13), 485 (FSS), 451 (MF), and 460 (SF) amino acids, respectively (Figure 3). The synthesis of the FSS and F13 proteins indicates that the translational machinery was able to recognize and initiate synthesis at normally internal methionine codons.

**Translation Inhibition by SRP.** Protein sequences which function as signal sequences bind SRP, often resulting in inhibition of translation in the absence of microsomal membranes (Walter et al., 1981c). To explore sequences in the fusion protein which can bind SRP, the mRNAs derived from the wild type and mutant cDNAs were translated in the presence of SRP. The NDV matrix (M) protein mRNA was added to each reaction to monitor nonspecific inhibition of translation by the SRP preparation. To control for consistancy in sample loading, the M protein mRNA was added to the reaction, and the mixture was split prior to the addition of SRP or SRP buffer. As shown in Figure 3, when SRP is added to the translation reaction, a significant reduction in wild-type F protein and SF protein synthesis occurs (lanes 2 vs. 3 and 10 vs. 11; Table I). This result is compatible with the existence of a signal sequence at the amino terminus of both proteins. The protein
F13 lacks the first 13 amino acids of the amino terminus and, as shown in Figure 3, the degree of translation inhibition due to SRP is significantly less than that of the products containing the wild-type amino terminus. The protein FSS lacks the entire amino terminal signal sequence but retains the internally located hydrophobic fusion peptide 48 amino acids downstream from the amino terminus. It was possible that this remaining domain might be recognized by SRP. However, when SRP is added to the FSS translation reaction, little inhibition of synthesis is observed (lane 6 vs. 7, Table I). Also, SRP had little effect on the synthesis of the MF product. Lack of inhibition of MF by SRP was somewhat unexpected. The position of the hydrophobic domain at the amino terminus of the MF protein is similar to the hydrophobic signal-anchor of type II membrane proteins. Since the membrane anchor of type II glycoproteins bind SRP, it was expected that in its new position, the fusion peptide might also bind SRP.

The extent of SRP inhibition of protein synthesis of the wild-type and the four mutant fusion proteins was quantitated in two separate experiments (Table I). These results indicate that the first 13 amino acids are required to maintain the degree of SRP-mediated translation inhibition seen with the fusion protein. Also, in the absence of an amino terminal signal sequence, the second hydrophobic region was not efficiently recognized by SRP as indicated by the lack of translational inhibition.

Membrane Insertion of the Fusion Protein. The translocation of the wild type and mutant proteins was explored by adding microsomal membranes (derived from pancreatic ER) to the translation reaction (Walter et al., 1981b). As shown in Figure 4, the addition of microsomal membranes stripped of SRP (KRM) has no significant effect on the wild type translation product (lane 1 vs. 2). However, when SRP is also added, several products of lower mobility are synthesized (lane 3). These
new products are likely glycosylated forms of the fusion protein. The fully glycosylated fusion protein was previously reported to migrate as a 66 kD protein (McGinnes et al., 1985). Translation of the SF mRNA in the presence of SRP and KRM (lane 15) also yielded a product with reduced mobility, indicating that this mutant protein has maintained the ability to be properly translocated and processed. Translation of the F13 mRNA in the presence of SRP and membranes also resulted in the synthesis of a species of lower mobility. The efficiency of F13 translocation was significantly lower than for the two constructs that retained an intact signal sequence. In contrast, translation of the FSS mRNA in the presence of KRM and SRP did not result in a new polypeptide (lanes 9-11). Thus, using glycosylation as an indicator for translocation, the FSS protein is not translocated.

To determine whether the FSS protein may behave differently in a more homologous system, the FSS mRNA was translated in the presence of membranes using a reticulocyte lysate system and again remained unglycosylated (not shown). Like the FSS protein, the MF protein behaved similarly in both the reticulocyte (not shown) and wheat germ translation system and did not appear to be translocated (lanes 17-19). However, on a longer exposure of the autoradiogram a very small amount of a glycosylated MF species was detectable.

**Glycosylation and Signal Sequence Cleavage of the F protein Constructs.**
The tripeptide Asn-Tyr-Thr (NYT) was used to explore signal sequence cleavage of the products of the cell free reactions. This tripeptide inhibits N-linked glycosylation by competition for the glycosylation machinery within the membrane vesicles (Welply et al., 1983). In the absence of translocation, NYT has no effect on the translation products (Figure 4, lanes 11 vs. 12 and 19 vs. 20). When NYT, SRP and membranes were present during translation of the F, F13 and SF mRNAs, the products of lower mobility were not synthesized, indicating that these polypeptides

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were indeed the result of glycosylation (Figure 4, lanes 3 vs. 4, 7 vs. 8, and 15 vs. 16). Translocation of the wild type, F13 and SF proteins in the presence of NYT resulted in products of increased mobility (lanes 4, 8 and 16). This result was consistent with the cleavage of the amino-terminal signal sequence and characteristic of secretory and integral membrane proteins. Signal sequence cleavage of the NDV fusion protein has been previously reported to occur between serine 31 and leucine 32 (Gorman, et al., 1988). The processed F and F13 products appear to have the same mobility, suggesting that the first 13 amino acids of the fusion protein are not necessary for recognition and cleavage by signal peptidase. Combined results from the F, SF and FSS proteins indicate that all of the signals necessary for targeting and translocation of the wild-type F protein are contained within the first 39 amino acids.

Taking into account signal sequence cleavage of 31 amino acids from the fusion protein, the difference in mobility between the most highly glycosylated and unglycosylated species of 15 kD is consistent with the addition of 4 to 5 high mannose oligosaccharides. The sequence of the fusion protein contains 5 N-linked glycosylation sites preceding the presumed cytoplasmic domain. Thus, most or all of the sites are likely used in the cell free reactions. A consistent observation was that the efficiency of glycosylation of the wild type F protein was less than that of the SF protein (Figure 4, lane 3 vs.15). However, the efficiency of signal sequence cleavage appeared comparable (Figure 4, lanes 4 and 16). The significance of these observations will be discussed below.

Protease Protection. To explore the topology of the wild-type F protein with respect to the membrane, translocated proteins were digested with proteinase K. For estimation of molecular weights, molecular weight markers were used to calibrate the gels. Digestion of the translocated and fully glycosylated form of the
fusion protein resulted in protection of a polypeptide reduced in size by approximately 3000 daltons (Figure 5, lane 4). In addition, a small fragment with an approximate molecular weight of 21 kD was also protected (see dot, Figure 5, lane 4). The product of translation in the absence of membranes and SRP was completely sensitive to protease (lanes 1 vs. 2). When detergent was added to solubilize the membranes, the protein was again entirely digested (lane 5). This result indicated that the fusion protein fragments seen in lane 4 were protected from digestion by the membrane vesicle and were not in a protease resistant conformation.

The second hydrophobic domain (fusion sequence) of the F protein appears hydrophobic enough to act as a stop transfer sequence, although previous reports indicate that during translocation of the Sendai virus nascent F protein, this domain is completely transported into the lumen (Davis and Hsu, 1986). To determine whether the membrane topology of the fusion protein would change in the absence of this domain, the SF protein was synthesized in the presence of membranes and digested with proteinase K. As shown in Figure 5, the fully glycosylated SF protein, like the wild type, was reduced in size by approximately 3000 daltons upon proteinase K treatment (10 vs. 11). However, the 21,000 dalton protected fragment seen with the wild-type F protein is not produced. Protease treatment of the MF and FSS products translated in the presence of membranes results in complete digestion of both proteins. The very small amount of glycosylated MF protein detected was protected from protease digestion (not shown).

As noted above, digestion of the wild-type F protein resulted in a small 21 kD polypeptide as well as the larger protected species. This observation led to the speculation that the fusion domain may make the vesicles permeable to the protease. In order to test this idea, the mRNA for the F and SF was cotranslated
with mRNA for the secretory protein preprolactin. As shown in Figure 6, the amount of prolactin recovered after proteinase K treatment is not significantly diminished in reactions containing either F or SF mRNA. Thus the existence of the 21 kD polypeptide after protease digestion cannot be attributed to membrane leakiness.

**Stop Transfer Function of the C-terminus.** After protease digestion, the existence of a species with only a small reduction in size from the fully glycosylated fusion protein and the SF protein indicated that some of these molecules contained an internal domain near one end of the molecule that stopped translocation. The third hydrophobic region of the fusion protein is comprised of 28 amino acids located 31 residues from the carboxy end of the molecule and is predicted to function as the stop transfer sequence. To verify that the carboxy terminus of the fusion protein is susceptible to protease, the 3' region of pSP6Fst DNA was altered resulting in synthesis of a protein missing the third hydrophobic domain, as well as the cytoplasmic domain. Transcripts from the resulting pSPdC91 were translated in the presence of membranes and SRP. Following translocation of the truncated fusion protein, digestion with the protease indicated that slower migrating glycosylated proteins are completely inaccessible to the enzyme (Figure 7, lane 3). Deletion of the carboxy terminus removes the protease-sensitive site found in the F protein. It is likely that the carboxy terminal region of the wild-type F protein and is exposed because the third hydrophobic domain serves as a stop transfer/membrane anchor sequence. There is a product produced in the presence of membranes that appears to be cleaved and unglycosylated (see dot, lane 2) and is considerably reduced in amount by protease treatment (lane 3). In addition, after protease digestion, a new polypeptide of approximately 21 kD appeared (dot, lane 3). The pSP6SF mutant (missing the fusion peptide) was similarly truncated, and the translocated products from this construct were fully
resistant to digestion (Figure 7, lanes 6 vs. 7). No small 21 kD fragment was
observed, and the signal sequence cleaved, yet unglycosylated form of the protein
was protease resistant.

Premature stop-transfer behavior of a mutant fusion protein. The previous
result suggests that a population of the wild-type F protein molecules are anchored
in the membrane at a position upstream from the proposed transmembrane region
near the carboxy-terminus. If the hydrophobic fusion peptide were functioning to
stop protein translocation, this population of molecules would halt translocation at
the fusion sequence as depicted by form 2 in Figure 10. As there is only one
glycosylation site prior to the fusion domain, translocation of this population would
result in a species of molecules whose molecular weight would increase by only
about 3 kD. Unfortunately, the loss of the cleavable signal sequence of about 3 kD
would "mask" this population, and they would comigrate with unmodified,
previously untranslocated, molecules. To test this hypothesis, a mutant was
designed to create an additional glycosylation site upstream from the fusion
domain. The resulting pSP6Fg mutant was translated in the presence of
membranes and subjected to the protease protection assay. As shown in Figure 8,
a small amount of the Fg molecules are fully glycosylated at 5-6 sites. These
molecules have a slight increase in mobility following the protease treatment. This
is expected from a population of fusion molecules anchored at the C-terminus and
having a small domain accessible to the protease. Secondly, close examination
revealed a novel band that runs slightly above the unglycosylated species and
appeared to be preferentially synthesized in the presence of membranes (lane 9).
Following protease digestion, this novel population of Fg molecules appears to be
completely accessible to the protease (lane 10).

As suggested above, if a population of these molecules were anchored at
the fusion sequence, they would be glycosylated at two sites. Following protease
digestion, only the amino terminal portion of the molecule would be translocated
(and glycosylated). This portion would show protease protection, and result in the
detection of a 24 kD species glycosylated at two sites for Fg or a 21 kD species
glycosylated at one site with the wild-type protein. Protein fragments of mobilities
consistent with this reasoning are protected from the protease as indicated in lanes
4 and 10. If translocation occurred in the absence of glycosylation, these
fragments from the F and Fg protein would have the same mobility. When the F
and Fg molecules are synthesized in the presence of both membranes and the
glycosylation inhibitor NYT, both F and Fg have two protected species of the same
mobilities, approximately 17 and 55 kD, protected from the protease (lanes 6 and
12), corresponding to the partially translocated and fully translocated molecules
respectively. Combined, these results indicated that translocation of the fusion
protein in vitro results in populations of molecules with differing membrane
topologies as shown by forms 1 and 2 in Figure 10. To estimate the percentage of
F molecules that were partially translocated, and taking into account that the larger
species would have 2-3 times the amount of $^{[35]S}$-methionine label, quantitative
densitometry of the two protected F species suggested that over 60% of the
molecules were partially translocated as depicted by form 2 in Figure 10.
Furthermore, translocation in the presence of NYT indicated that glycosylation was
not a factor in determining membrane topology.

Topology of proteins synthesized in vivo. To determine whether the fusion
protein behaves similarly in cells, intact membrane vesicles from $^{[35]S}$-methionine-
labelled, NDV-infected cells were isolated and subjected to protease digestion. By
using pulse-labelled extracts, only the newly synthesized, or ER-associated,
molecules would be detected. As shown in Figure 5, the fusion protein synthesized
in infected cells (see dot, lane 6) has a small terminal domain that is sensitive to digestion at 20 ug/ml protease (compare lanes 6 and 7). The remainder of the molecule is resistant to digestion even at 200 ug/ml of protease (not shown). Protection of a 21 kD band could not be detected.

Since it was possible that the previous experiment might not detect the 21 kD protected fragment, the topology of the protein was further investigated. The mutant DNA, dC91, was subcloned into the pSVL vector and the protein was expressed in Cos cells. As noted previously, the dC91 mutant was found to be anchored in membranes by the fusion sequence when synthesized in the cell free system. If this occurred in the cell, the protein might be transported through the cell and be detectable at the cell surface. When dC91 was tested for surface expression by immunofluorescence, it was detected although at very low levels (not shown). To further evaluate the surface expression of dC91-expressing Cos cells, the cells were subjected to a cell surface assay. In this assay, the cells were radiolabelled, chased with cold medium and the cell surface was probed with a polyclonal antibody generated against virion F protein. The immune complexes were precipitated and the samples were analyzed by SDS-PAGE. Under these conditions, no dC91 was detected on the cell surface (not shown).

If dC91 was not membrane-anchored, it would likely be secreted from the cell. To determine if dC91 is secreted, transfected cells were subjected to a pulse-chase experiment. Both the cell-lysate and supernatants were analyzed by immunoprecipitation with an anti-F monoclonal antibody (Fu1a). As shown in Figure 9, lane 10, the protein can be found in the supernatant by 2 hours. There is a species that remains cell-associated even with a 6 hour chase (not shown), but, it migrates consistent with a fully glycosylated molecule, indicating that the molecule is completely translocated. The reason for the decreased mobility of the secreted
form is unknown. In addition, the wild-type protein is totally cell-associated (lanes 14 and 16).

Proteins precipitated with anti-F antisera and electrophoresed under reducing conditions resolve into two major bands as shown in Figure 9, lanes 9-16. This result would occur if the fusion proteins are cleaved into the F1-F2 subunits as occurs with the wild-type protein as it leaves the Golgi. As opposed to the wild-type F, the dC91 is secreted (lanes 10 and 12 versus 15 and 17). The fully processed F1 subunit of dC91 would be predicted to have the same electrophoretic mobility as an uncleaved, underglycosylated dC91 species which would result from partial translocation. To distinguish between the F1 subunit and an underglycosylated, partially translocated form, the immunoprecipitates can be resolved without reducing agents. This will allow any F1-F2 subunits to remain disulfide-linked and will have the same mobility as any uncleaved precursor dC91 protein (60 kD). If an underglycosylated species (form 2 in Figure 10) is detected, it will have a predicted molecular weight of about 48 kD, and will also be detected under nonreducing conditions. When the immunoprecipitates from both the cell lysates and the supernatants were run under nonreducing conditions, only one band, migrating consistent with a 60 kD species, was detected (Figure 9, lanes 1-3). These results suggest that the F protein synthesized in vivo undergoes only one form of membrane insertion.

As mentioned above, the in vivo-expressed wild-type F1 subunit may comigrate with partially translocated, underglycosylated, precursor molecules. Thus, it would be useful to employ a mutant fusion protein that does not undergo cleavage during transport through the cell. The mutant fusion protein F117L was found to be suitable for this analysis (Morrison et al, 1993). By using western blot analysis of cells expressing this non-cleavable mutant, any F1-sized molecule
would represent an underglycosylated species of F. When this mutant was examined, only the species consistent with a fully glycosylated molecule was detected (see Figure 28, p. 110).
DISCUSSION

Cell-free translation of mRNAs derived from a full length fusion protein gene and deletion mutants has allowed characterization of the topogenic signals of the NDV fusion protein. Translation of the wild type mRNA in the presence of SRP resulted in an inhibition of fusion protein synthesis, indicating the binding of SRP to a signal sequence in the nascent chain. The presence of both SRP and membranes in the reaction resulted in the appearance of a new product which comigrated with the fully glycosylated protein synthesized in NDV infected cells. The use of the glycosylation competitor, NYT, revealed that this new product was the glycosylated form of the fusion protein. The use of NYT also demonstrated signal sequence cleavage of the fusion protein. Thus, in the cell-free system, the fusion protein is translocated and glycosylated with concomitant signal sequence cleavage.

Removal of the first 13 amino acids from the fusion protein, including three proline and two basic residues, reduced the ability of the F13 product to interact with SRP, and loss of this property may be directly responsible for the reduced amount of translocation of F13 as indicated by glycosylation. The addition of membranes and NYT to the reaction indicated that the cleavage site recognized by signal peptidase is not dependent on the first 13 amino acids of the fusion protein. This mutant as well as FSS, discussed below, show that the first hydrophobic domain of the fusion protein is an ER-targeting signal sequence.

The mutants FSS and MF were constructed primarily to determine whether the second hydrophobic domain, the fusion sequence, could function as a signal
sequence in the absence of the normal amino terminal signal sequence. The fusion domain of the FSS construct is preceded by 48 amino acids, so the protein encoded resembles a type II membrane protein with respect to the location of the first hydrophobic domain. The signal/anchor domain of type II membrane proteins remains uncleaved upon membrane integration and serves to anchor the protein in the bilayer with the amino terminus of the protein exposed to the cytoplasm (for a review see Wickner, 1985).

In contrast to the wild type fusion protein, synthesis of the FSS protein was not significantly inhibited by the presence of SRP. Thus the fusion sequence in this construct likely has little ability to bind SRP. Further, the protein was not glycosylated nor protected from protease digestion when membranes were added to the reaction. The lack of SRP inhibition of FSS synthesis could have been due to the length of the sequence preceding the hydrophobic fusion domain which might have sterically hindered an interaction with SRP. This possibility was tested with the pSP6MF construct which encodes a protein with only 14 amino acids preceding the fusion domain. Results with this construct were similar to that obtained with the FSS protein. Inhibition by SRP was not detected and only barely detectable amounts of glycosylation were observed. Thus, the hydrophobic fusion domain in its natural context is not recognized by SRP and does not direct significant amounts of membrane insertion. Therefore, these two mutants are depicted as cytoplasmic proteins in Figure 10.

A recent study replaced the signal/anchor domain of the influenza NA protein with the hydrophobic fusion peptide of a paramyxovirus, SV 5 (Paterson and Lamb, 1990). Cellular expression of the resulting NAF construct resulted in a molecule which was translocated and cleaved by signal peptidase. The MF construct described here has the fusion domain similarly positioned near the
amino terminus and results in translocation in vitro but at an exceedingly low efficiency. It is possible that a cellular component assisted the translocation of the NAF protein and this component is lacking in the cell-free system. It is also possible that membrane targeting of the NAF protein was assisted by the NA sequences flanking the fusion sequence. It has been previously shown that the amino terminal flanking region of the NDV HN protein is very important in efficient and proper membrane insertion (Wilson et al., 1990). Substitution of two basic amino acids preceding the hydrophobic signal of cytochrome P450 reversed the normal topology of the protein (Szczesna et al., 1989). Also, it appears that the hydrophobic sequences within type II glycoproteins do not specifically direct translocation. Zerial et al., (1987) has found that several hydrophobic sequences can replace the signal/anchor of a type II glycoprotein, the transferrin receptor, and still maintain its ability to be translocated. Consequently, the absence of translocation of the MF construct may be due to an inability of the flanking sequences to promote translocation.

The second and third hydrophobic domains of the NDV fusion protein sequence resemble stop transfer/membrane anchor sequences. Results of protease digestion of the cell free products support the notion that the third hydrophobic domain near the carboxy terminus has stop transfer activity. Following translocation, protease digestion resulted in species of F and SF proteins only slightly reduced in size. The exposed protease sensitive region was shown to be the carboxy terminus by digesting translocated truncated forms of the protein that lacked the third hydrophobic segment. The slower migrating, fully glycosylated, products of the dC91 and SFdC91 messages were completely protected from protease.

The SF mRNA-directed reactions have consistently been observed to
contain a higher percentage of glycosylated product than was seen with the F protein. One possible explanation for this discrepancy is that the less hydrophobic SF molecules are more easily glycosylated than the fusion protein. However, a population of only partially translocated fusion molecules would also reduce the amount of fully glycosylated species produced by the F mRNA relative to the SF mRNA. It therefore seems probable that the wild type F protein may be associated with membranes in two different ways in the cell free system and that the fusion sequence may function as a weak stop transfer signal as depicted as form 2 in Figure 10. This result would be consistent with results from Kretzschmar et al (1992) who found that the fusion peptide of an influenza HA molecule could serve as a membrane anchor under certain conditions.

Examination of the protease protected fragments derived from the F and dC91 proteins again suggested that a second population of molecules exist which are only partially translocated and may be anchored in the membrane by the second hydrophobic domain, the fusion peptide. Translation of the dC91 mRNA in the presence of membranes and SRP resulted in a translation product with a mobility that is expected to result from processing by the signal peptidase and lacking glycosylation as seen dotted in Figure 7, lane 2. Proteinase K digestion revealed that this population of unglycosylated, but cleaved dC91 molecules are susceptible to digestion and, consequently, must be only partially translocated. Further, if this unglycosylated product was anchored by the fusion domain, the fragment protected from proteinase K would be predicted to have approximately 123 amino acids, and have a molecular weight of about 16 kDa after glycosylation. Indeed, a fragment with approximately that mobility was consistently produced by digestion of the F or dC91 proteins (Figure 5, lane 4, Figure 6, lane 5, and Figure 7, lane 3, Figure 8, lane 4). From a calibration curve derived from the migration of
molecular weight proteins in gels, this fragment migrated as a 21 kDa species. In contrast, digestion of the SFdC91 protein, which is missing both the fusion sequence and carboxy-terminus, showed a small population of unglycosylated, cleaved species that was protected and evidently fully translocated into the lumen of the vesicle (Figure 7, lanes 6 and 7). No 21 kDa species was produced following digestion of the SF or SFdC91 proteins, proteins missing the fusion peptide.

Analysis of the Fg mutant verified the existence of the two topological forms of the fusion protein. This mutant was constructed to "tag" the protected fragment with an additional glycosylation site. Protease digestion of the translocated Fg mutant indicated that the protected fragment was from the amino terminus of the protein.

The 21 kD protected species is 5 kD larger, or roughly, 50 residues longer than predicted. The 50 residues following the fusion peptide (129-182) consist of a heptad repeat and have been predicted to form an alpha helical structure with an apolar face (Figure 21; Lamb, 1993). A comparably placed alpha helix of the influenza HA monomer has been predicted to be associated with the membrane prior to oligomerization (Gething, 1986). Thus, the NDV alpha helix may be part of the protease-resistant 21 kD fragment protected in the cell-free assays. Complete digestion of the 21 kD fragment in the presence of detergents suggested that it was associated with the membrane and inaccessible to the protease. Similar results were obtained from studying the HIV env protein in a cell-free system and revealed that gp160 had a potential alpha helical domain near the C-terminus that associated with the membrane surface (Haffar, 1988). A deletion mutant was designed to determine if this potential helical structure was associated with the membrane. Preliminary results have indicated that the residues within the potential
helix are protease resistant due to a membrane association (not shown).

Studies of protein translocation have identified other proteins that can adopt more than one topology during biosynthesis in cell-free systems (Garcia et al., 1988). A striking example is the scrapie prion protein that was shown to be present in both a membrane integrated, and fully translocated form after cell-free biosynthesis (Lopez et al., 1990).

The context in which a hydrophobic domain resides appears to be crucial to the topological function. Previous reports suggested that the fusion domain is on the threshold of hydrophobicity required to arrest translocation (Paterson and Lamb, 1987) but passes this threshold and is able to halt translocation when located at the carboxy end of a molecule. When placed near the amino terminus, as in the NAF construct, a fusion peptide functioned as a cleavable signal sequence in vivo (Paterson and Lamb, 1990). These studies suggest that the regions flanking the fusion domain in the nascent fusion protein may contribute to the inefficient anchoring ability. This behavior may not be restricted to the hydrophobic fusion peptide, as alteration of the sequence upstream of the first transmembrane region of the prion protein allows translocation of the downstream hydrophobic domain (Yost et al., 1990).

The wheat-germ translation system has been useful in faithfully synthesizing several viral fusion glycoproteins (Haffar, 1988; Chao, 1987). Curiously, these studies have not reported the ability of a hydrophobic fusion domain to halt translocation. It is not known whether this discrepancy is due to a difference in fusion proteins or a difference in methods. As the microsomal membranes and wheat germ extract are independently prepared, it seems possible that the components necessary for faithful translocation may vary with the laboratory. To
test this idea, the protease protection assay was performed following translocation of F in a rabbit reticulocyte extract as described in Materials and Methods. Results from this experiment were identical to those obtained with the wheat germ system (not shown), suggesting that the transcription and translation components do not account for the differing topologies. Alternatively, the preparation of the salt-washed membranes may vary between laboratories. Nichhitta and coworkers have shown that partial translocation of the prolactin precursor can occur when the KCl concentration is not between 0.4-0.7 M during preparation of the membranes (1990) and the net transfer of a nascent protein does not occur in the absence of lumenal proteins (1993).

When the dC91 and wild type fusion proteins were expressed in Cos cells, the molecules detected by immunoprecipitation migrated consistent with fully glycosylated molecules, suggesting complete membrane translocation. This result was not comparable with the results obtained from the cell-free system. As the monoclonal antibody used for these assays was generated against mature, virion F protein, it was likely that it would not detect underglycosylated molecules. In an effort to generate new anti-F sera, fragments of the F protein were synthesized in bacteria using the pATH vectors as described in Materials and Methods. This unglycosylated protein was used to generate polyclonal antisera in rabbits. Three different antisera were tested. None of the three antibodies were able to detect a partially translocated form of the fusion protein expressed in Cos cells. This suggested that the inability to detect unglycosylated forms of cellular dC91 or the wild type protein is not due to inadequacies of the antisera used for immunoprecipitations.

An additional piece of evidence that supports the notion of one topological form of the cellular fusion protein, comes through the use of a mutant protein which
does not become cleaved during cellular transport. When this F117L mutant was expressed in Cos cells and subjected to western blot analysis, only one species was detected under both reducing and nonreducing conditions. Under the conditions used for the analysis, all forms of the molecules should have been detected. However, these results do not rule out the possibility that partially translocated, underglycosylated fusion proteins are unstable or quickly degraded in vivo.

The topological forms of the fusion proteins detected in the cell-free system may reveal a novel intermediate form of the fusion protein that is undetectable with in vivo expression systems. Chuck and Lingappa have recently described a specific pause transfer sequence contained within apolipoprotein B, a ligand for the low density lipoprotein receptor (1993). This sequence mediates discrete pauses in the translocation of the protein and can confer translocational pausing to an otherwise secretory chimeric protein (Chuck and Lingappa, 1992). Further, translocation was found to restart post-translationally. Generally, protein translocation can occur following translation (Schatz, 1986; Perara, 1986). However, as shown by Collier et al, (1988) precursor proteins must be unfolded in order for translocation to occur post-translationally in E. coli and SecB may assist in this unfolding. Furthermore, it seems likely that the partially translocated fusion protein species may represent a population of molecules in which translocation has paused, possibly due to the hydrophobic fusion sequence, and been unable to resume. It would follow that the C-terminal regions of the protein may prematurely fold and, unlike lipoprotein B, inhibit further translocation. Consequently, the topological forms of the fusion protein detected by the in vitro system may represent a translocation intermediate. Some feature of cellular translocation makes this intermediate form undetectable.
If the discrepancies in protein topology found between the cell-free and cellular expression systems are due to a translocational pause that causes the protein to become trapped during in vitro translocation, then it may be that additional cellular processes or cytosolic factors, such as the SecB of E. coli, are required to allow proper translocation. The system described in this work may be useful to test the translocation-promotion potential of cellular factors. In addition, the requirement for unfolding may be tested by the addition of mild denaturants or cellular "unfoldases."

In summary, in a cell-free system, the fusion protein can adopt two different topologies with respect to membranes. In one population, translocation results in cleavage of the signal sequence and continues until a stop-transfer sequence is reached near the carboxy terminus. The second hydrophobic region, the fusion sequence, of the fusion protein appears to be translocated. However, in a second population this fusion domain may act as the stop transfer sequence resulting in a protein anchored by the fusion sequence. Although the fusion domain has characteristics similar to type II signal/anchor sequences, when placed near the amino terminus of a protein it has little ability to direct translocation.
Figure 1. Construction of full-length fusion gene.

(A) The overlapping clones pTL 831 and 438 were derived as previously described (McGinnes et al., 1986) and cloned into pBR322. The 5' sequence of the fusion protein contained within the Pvul and Xmal sites of pTL831 was excised and ligated to the Pvul-Xmal fragment from pTL438 containing the 3' region of the fusion gene (described in Materials and Methods). Ligation of the two fragments, using the common Xmal site, resulted in clone pTLF which contains the entire coding region for the fusion gene. Circles represent vector plasmids. Legend to Figure 1B defines the notation for restriction enzymes.

(B) Removal of G-C tails at ends of the fusion gene and assembly of the full-length gene in pSP64. From the plasmid pTLF, the 52-bp BamHI-BamHI, the 1545-bp BamHI-SphI, and the 97-bp SphI-Mnl I fragments were ligated into the BamHI and Smal sites of the cloning cassette of pSP64 as described in Materials and Methods to produce pSP6Fst. P, PstI; X, Xmal; B, BamHI; Sph, SphI; H, HindIII; S, SalI; Sm, Smal; E, EcoRI.
Figure 2. Schematic diagram of precursor form of wild-type and mutant fusion proteins. Panel A. Relative positions of restriction enzyme sites in pSP6Fst used for construction of mutant genes. Ps, PstI; B, BamHI; Pv, PvuII; Hf, HindIII. Panel B. Schematic diagram of protein structures predicted from amino acid sequence of the wild type and mutant fusion protein genes. Vertical bars on wild-type indicate methionine codons used for translation initiation of F13 and FSS. Filled boxes represent the relative position of the hydrophobic domains (signal, fusion and anchor sequences, respectively) of the fusion and mutant proteins. X's represent positions of potential glycosylation sites. Construction of plasmids is described in Materials and Methods. The MF gene results from deletion of the sequences 5' to the HindIII site of pSP6Fst followed by insertion of a methionine codon from the NDV matrix protein cDNA. The fusion peptide was deleted from the F gene by removal of sequences between the two PvuII sites to produce the SF cDNA. The sequence for the membrane anchor was removed from the fusion and SF cDNAs by deletion of sequences between the EcoRV and SphI sites. Ligation of the two blunt ends produced a translational frame-shift resulting in a termination site. Vertical arrows identify cleavage sites described in the text.
Table 1.

Figure 3. In vitro expression of proteins in the presence and absence of SRP.

In vitro synthesized transcripts were used to direct protein synthesis in a wheat germ extract and are indicated above their respective lanes. Products are synthesized, labelled with $[^{35}S]$-methionine, and electrophoresed under reducing conditions as described in Materials and Methods. Lane 1, no added mRNA; lanes 2 and 3, F mRNA; lanes 4 and 5, F13 mRNA; lanes 6 and 7, FSS mRNA; lanes 8 and 9, MF mRNA; lanes 9 and 10, SF mRNA. Lanes 2-13 contain mRNA for the NDV matrix protein to control for nonspecific inhibition of the SRP preparation. Lanes 3, 5, 7, 9, 11, and 13 contain SRP; all other lanes were supplemented with SRP buffer.
Figure 4. Translocation in the presence of membranes, SRP, and NYT.

The mRNAs used to direct cell-free protein synthesis are indicated. Lanes 1-4, F mRNA; lanes 5-8, F13 mRNA; lanes 9-12, FSS mRNA; lanes 13-16, SF mRNA; lanes 17-20, MF mRNA. Translation reactions were supplemented with components as indicated above each lane. Salt-washed microsomal membranes are indicated as KRM. The tripeptide NYT was added during the reaction at a final concentration of 400 μM to inhibit glycosylation.
Figure 5. Protease digestion of proteins synthesized in vivo and in cell-free reactions.

Wheat germ translation reactions were programmed with mRNA for the F protein in lanes 1-5 and mRNA for the SF protein, which is missing the fusion domain, in lanes 9-12. RM indicates the addition of membranes and SRP to the reaction. Lanes 6-8 are products from isolated vesicles from NDV infected chick embryo cells labelled with $^{35}$Smethionine for 5 minutes. Proteinase K, used at 20 $\mu$g/ml for 60 minutes at 4°C, and Triton X-100, used at 0.5%, were added following the translation reaction as indicated above each lane. The fusion protein synthesized in infected cell extracts is indicated by the dot in lane 6. Reference to the protected fragment indicated by a dot in lane 4 is found in the text.
Figure 6. Membrane integrity during proteinase K digestion.

The fusion and SF proteins were synthesized along with the bovine secretory protein preprolactin. Lanes 1-5 contained mRNA for the fusion protein. Lanes 3-10 contained mRNA for preprolactin. Lanes 8-12 result from reactions programmed with mRNA for the SF protein. Translation of the F and SF mRNA proceeded for 90 minutes. Total pituitary RNA, containing preprolactin mRNA, was added to the reaction for the final 30 (lane 3) or 60 minutes (lanes 4-10). RM indicates the addition of a microsomal membrane preparation containing SRP. Proteinase K concentration was 20 μg/ml and Triton X-100 was used at a final concentration of 0.5%. Reference to the dot by the low molecular weight protected fragment in lane 5 is found in the text.
Figure 7. Translocation and proteinase K digestion of the truncated F and SF proteins.

The wheat germ translation reaction shown in lanes 1-4 contained mRNA for the dC91 protein; lanes 5-8 contained SFdC91 mRNA. Membrane additions, proteinase K and detergent treatments are shown above each lane and added as described in Figure 5. The species indicated by dots in lanes 2 and 3 are discussed in the text.
Figure 8. Protease Protection analysis of Fg mutant.

Transcription-linked wheat germ translation reactions were programmed with mRNA for the fusion protein in lanes 1-7 and mRNA for the Fg protein in lanes 8-13. The reactions were supplemented with components as indicated above each lane. NYT, detergent, and proteinase K were added as described in previous figure legends. The approximate molecular weight of the smaller membrane-protected species, identified as 21 and 17 kD, were estimated from the electrophoretic migration of molecular weight protein markers (not shown).
Figure 9. In vivo expression of the wild type and truncated fusion proteins.

Cos cells transiently transfected with either the dC91-pSVL (lanes 1-12), F-pSVL (lanes 13-17) or parental vector pSVL (lanes 18-20) were lysed in RSB, Triton-X 100 and deoxycholate as described under Materials and Methods. Cells were radioactively pulse-labelled for 30 minutes (lanes P; 6, 13 and 18) or chased with cold methionine for 1, 2 or 3 hours as indicated above each lane. Cell lysates (lanes C) or culture supernatents (lanes S) were immunoprecipitated with a monoclonal antibody directed against F (Fu1a) and resolved in the absence (lanes 1-3) or presence of β-mercaptoethanol (lanes 6-20). The dots indicate the position of the uncleaved, glycosylated, precursor form of dC91 and F.
Figure 10. Schematic of the topology of fusion and mutant proteins synthesized in the presence of microsomal membranes.

Hydrophobic domains are indicated as described in Figure 2; C, carboxy terminus; N, amino terminus; *, potential glycosylation sites; dotted region represents the fusion peptide; horizontal marks indicate the anchor; vertical marks indicate the signal sequence. Positioning of carbohydrates and cleaved signal sequences is for illustrational purposes only. The SF protein and form 1 of the wild type fusion protein, F, behave like class I glycoproteins. The amino terminus is inserted into the membrane, the signal sequence is cleaved, translocation continues until a stop-transfer sequence is reached near the carboxy terminus and exposing part of the molecule. In form 1 of the anchorless protein, dC91, no part of the molecule is exposed. A portion of the F and dC91 molecules, molecules containing the hydrophobic fusion sequence, are partially translocated as indicated by forms 2. They may undergo signal sequence cleavage and, except for approximately 21 kD, a majority of the molecule is exposed on the cytoplasmic side of membranes. In the absence of the complete signal sequence, the fusion domain cannot target a nascent chain to membranes as indicated by the untranslocated FSS and MF proteins.
Table I. SRP-Mediated Reduction of Protein Synthesis.

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<th>Experiment 1</th>
<th>Experiment 2</th>
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<td>0.49</td>
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Table I. Protein synthesis was quantitated from a Betascope scan of [35S]-labelled products. The cpm of full-length products was normalized to that of the matrix protein synthesized in each reaction.

- SRP was added to the translation reaction as indicated in legend to Figure 3.
- The percent decrease in protein synthesis due to inhibition by SRP was determined by using the following calculation: % Reduction = 1 - (normalized cpm from the F protein synthesized in the presence of SRP/ normalized cpm from F protein synthesized in absence of SRP). The gel shown in Figure 3 corresponds to experiment 2.
CHAPTER IV
AN ANALYSIS OF STRUCTURAL AND FUNCTIONAL DOMAINS

INTRODUCTION

Fusion between a viral membrane and a target cell membrane is an essential step in the infectivity of the Paramyxoviruses. The membrane of the Newcastle disease virus (NDV) contains spike structures made up of two types of glycoproteins which facilitate this process, the hemagglutinin-neuraminidase (HN) and fusion glycoproteins. The HN glycoprotein is responsible for attaching the virion to the target cell, whereas the fusion glycoprotein is believed to disrupt the target cell membrane and induce membrane fusion (reviewed in Morrison and Portner, 1991). The fusion protein monomer is synthesized as a precursor, denoted F₀. It is inserted into the rough endoplasmic reticulum membrane (RER, reviewed by Lingappa, 1989) and cotranslationally glycosylated as a precursor. Following exit from the RER, the fusion protein is cleaved during transport through the Golgi apparatus into two disulfide-linked subunits, F₁ and F₂. This processing exposes the hydrophobic domain at the amino terminus of the F₁ subunit and is essential for the biological activity of the mature protein (Scheid and Choppin, 1974). This hydrophobic domain is highly conserved among paramyxovirus fusion proteins and is considered to be directly involved in mediating membrane fusion (White, 1990; Morrison and Portner, 1991). All the paramyxovirus fusion proteins are type I glycoproteins that have one hydrophobic transmembrane region near the carboxy terminus of the F₁ subunit which functions to anchor the protein in the bilayer.

An examination of the primary sequence of the fusion protein revealed general structural features of the protein. A comparative analysis of the fusion
protein sequences within the family of paramyxoviruses substantiated this information by revealing areas of conservation that may identify core regions of the protein pertinent to the fusogenic structure of the NDV molecule.

A Garnier-Robson structural prediction of the primary sequence has identified several regions within the fusion protein that have the potential to form alpha helices. The longest potential helix (Garnier et al, 1978) of the NDV fusion protein lies adjacent to the hydrophobic fusion peptide at the amino terminus of F₁ and involves residues 129-180. When the amino acid sequence of this region is displayed on an ideal alpha-helical wheel, the charged residues line up on one face of the helix, while a hydrophobic face is created on the opposing side of the helix. Mutations introduced into this helix often result in proteins that are either unable to induce fusion or not transported to the cell surface (Wang et al, 1992, Sergel et al, 1994). Since misfolded surface proteins are often retained in the ER (Segel, 1992), this helix in the fusion protein may be important for the proper folding of the molecule.

A second potential alpha helical domain lies immediately upstream of the transmembrane region and includes residues 476-491. This sequence consists of a series of highly conserved leucine or isoleucine residues which occur at every seventh residue (Figure 11) and was first described by Buckland and Wild (1989). When the amino acid sequence of this region from the NDV fusion protein is displayed on an ideal alpha-helical wheel, the periodic repeat of leucine or isoleucine residues line up on one face of the helix for 6 helical turns (Figure 12). All the charged residues face opposing sides of the helix. This type of leucine repeat within an alpha helix has been identified in several DNA binding proteins and has been termed the leucine zipper motif (Landshultz, 1988). This type of alpha helix has been proposed to associate proteins by coiling around each other and contact along the hydrophobic faces, resulting in a coiled-coil
structure (O'Shea, 1989). The potential to form a coiled-coil predicts that this leucine region of the fusion protein may be involved in the oligomerization of the molecule. The oligomeric structure of the mature form of the NDV fusion protein has recently been suggested to be a trimer by cross-linking studies (Russell et al., 1994).

The leucine zipper helix is of interest for two reasons. First, several reports suggest that the zipper motif of fusogenic proteins is necessary for infectivity and syncytium formation of enveloped viruses (Buckland, 1992; Chen, 1993). As this domain is expected to be far removed from the site of fusion on a target cell, information leading to the function of this domain will assist in elucidating the mechanism of the fusion process. Also, as previously suggested, the leucine motif has properties similar to that of other proteins known to form coiled-coil structures, and this motif may maintain an oligomeric form of the protein that is necessary for membrane fusion.

In order to define the requirements for the leucine residues in this motif for the structure and biological activity of the NDV fusion protein, three of the highly conserved residues were each replaced by an alanine. The wild-type and mutant F DNAs were expressed from an SV40-based vector in Cos-7 cells and tested for fusion activity. Although single leucine-alanine substitutions did not significantly affect the fusion activity, substitution of two or three leucines abolished the ability to cause syncytium formation. In contrast, nonconservative substitutions on polar sides of the potential helix did not significantly affect the fusion activity. Molecular weight estimations using centrifugation through sucrose gradients indicated that the leucine mutants retained the ability to oligomerize. Deletion mutations were generated to demonstrate the requirement for the entire helix and for the carboxy terminal hydrophobic domain for stability of this oligomer. F proteins produced from these deletion mutants were detected extracellularly in an
oligomeric form. A model is presented which suggests that fusion activity requires the conserved leucine residues to interact with and disorganize the infected cell membrane.
RESULTS

**Mutagenesis of heptad leucines.** The amino acid sequence of the NDV fusion protein in the region of the leucine zipper motif is shown in Figure 13. To investigate the structural and functional role of the conserved leucines, the third, fourth, or fifth repetitive leucine (L3, L4, L5, respectively) at residues 481, 488, and 495 respectively, were changed to alanine. Mutations were introduced singly and in several combinations as indicated in Figure 13. The location of mutations on the predicted helix is shown in Figure 12. In order to allow an examination of the function of the leucines and not the potential helical structure, alanine was chosen for the substitutions.

**Expression of mutants.** To characterize these mutant proteins, the mutated fusion cDNAs were subcloned from M13mp18 into the SV-40 based expression vector, pSVL. This vector allows transient expression in cells that express the large T-antigen. Cos 7 cells were transfected using a DEAE-dextran protocol described in Materials and Methods. At 48 hours post-transfection, proteins in the post-nuclear extracts were separated on a nonreducing gel and subjected to western analysis. Western blots were probed with a rabbit antibody generated against 29 residues at the C-terminus of the F protein (Wang et al, 1992). The extract from cells transfected with only the pSVL vector was included to control for nonspecific antisera binding (Figure 14, lane 1). The extract from cells expressing the wild-type protein resulted in one novel band (lane 2). Expression of all the mutant DNAs resulted in products that migrated with the wild-type protein, at amounts similar to wild type. Curiously, the proteins that have the L3A mutation ran slower than the wild-type protein (lanes 3, 6, 7, 9).
This decreased mobility was also seen in the presence of reducing agents and when the L3A mRNA was translated in a cell-free system in the absence of membranes and in the presence of reducing agent, indicating it was not due to differential glycosylation or alterations in intramolecular disulfide bonds (not shown). In addition, all fusion proteins resulted in minor species of lower mobility.

Mutations in viral glycoproteins may result in proteins which are improperly folded. To examine the effect these mutations may have on the folding of the molecule, the panel of mutants was subjected to a two hour pulse label and immunoprecipitated with a monoclonal antibody directed against the virion fusion protein. This Fu1a antibody has been shown to bind only relatively mature antigen (Morrison et al, 1987). Figure 15 shows that the antibody precipitated all the mutant fusion proteins in approximately equivalent amounts. This result suggested that the folding of the mutant molecules is not significantly different from that of the wild-type protein.

Surface expression of wild-type and mutants. For the fusion protein to be functional, it must be transported through the cell and properly expressed at the cell surface. When the surface expression of the proteins was evaluated by immunofluorescence, all proteins were expressed on the cell surface (not shown). To further examine the surface expression, an assay designed to quantitate surface expression of an antigen was employed (Morrison et al, 1990). The transfected cells were labeled with $^{35}$S-methionine and chased with cold methionine to allow the labelled proteins to accumulate at the cell surface. The intact cells were incubated with a polyclonal antibody generated against virion protein. After an incubation on ice the unbound antibody was removed by thoroughly washing the plates in ice-cold PBS. The cells were lysed in 1% Triton and 0.5% sodium deoxycholate. The immune complexes were precipitated with
beads conjugated to anti-rabbit IgG. The cell surface proteins were electrophoresed under nonreducing conditions. To control for any precipitation of intracellular F protein, unlabelled F-expressing cells were incubated with the primary antibody, washed, and the lysate was combined with a cell lysate from labelled, unprobed F-expressing cells. The results from this assay are shown in Figure 16A and indicated that all mutant fusion proteins were expressed at the cell surface.

The autoradiogram from this experiment and two other comparable experiments were scanned with a microdensitometer to quantitate the amount of protein immunoprecipitated. The averages of the results obtained, expressed as the percentage of wild-type protein, are: WT=100, L3A=147; L4A=84; L5A=83; L34A=240; L35A=246; L45A=131; L345A=280.

**Precursor processing.** For the mutant fusion proteins to have biological activity, the precursor must become cleaved and the disulfide-linked F₁ and F₂ subunits must exist at the surface (Scheid and Choppin, 1977). To evaluate the form of the proteins at the cell surface, the precipitated proteins were electrophoresed in the presence of reducing agent. Under these conditions, the subunits should dissociate, and the F₁ subunit should be detected. The results shown in Figure 16B indicated that all proteins become cleaved and result in the faster migrating F₁ species. In all cases, precursor cleavage appears to be inefficient as the uncleaved precursor species is also detected at the cell surface.

**Fusion ability with single leucine mutations.** Since all six mutant fusion glycoproteins were expressed at the cell surface, the biological function of the proteins was assessed. To determine whether these conserved leucines are required for the fusion activity of the NDV fusion protein, cells expressing the proteins were subjected to a fusion assay. In this assay, the fusion and HN cDNAs are cotransfected into subconfluent Cos cells. During optimization of this
assay, the use of Lipofectin (Gibco) was determined to give preferential results over the DEAE-dextran protocol which required harsher incubation conditions (not shown). When the wild-type HN and F proteins are co-expressed, multinucleated giant cells form within 24 hours and the size of syncytia increases linearly with time for over 72 hours (Figure 17). To quantitate this activity, the plates were scanned for fusion and the number of nuclei in forty syncytia were counted at 24, 48 and 72 hours post transfection for each sample. Each protein in question was assayed three times and the resulting values were averaged.

Cos cells show background fusion at low frequency with an average of 3-4 nuclei per syncytia. This background value is also obtained from cells transfected with only the parental vector and was subtracted from each assay.

To define the requirements for the individual leucine residues for the process of fusion, the activities of mutants L3A, L4A and L5A were determined. When any of these mutant proteins were expressed alone, they had near background levels of fusion, again demonstrating the requirement for the presence of the HN protein (not shown). As shown in Figure 18, when these mutant fusion proteins were co-expressed with HN, all showed fusion activity. Curiously, there appeared to be a gradient effect on the fusion activity with changes from the third to fifth leucine. Although the fifth leucine is least conserved among the other paramyxovirus fusion proteins, substitution of this leucine resulted in the most disruption of the syncytia-forming ability of any of the single substitutions. Although the three mutants showed a slight decrease in fusion activity, these results suggested that a single leucine substitution does not significantly alter the fusion ability of the protein.

**Fusion activity of proteins with multiple leucine substitutions.** To test further the requirement of the conserved leucines for the biological function of the protein, the double and triple leucine mutants were cotransfected with HN cDNA.
Results are shown in Figure 19. When two or more of the conserved leucines were changed, the ability of the protein to induce fusion was abolished. These results suggested that the individual leucines do not have importance but that the fusion activity of the protein depends on repetitive leucines.

**The S473A, E482K, and N485K mutants.** The lack of fusion activity of proteins with multiple leucine substitutions indicated that this region of the fusion protein is required for its biological activity. To assess further the sensitivity of this region to mutations, three nonconservative substitutions were introduced into the polar face of the potential helix. The asparagine at position 485, which falls between leucines 3 and 4, is predicted to be on a polar face of the alpha-helix as shown in Figure 12. This residue was changed to a basic residue, lysine, to create the N485K mutant. The predicted alpha helix has acidic residues in the b positions on the helix. To alter this region, a basic residue replaced the acidic glutamine at residue 482 to produce the E482K mutant. An additional mutant was created to test the functional requirements of the partially conserved N-linked glycosylation consensus site that falls between the first and second heptadic leucine residues (Figures 12 and 13). This mutant, S473A, abolished the potential for glycosylation of the asparagine at position 471 by replacing the polar serine residue with the nonpolar alanine residue.

The expression of these three mutant genes was examined by Western analysis. The E482K and N485K proteins had an electrophoretic mobility similar to that of the wild-type protein (Figure 20). The S473A protein had a slight increase in mobility as compared to the wild type protein, suggesting that the N-linked glycosylation site at residue 471 is normally used. In addition, the three mutants were found to be present at the cell surface when the surface antigens were immunoprecipitated as described above (Figure 16). Quantitation of the
surface expression, relative to the wild-type are: S473A=92%, E482K=31% and N485K=68%.

When the N485K, E482K and S473A mutants were subjected to the fusion assay, all mutants had fusion activity, as shown in Figure 21. While levels are reduced from wild type, none of the mutations abolished fusion to the extent of the double or triple leucine mutations. These results, along with the finding that the fusion activity is abolished only by multiple leucine substitutions, suggested that only the nonpolar side of the helix involving the conserved leucine residues is specifically required for the function of the protein. Single substitutions along the hydrophobic face or alterations that disrupt the charge distribution or glycosylation on other sides of the potential helix do not appear to affect significantly the biological activity.

Expression of HN in the presence of F mutants. For cell fusion to occur, both the HN and F glycoproteins must be expressed at the surface of the cell. The mutants unable to induce fusion may alter the cellular transport or expression of HN and the absence of fusion activity might be due to an absence of the HN protein at the cell surface. To establish the co-expression of HN and the mutant fusion proteins at the surface of the cell, dual immunofluorescence was employed. This examination revealed that HN was expressed in all cases, but at much lower levels than when expressed alone (not shown). This apparent decrease in HN expression following co-expression was confirmed by immunoprecipitating the cell surface antigens as previously described. However, the decrease in detection of HN expression was comparable between the wild-type and mutant fusion proteins indicating that there was not a selective lack of HN protein with the multiple leucine mutants (not shown).

Oligomer Formation. The lack of fusion activity for the multiple leucine mutants could not be accounted for by any obvious defect in the processing,
steady-state level, or cell surface expression of the mutant fusion proteins. Since the leucine zipper motif has been implicated in the oligomerization of proteins (O'Shea et al, 1992), it follows that the leucine mutants might be defective in fusion activity because of faulty oligomerization.

To examine the oligomeric structure of the wild-type fusion protein, sucrose gradients were employed. Cos cells expressing the F protein were lysed in a low salt buffer containing a non-ionic detergent (1% Triton-X 100). Iodoacetamide was added (10 mM) to block any free sulfhydryl groups from forming aberrant disulfide bonds during lysis. The post-nuclear extract was fractionated on an 10-25% linear sucrose gradient. This gradient was optimized to separate proteins in the 60-300 kD range. To reduce pelleting of larger species, a 2M sucrose pad was positioned at the bottom of the gradient. Twenty-one 0.5 ml fractions were collected from the top of the tube, TCA precipitated, solubilized and subjected to western blot analysis. Molecular weight markers were used to calibrate the gradient and indicated that an ideal monomer, dimer, trimer and tetramer of F would sediment in fractions 15, 11, 7, and 2, respectively. However, the molecular weight positions were only intended to serve as a reference point for the oligomeric structure of the fusion protein, which sedimented in fractions 8-10 at approximately 190 kDa (Figure 22A). Very little protein sedimented in the monomer region or towards the bottom of the gradient.

To examine the stability of this oligomer, a lysate of Cos cells expressing the F protein was supplemented with either 0.5% sodium deoxycholate or 5% SDS prior to centrifugation. Sodium deoxycholate had no effect on the sedimentation pattern of the protein, whereas a majority of the protein dissociated into a monomer and sedimented in fractions 14-16 in the presence of SDS (not shown). This result indicated that the mature F protein expressed in
Cos cells forms a Triton X-100 insensitive oligomeric structure that can be disrupted by solubilization in SDS and, as expected, is not covalently associated.

To examine the oligomeric structure of the triple leucine mutant, cells expressing the L345A mutant were similarly lysed and fractionated on a sucrose gradient. As shown in Figure 22B, this protein formed a Triton X-100-insensitive structure that sedimented similarly to that of the wild-type protein. Again, very little protein sedimented in the monomer region or pellet. Thus the conserved leucines are not required for the maintenance of the Triton X-100-insensitive oligomeric structure of the fusion protein. Furthermore, the lack of biological activity of the multiple leucine mutant cannot simply be accounted for by the leucine zipper and its possible contributions to disruption of this apparent oligomeric structure.

Molecular domain requirements for oligomerization. Given this finding, an attempt was made to identify the region of the protein that is responsible for the oligomerization. The NDV fusion protein has two potential amphipathic alpha helices (AAH) that could be involved in the oligomeric structure. The first potential AAH on the F1 subunit encompasses the sequence immediately downstream from the fusion peptide. As the previous results indicated, the leucine zipper region is not required for the maintenance of the oligomeric structure of the protein, and it seemed possible that the first helix may assist in the formation of the oligomeric structure. To test this hypothesis, two mutants in this region were added to the triple leucine mutant. The K155L mutation removes a charge from one face, whereas the A140K substitution adds a charged residue to a hydrophobic face of the predicted helix (Figure 23). These substitutions produced the K155L/L345A and A140K/L345A mutants, respectively. Since these mutant proteins had both potential amphipathic helices altered, it was expected that they might sediment as monomers when
fractionated on a gradient. Surprisingly, that was not the case as is apparent from Figure 24. The K155L mutant protein was inefficiently processed and resulted in sedimentation of the protein towards the bottom of the gradient (Panel B). The addition of the A140K mutation did not seem to significantly disrupt the oligomeric structure of the wild-type (not shown) or the L345A mutant as shown in Figure 24A. These results suggested that the oligomeric structure of the F protein is stable after the introduction of nonconservative mutations into the first potential alpha helical region.

To further delineate the region responsible for oligomerization, a termination codon was inserted immediately upstream of the coding sequence for the transmembrane region. This deletion removed the cytoplasmic tail and the transmembrane region while retaining the leucine zipper (dC49; Figure 13). Also, the protein was truncated to delete the leucine zipper motif and the transmembrane and cytoplasmic regions as previously described (dC91). Since neither of these mutant proteins have the membrane anchor region, they were secreted from the cell. Neither mutant was able to induce fusion when co-expressed with HN (not shown). Neither mutant was able to induce fusion when co-expressed with HN (not shown). To examine the oligomeric structure of these mutants, cells expressing these mutant genes were radioactively labelled, and the cell lysate or supernatants were fractionated on gradients previously described. The protein in the resulting fractions was immunoprecipitated with a monoclonal antibody generated against mature virion F protein. As shown in Figure 25, both of the truncation mutant proteins sedimented on the gradient consistant with having an oligomeric structure. No fusion protein was detected in the monomer region. The sedimentation pattern of proteins derived from the cell lysate and supernatant were similar and only one gradient of each mutant is
shown. The antibody immunoprecipitated nonspecific species that sediment near the top of the gradient (Figure 25A).

If these oligomeric forms were due to an association with host protein, host proteins would be expected to co-immunoprecipitate with these molecules. No host proteins co-immunoprecipitated in the fractions with the fusion proteins. These results indicated that the carboxy-terminal 91 amino acids of the fusion protein molecule do not function in the stability of this oligomeric structure.

**Heteroligomer formation.** Several laboratories have suggested that the F and HN proteins must interact for membrane fusion to occur (Sergel et al, 1993, Hu et al, 1992). It was possible, therefore, that the leucine zipper motif might function in an interaction of the F and HN proteins and the leucine mutations may disrupt this process. Therefore, an attempt was made to develop an assay that may identify an association between the two proteins. With this tool, it would then be possible to determine whether a correlation existed between an inability to promote membrane fusion by the leucine mutants and an inability to associate with the HN protein.

Initially, various pulse-chase protocols were used to co-immunoprecipitate F and HN from cotransfected or infected cells. Using antibodies directed against either protein and against both mature and immature molecules, no associations were detected. It was possible that the conditions used to solubilize the cells disrupted an association between the two proteins. To overcome this obstacle, the cell-surface molecules were cross-linked prior to immunoprecipitation. Several homobifunctional cross-linking agents with a range of linker-lengths were tested. Under no conditions were the attempts to appreciably co-immunoprecipitate cross-linked F and HN proteins reproducibly successful.
DISCUSSION

A leucine zipper-type amphipathic alpha helix is found adjacent to the transmembrane region in paramyxoviruses (Buckland, 1989) and coronaviruses (Britton, 1991). When Buckland and coworkers replaced four of the heptadic leucines in the measles virus fusion protein, fusion was abolished. Similarly, fusion activity of the NDV fusion protein containing two or more leucine substitutions in this region is abolished.

The leucine zipper motif is spatially placed well away from the presumed site for the fusion-promotion region of the proteins. It was expected, therefore, that this region does not directly participate in membrane fusion. However, mutations of conserved leucine residues resulted in proteins whose transport and folding was similar to that of the wild-type protein, as indicated by the ability to be detected by conformation sensitive monoclonal antibody. The lack of fusion activity for the double and triple leucine mutants also cannot be accounted for by a lack of cell surface expression or precursor processing. When cells expressing the panel of mutant proteins were examined, the faster migrating F₁ species was detected at the surface in all cases. Thus, the leucines are not conserved for these functions. Indeed, the helix and all subsequent residues can be deleted causing no apparent defect in transport, cleavage or antibody reactivity, suggesting that this region may have little role in the actual folding of the molecule.

The prediction that a leucine zipper motif may function in oligomer formation (Buckland and Wild, 1989) led to an examination of the oligomeric structure of the F protein. When the wild-type F protein is sized on sucrose gradients, the mature protein consistently sedimented as a 190 kD species, between the aldolase (158 kD) and catalase (240 kD) molecular weight markers.
The calculated molecular weight of F₀ is 56 kD after signal sequence cleavage. Glycosylation at 4 sites may add 12 kD (prior to trimming in the Golgi) resulting in an F monomer with an estimated molecular weight of 68 kD. The sedimentation profile would suggest that the F oligomer is a trimer. The gradients gave no indication that the mature protein exists as a 68 kD monomer, dimer (136 kD), or tetramer (272 kD). This result is in agreement with the recent report from Russell and coworkers (1994) that the electrophoretic migration of cross-linked SV5, NDV and HPIV-3 fusion proteins is consistent with trimeric structures (Russell et al., 1994). In contrast, crosslinking studies with the Sendai F protein (Sechoy et al., 1987) and sucrose gradient analysis of the respiratory syncytial virus F protein (Collins and Mottet, 1991) suggested that the paramyxovirus F proteins exist as a tetramer.

There may be several explanations for this discrepancy. The protein Sechoy and coworkers used for these studies was iodinated in the presence of H₂O₂, and it is possible the protein conformation was altered by this strong oxidizing agent. The gradient conditions used by Collins and Mottet of pH 5.8 run at 40°C differ significantly from the conditions used in this study (pH 7.4 and 17°C) and may account for the differing sedimentation results.

The exact number of monomers within the F oligomer cannot be determined without x-ray crystallographic data. Even the oligomeric form of the intensively studied HIV envelope glycoprotein has not been elucidated by these methods as it has been reported to form both a trimer (Weiss et al., 1990) and tetramer (Earl, 1990). However, the existence of a single homogenous species of the F protein following centriguation through sucrose gradients suggests that only one of the several forms of F detected following SDS-PAGE are native structures. Furthermore, the oligomeric species of the fusion protein was not stable in 5% SDS, indicating that it is held together by noncovalent forces. Since
monomer release is dependent on SDS, it is likely that the larger structures of F detected following electrophoresis in SDS represent aberrantly aggregated and/or incompletely solubilized monomers (Figures 14 and 15).

The fusion protein containing three leucine to alanine changes sedimented on sucrose gradients in the same position as the wild type protein. This result shows that the leucine zipper motif is not required for oligomer formation. Furthermore, the sedimentation profile of the truncation mutants indicated that the leucine motif and all subsequent residues are not necessary for assembly of the Triton X-100 insensitive oligomeric structure. These results are comparable to those found by Singh et al (1990) and Crise et al (1989), who found that truncation mutants of the fusogenic influenza HA and VSV G proteins, respectively, lacking the membrane anchor and cytoplasmic domains, could form trimers. Cellular transport of many viral glycoproteins has been shown to be dependent on prior oligomerization (for review see Doms et al, 1993). Since the truncated F protein is secreted as an oligomer, the portion of the NDV F protein required for oligomerization and, therefore, cellular transport, involves regions upstream from the leucine zipper. Additional mutational analysis will be required to identify the domains within the protein that are responsible for proper oligomerization.

The absence of fusogenic activity of the fusion protein with the multiple leucine mutations may be due to an inability to properly interact with the NDV HN glycoprotein. Reports from our laboratory suggested that a specific interaction between F and HN proteins must occur to promote membrane fusion (Sergel et al, 1993). Also, a study that substituted the PIV2 HN for a HN protein from a genetically similar virus found that fusion by the PIV2 F protein strictly required the PIV2 HN protein (Hu, 1992). The HN protein has previously been shown to have a distinct fusion promotion function (Sergel, 1993a). One way that these proteins could associate is by the leucine-zipper motif that they both contain near
their transmembrane region. When the HN residues 96-116, just past this transmembrane region, are positioned on an ideal alpha-helix, a hydrophobic face of the helix contains a Leu-Ile-Leu strip on one side. The HN molecule, a type II glycoprotein, is anchored in the membrane in the opposite orientation with respect to the fusion protein and this orientation would fit the criteria of dimerization originally proposed for the C/EBP leucine zipper by Landschultz et al. (1988). Since side chains tend to angle towards the amino terminus of a helix, the authors suggested that an antiparallel arrangement is better suited for side chain interlocking between leucine zippers than a parallel orientation. Specificity of the interaction could be rendered by an attraction between polar side chains of the helix (O'Shea et al, 1992). According to this model, two transmembrane proteins could interlock only if they were anchored in opposite orientations.

All the paramyxoviruses encode a glycoprotein with the unusual type II topology that would conveniently fit this model of dimerization. However, efforts to cross-link F and HN have been unreported as well as personally unsuccessful. However, this inability to crosslink the two proteins may have several explanations. It is possible that there is an absence of basic residues in positions appropriate for cross-linking the two proteins. This is supported by the observation that even the disulfide-linked HN dimer is difficult to appreciably crosslink using noncleavable agents (Nagai, 1978, and personal observations). It may also be possible that the required association of F and HN is very transient. However, it would be unlikely that an association via hydrophobic interactions would be so fleeting as to be undetectable. Another possibility is that an activation of the fusion protein by HN may occur at such a low frequency that it would be undetectable. Spruce and coworkers (1991) have reported that only a few molecules are required for fusion pore formation, and it seems reasonable to expect that this activity need only occur at points of cell-cell contact.
It was possible that the multiple leucine mutants altered the expression of HN protein. To establish the co-expression of HN and the mutant fusion proteins at the surface of the cell, the cell surface antigens were immunoprecipitated and revealed that HN was properly expressed in all cases.

What is especially puzzling about these results is that the alanine substitutions were extremely conservative. Chou and Fasman (1974) have indicated that the alanine and leucine amino acids have a comparable potential to form an alpha helix, suggesting that the hypothetical alpha helix would not be disrupted by the alanine substitutions. The alanine residues may be less able to participate in hydrophobic interactions, as the residues differ significantly in their hydrophobicity (Kyte and Doolittle, 1982). Leucine residues have a long side-chain and the two methyl groups branch out to form a Y-shaped structure. This side chain is optimal for filling space between helices in a knobs-into holes fashion as is predicted for stabilizing dimer formation (Landshultz, 1988). The alanine residue has only one methyl group, and this side chain may not be able to interact with longer side-chains. However, this thesis work gave no indication that these residues play any role in the oligomeric structure of the protein.

It is entirely possible that this region of the fusion protein plays an unknown role in the biological activity of the protein. A diverse group of proteins contain a leucine-zipper motif which do not function in oligomerization. For instance, a family of voltage-gated K+ channels contain a conserved leucine zipper adjacent to a proposed transmembrane region this is expected to play a role in the transduction of charge (McCormack, 1991). The same motif in soluble proteins also may have diverse functions. The Drosophila poly(ADP)-ribose polymerase protein contains heptadic leucines on an alpha-helix of its auto-modification domain that may regulate protein-protein interactions (Uchida, 1993). The apo B mRNA editing protein requires the leucine zipper motif for its editing
function (Teng, 1993), whereas the cytoplasmic zipper of HIV gp160 has been shown to bind calmodulin (Srinivas, 1993). Lastly, the heat shock transcription factor monomer has four leucine zipper motifs which regulate the heat-dependent trimer formation (Rabindran, 1993).

Amphipathic alpha-helices may exhibit an additional behavior. The fusogenic and hemolytic peptide melittin has been reported to lie parallel to the membrane surface by virtue of a hydrophobic face of an alpha-helix (Altenbach et al, 1989; Dempsey, 1990) as have portions of the apolipoproteins and apolipoprotein analogs (Segrest, 1992). The hydrophobic photolabeling studies of Harter and coworkers suggested that the influenza HA fusion peptide may also have this topology (1989). In addition, the protein components of plant storage lipid bodies, the oleosins, are predicted to have domains with this structure and membrane topology (Murphy et al, 1991).

These examples of membrane interactions of amphipathic helices along with results from this work has led to the model depicted in Figure 26. The premise of this model is that the conserved leucines do not function in oligomerization but the long leucine side chains interact with the hydrophobic milieu of the membrane and would effectively increase the area of the protein that is anchored in the membrane. The multiple zipper segments may radiate from the oligomer in a propeller-like fashion.

Several observations support this model. Insertion of the leucine zipper domain through the ER membrane may be incomplete due to its hydrophobic nature and proximity to the transmembrane domain. As potential amphipathic alpha helices have been shown to attain a helical secondary structure in the presence of lipids with the hydrophobic face of the helices spontaneously associating with the hydrocarbon chains of the bilayer (Segrest, 1990; DeGrado et al, 1993), the zipper motif may form a helical structure and associate with the
bilayer. If the conserved leucine residues in the NDV F protein are replaced with an amino acid with short side-chains, such as alanine, the hydrophobic interaction with the bilayer would be diminished. In such a model, the charged residues on polar sides of the helix would interact with the polar extracellular solvent and the polar head-groups of the membrane. It is likely that these interactions with the polar face of the helix would not be specific, and therefore, could tolerate substitutions as was suggested with the E482K and N485K mutants. However, Anantharamaiah and coworkers have used an 18-residue peptide to show that positively charged residues at the hydrophobic-hydrophilic interface of an amphipathic helix assists the helix in binding to the phospholipid and promoting lipid hydration (1985; Epand et al, 1989). Thus, the lysine-rich strip (or g positions, Figure 12) may be less able to tolerate substitutions. Mutations of these residues would address this point.

The residues between the transmembrane domains and leucine zipper motifs of the paramyxovirus's are rich in serines and glycines, and these residues may allow the protein to bend between the two segments. Both residues have a high bend potential and frequently occur at β-turn regions in a polypeptide chain (Chou and Fasman, 1977).

The induction of membrane fusion by amphipathic helices such as melittin has been attributed to a local disruption of the bilayer (Dempsey, 1990). Thus, the presence of the leucine zipper helices inserted into the cell membrane may similarly perturb the membrane and induce fusion. The transmembrane domains themselves have also been proposed to destabilize the bilayer and may contribute to the process (Siegel, 1993). During membrane fusion with a target cell, the fusion peptide inserts obliquely into the target cell bilayer (Ishigura, 1993) and is thought to disrupt target membrane. At this point, the two membranes are tethered together by the two hydrophobic domains on the F1
polypeptide. The leucine "propeller" may function to disrupt the host cell monolayer in response to any conformational changes in the ectodomain of the protein. As suggested in Chapter 3, the first heptad repeat may also associate with membranes. Consequently, a disruption in both the infected cell and target cell membranes should assist in the fusion process (Helm, 1989). Alternatively, the helices may nucleate a channel allowing lipid mixing between the two membranes.
Figure 11. Alignment of the leucine zipper motif of seven paramyxovirus fusion proteins. Sequences were adopted from Morrison and Portner (1991). NDV F protein residues 462-502 were manually aligned with those of SV5 (Simian virus 5), MuV (mumps virus), RV (Rinderpest virus), MV (measles virus), SV (Sendai virus), and RSV (respiratory syncytial virus). The beginning of the potential membrane spanning region of the NDV strain is the last alanine residue shown (#500). The glycosylation consensus site is underlined.
Figure 12. Helical wheel representation of the amphipathic structure of residues 467-515 of the NDV fusion protein. The most amino terminal residue is placed at position number one of the idealized helix. All subsequent residues are rotated by 100° from this position. The distribution of charged side-chains is indicated. Potential helix-breaking glycine residues are indicated by the lower case g. Position of substitutions are marked by arrow. The glycosylated asparagine residue is underlined.
Figure 13. Description of mutations in the leucine repeat region. The amino acid sequence for the wild-type protein, residues 467-504 are shown in A. The nomenclature used for individual mutants is shown at left. Conserved residues of leucine zipper are indicated by vertical boxes. Substitutions are indicated below the wild-type sequence. Panel B shows structurally significant domains in the F₁ and F₂ subunits of the mature protein and the relative position of the termination sites used to produce the dC49 and dC91 mutants. FP, fusion peptide; AAH, amphipathic alpha helix; LZ, leucine zipper motif; TM, transmembrane domain; *, potential glycosylation sites. S-S represents a proposed disulfide linkage although the actual cysteine residues involved are unknown. Generation of the mutants is described under Materials and Methods.
Figure 14. Western blot analysis of mutant fusion proteins. Cells transfected with wild type and mutant DNAs were lysed at 48 hours post-transfection and the post-nuclear extracts were mixed with sample buffer and incubated at 85° for two minutes and electrophoresed under nonreducing conditions on a 10% gel and subjected to western blot analysis as described in Materials and Methods. Blots were probed with an antibody raised against the C-terminus of the NDV fusion protein (Wang et al, 1992). Lane 1, pSVL; lane 2, pSVL-F; lane 3, pSVL-L3A; lane 4, pSVL-L4A; Lane 5, pSVL-L5A; lane 6, pSVL-L34A; lane 7, pSVL-L35A; lane 8, pSVL-L45A; lane 9, pSVL-L345A. Fnr indicates the monomeric, nonreduced, form of the fusion protein.
Figure 15. Metabolic labeling of F proteins expressed in Cos cells. At 48 hours post-transfection, transfected cells were radioactively labeled for two hours with \(^{35}\text{S}\)methionine and proteins in the post-nuclear extract were immunoprecipitated with an anti-F monoclonal antibody (Fu1a). The precipitated proteins were mixed with sample buffer and heated at \(85^\circ\text{C}\) for two minutes prior to loading gel. Lane 1, pSVL; lane 2, pSVL-F; lane 3, pSVL-L3A; lane 4, pSVL-L4A; lane 5, pSVL-L5A; lane 6, pSVL-L34A; lane 7, pSVL-L35A; lane 8, pSVL-L45A; lane 9, pSVL-L345A. Fnr, nonreduced, monomeric form of the protein.
Figure 16. Cell surface expression and precursor processing of mutant proteins. At 40 hours post-transfection, the cells were radioactively labelled for 2 hours and chased with nonradioactive media for 4 hours. The cells were washed in ice-cold PBS and treated with anti-NDV polyclonal antibody as described in Materials and Methods. Immune complexes containing cell surface proteins were precipitated and electrophoresed in the absence of reducing agent (Panel A) or under reducing conditions (Panel B). (A) Lane 1, pSVL; lane 3, pSVL-F; lane 4, pSVL-L3A; lane 5, pSVL-L4A; lane 6, pSVL-L5A; lane 7, pSVL-L34A; lane 8, pSVL-L35A; lane 9, pSVL-L45A; lane 10, pSVL-L345A; lane 11, pSVL-S473A; lane 12, pSVL-E482K; lane 13 pSVL-N485K. Lane 2 controls for antibody released during cell lysis and is from a lysate of unlabeled F-expressing cells incubated with anti-NDV antibody, mixed with an extract from labeled, F-expressing cells not treated with antibody. Fn, nonreduced form; F0, precursor form; F1, F1 subunit.
Figure 17. Quantitation of fusion activity of wild-type glycoproteins. The number of nuclei in 40 fusion areas was determined at 24, 48 and 72 hour post-transfection to indicate the average size of syncytia at each time point. The value obtained from pSVL alone has been subtracted. The standard deviation (vertical lines) and average values from three separate experiments (column) are shown. The values from transfections with pSVL-F, pSVL-HN, and pSVL-F and pSVL-HN are shown.
Figure 18. Quantitation of fusion activity with single leucine-to-alanine mutations. The standard deviation and average values were obtained as described in the legend to Figure 17 and in Materials and Methods. Values shown were obtained from co-transfections of pSVL-F and pSVL-HN, pSVL-L3A and pSVL-HN, pSVL-L4A and pSVL-HN, and pSVL-L5A and pSVL-HN.
Figure 19. Quantitation of fusion activity with multiple leucine substitutions. The values and standard deviation were obtained as described in the legend to Figure 17 and under Materials and Methods. Values shown were obtained from co-transfections of pSVL-F and pSVL-HN, pSVL-L34A and pSVL-HN, pSVL-L35A and pSVL-HN, pSVL-L45A and pSVL-HN, pSVL-L345A and pSVL-HN.
Figure 20. Western analysis of fusion proteins with nonconservative substitutions within the zipper motif. Proteins in transfected cells were prepared, electrophoresed in the absence of reducing agent, and detected as indicated in legend to Figure 14. Lane 1, pSVL; lane 2, pSVL-F; lane 3, pSVL-S473A; lane 4, pSVL-E482K; lane 5, pSVL-N485K. Fnr, nonreduced, monomeric form.
Figure 21. Quantitation of fusion activity with nonconservative substitutions.

The values and standard deviations were obtained as described in the legend to Figure 17 and under Materials and Methods. Values shown were obtained from co-transfections of pSVL-F and pSVL-HN, pSVL-S473A and pSVL-HN, pSVL-E482K and pSVL-HN, and pSVL-N485K and pSVL-HN.
Figure 22. Sucrose gradient analysis of wild-type and L345A fusion proteins. At 48 hours post-transfection, cells were lysed and the extracts layered on a 10-25% sucrose gradient as described under Materials and Methods. Proteins in each fractions were TCA precipitated, solubilized and electrophoresed as described under Materials and Methods. Blots were probed with an antibody generated against the C-terminus of F. Panel A, fractionation of wild-type cell lysate. Panel B, fractionation of cells expressing the L345A mutant. Lanes 1, M, AV virion proteins; Lanes 2, one-third of total solubilized pellet; lanes 3-20, fractions 1-15, 17, 19, and 21, respectively. Positions expected for an ideal monomer, dimer, trimer and tetramer are indicated above lanes 18, 14, 10, and 5, respectively.
Figure 23. Helical wheel representation of the amphipathic helical structure of residues 123-192 of the NDV fusion protein. The most amino terminal residue is placed at position number one of the idealized helix and is the sixth residue of the fusion peptide. All subsequent residues are rotated by $100^\circ$ from this position. The distribution of charged side-chains is indicated. Potential helix-breaking glycine residues are indicated by the lower case g. Position of mutational substitutions are marked by an arrow.
Figure 24. Sucrose gradient analysis of mutants with disruption of two potential alpha helices. At 48 hours post-transfection, cells expressing the A140K/L345A or K155L/L345A proteins were lysed and the post-nuclear extracts were centrifuged through a 10-25% sucrose gradient and the resulting fractions were electrophoresed in the presence of reducing agent as described under Materials and Methods. Blots were probed with an antibody generated against the C-terminus of F. Panel A, fractionation of A140K/L345A cell lysate. Panel B, fractionation of K155L/L345A cell lysate. Lanes 1, M, AV virion proteins, lanes 2, one-third of total solubilized pellet, lanes 3-20, fractions 1-15, 17, 19 and 21. Positions expected of an ideal monomer, dimer, trimer and tetramer are indicated above lanes 18, 14, 10, and 5, respectively.
Figure 25. Sucrose gradient analysis of truncated mutants. Panel A, at 48 hours post-transfection, cells expressing the dC49 mutant protein were radioactively labelled for 2 hr and chased with nonradioactive methionine for 6 hours. The culture media was removed, layered over a gradient, and fractionated as described under Materials and Methods. The resulting fractions were immunoprecipitated with a monoclonal antibody directed against mature F protein. Lane 1, total extract from AV infected and radiolabeled cells; lane 2, immunoprecipitate from solubilized pellet; lanes 3-20, immunoprecipitated proteins in fractions 1-5, 17,19, and 21, respectively. Panel B, at 48 hours post transfection, cells expressing the dC91 mutant were radioactively labelled for 2 hours. The cells were lysed and the post-nuclear fraction was layered over a gradient as described previously. The resulting fractions were immunoprecipitated as in Panel A. Lanes are identical as those described for Panel A. Relative positions expected for an ideal monomer, dimer, trimer and tetramer are indicated above each figure.
Figure 26. Schematic of hypothetical model depicting the membrane topology of the leucine zipper motif. (A) represents the oligomeric structure of the mature fusion protein along with the exposed fusion peptides of the F₁ subunits. (B) represents the bilayer of the infected cell membrane. (C) represents the amphipathic helix of the conserved leucines. The heptadic leucine and isoleucine residues are shown and are expected to be in contact with the hydrophobic portion of the monolayer. (D) and (E) represent the transmembrane domain and cytoplasmic tail, respectively. Only two subunits of the proposed oligomer are shown.
CHAPTER V

THE ROLE OF THE AMINO TERMINUS OF THE F₁ SUBUNIT OF THE NDV FUSION PROTEIN

INTRODUCTION

There are a variety of strains of the Newcastle disease virus which differ widely in virulence for its avian host. Infection with a virulent strain can result in 100% mortality in an avian population, whereas avirulent strains result in very low mortality rates. Originally, the virulence of an NDV strain was determined by its ability to kill chick embryos following allantoic inoculation. By definition, virulent strains will kill 100% of embryos within 48 hours (Granoff, 1964).

The strain-specific difference in virulence is determined primarily by a structural variation of the viral fusion glycoprotein. The fusion protein mediates fusion of the viral envelope with the host cell plasma membrane, thereby enabling the penetration of viral genome into the cytoplasm. The fusion protein is initially synthesized as a biologically inactive precursor termed F₀. During transport through the cell, the precursor may be cleaved by a host endoprotease. The resulting two polypeptides, F₁ and F₂, remain disulfide-linked through cysteine residues. This cleavage, which is required for the fusion activity, positions the hydrophobic fusion peptide at the new amino terminus of F₁. In virulent strains, cleavage of the precursor occurs in many cell types after transport through the Golgi apparatus and results in a systemic infection. In avirulent strains, cleavage of the fusion protein only occurs in the presence of a few cell types (such as those of the respiratory tract), which secrete a virus activating protease, such as VAP, resulting in a localized infection (Ogasawara et al, 1992). In
he absence of cleavage, the F protein cannot promote membrane fusion. Consequently, the cleavability of the F protein determines the virulence of the virus and the availability of an appropriate protease determines the host range and tissue tropism.

The difference in cleavability of the fusion proteins derived from different strains of NDV has been attributed to an amino acid sequence difference at the cleavage site (Glickman et al, 1988; Toyoda et al, 1989). A comparison of the sequence around the cleavage site of the F protein from a number of different strains of NDV is shown in Figure 27A. The fusion proteins from the readily cleaved virulent strains contain two pairs of basic amino acid residues at the cleavage site and are recognized by an intracellular protease, furin, commonly present in eukaryotic cells (Barr, 1990). The fusion proteins from avirulent strains have a pair of single basic residues at the cleavage site. In cell culture, these avirulent strains require the addition of a protease such as trypsin to cleave the monobasic site, allowing plaque formation (Gotoh et al, 1990).

Figure 27B shows the sequence around the cleavage site of many different paramyxoviruses. The cleavage site sequences are shown along with the beginning of the fusion sequence at the amino terminus of F1. The fusion sequence is well conserved across the family and begins with a phenylalanine in virtually all paramyxoviruses. The Phe-X-Gly motif is frequently found in the fusion protein in other families of enveloped viruses (Figure 27C). A striking exception to this motif is the avirulent strains of NDV, which all have a leucine at the initial position.

The requirement for a specific residue adjacent to the cleavage site has not been examined. The question this work addressed was what role the leucine residue plays in this avirulent phenotype. The results indicated that this leucine residue influences cleavage and suggested that a single nucleotide change can abolish the
virulence of NDV. These results may have implications with respect to the origination of avirulent strains of NDV.

RESULTS

To evaluate the requirement of the conserved phenylalanine at the cleavage site of the F protein of virulent strains, residue 117 was changed to a leucine to mimic the residue in the avirulent fusion proteins. A single nucleotide change created this F117L mutant. The phenylalanine was also changed to a glycine to create the F117G mutant. Both of these mutations keep the upstream cleavage site of this virulent AV strain unaltered. Following cleavage of the precursor, residue 117 of the nascent protein becomes the amino terminus of the F₁ molecule.

To examine the properties of these mutants, the DNAs were subcloned into pSVL, an SV40-based expression vector. These clones were expressed in Cos 7 cells using a DEAE-dextran transfection protocol. By immunofluorescence, both mutants, as well as the wild-type protein, are found expressed at the cell surface (Morrison et al, 1993).

To investigate the ability of these two mutants to promote fusion, they were tested in a fusion assay developed in this lab. In this assay, the F cDNAs are co-transfected with HN cDNA. At 24, 48, and 72 hours post-transfection, the average number of nuclei per syncytia were determined. When compared to wild type, the substitution of phenylalanine with glycine maintained the ability of the protein to induce fusion. This result suggested that the conserved phenylalanine at the amino terminus of F₁ is not required for the fusion activity of these virulent viruses. However, substitution of the phenylalanine with leucine virtually eliminated the fusion activity of

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The absence of fusion activity with the leucine mutant could be explained if this fusion protein is not cleaved. To determine whether or not this mutant becomes cleaved in the cell, proteins present in lysates from transfected cells were examined by western blot analysis. The blot was probed with a polyclonal antibody directed against the C-terminus of F (obtained from Mark Peeples; Wang et al, 1992). When the mature fusion protein is treated with reducing agents, the disulfide linked polypeptides separate. Under these conditions, only F₁, the larger of the two subunits, is detected as well as the uncleaved precursor. As shown in Figure 28, both the wild type and glycine mutant proteins are readily cleaved and the cleaved forms migrate with the viral F₁ molecule. Under these conditions, the leucine mutant shows no cleavage. This result indicated that the cellular protease is unable to cleave the arginine-leucine bond at the processing site.

The absence of cleavage of the F₁₁₁₇L mutant could account for its lack of fusion activity. Therefore, an attempt was made to activate the fusion activity of this leucine mutant by cleavage with trypsin. In this assay, trypsin should cleave any susceptible protein expressed on the cell surface. When trypsins was added to an agar overlay over cotransfected cells, the leucine mutant was able to stimulate the formation of syncytia (Morrison et al, 1993). This result indicated that the presence of a leucine residue at the amino terminus of the F₁ portion of the fusion protein is not detrimental to the fusion activity of this protein. Once the precursor is cleaved, the F₁ protein beginning with a leucine, glycine, or phenylalanine is functional in mediating fusion.

DISCUSSION

The amino terminus of F₁ begins with a Phe-X-Gly in all virulent NDV fusion
glycoproteins in the family of paramyxovirus and this motif is found in the glycoproteins from other viral families (Gallaher, 1987). This conserved region is at the amino terminus of the hydrophobic fusion peptide of NDV and was expected to be crucial for the biological activity of the protein. Mutational analysis of the first amino acid in this motif revealed that this position can accept mutations and still have syncytium-inducing activity.

An unexpected result from this work is that this initial residue of F1 does not affect fusion but affects the precursor processing site. There have been several reports that viral glycoprotein precursors are processed by furin, a member of the subtilisin family of proteases, that cleaves after the RxR/KR sequence of amino acids (Steineke-Grober et al, 1992; Gotoh et al, 1992). This ubiquitous enzyme cleaves proteins as they are transported through the constitutive secretory pathway of the Golgi apparatus (Steiner et al, 1992). There have been no reported restrictions about the amino acid immediately following the furin processing site. The work presented here indicates that the amino acid at the C-terminal side of the cleavage site can alter the ability of the cell-associated protease to process a precursor. Although a glycine or phenylalanine residue in this position allows processing, it is possible that the leucine residue may cause a change in conformation that makes the cleavage site inaccessible. Furthermore, these results show that the cleavage of FO can be eliminated by a single nucleotide change which converts the phenylalanine (TTT) to a leucine (CTT). In otherwords, this single change converts the virulent form of the fusion protein to an avirulent form.

These results further suggest that avirulent strains may have evolved from virulent strains by a single nucleotide mutation. When viral stocks are passaged in chick embryos, the fusion protein in avirulent viruses is cleaved and viral growth is as vigorous as that of the virulent viruses (Granoff, 1964). This indicates that the cell
types in the embryo produce a protease that can recognize the RxxR-L processing site of the avirulent strains. Gotoh and coworkers have isolated from chick embryos a calcium-dependent serine protease designated VAP, that is highly homologous to the activated form of the blood clotting factor X (FXa) (Gotoh et al, 1990). FX is a member of the prothrombin family and circulates as an inactive precursor until the blood clotting cascade takes place. The activated form of this enzyme is secreted into the amniotic and allantoic fluids and may be activated at sites of tissue damage (Ogasawara et al, 1992). This protease cleaves after the arginine residue at the Glu-Gly-Arg processing site in prothrombin (EGR-T/I) and in monobasic processing sites of glycoprotein precursors such as found in the avirulent NDV strains. The specificity of this protease suggests that the VAP/FXa protease is a determinant of viral tropism of the avirulent strains.

The existence of this type of protease allows for the replication and for the natural maintenance of the avirulent strains. This protease may also allow for the origination of avirulent isolates. In the event that a virulent viral F protein undergoes a spontaneous phenylalanine-leucine substitution, the altered protein can no longer be activated by the furin protease. For this mutation to become established, the protein must be cleaved at some point to allow viral infection and subsequent replication. In the presence of VAP/FXa, the mutant can be cleaved and may then subsequently evolve to be more efficiently recognized by this enzyme. In the presence of this enzyme, the pair of dibasic residues are no longer required and further evolution may result in a loss of the second basic residue and create the sequence found at the cleavage site of the avirulent strains.

For the virulent strains to arise from avirulent, they would have to be in an environment which selects for the RXRR-F motif. Of the known proteases that cleave at monobasic sites, only FXa appears to cleave an arginine-leucine bond, while several
proteases will cleave if a glycine, alanine or serine are on the C-terminal side of the site (Devi, 1991). As the Sendai virus F protein having an XXXR-F site is cleaved in the same embryonic fluids that the avirulent NDV strains are cleaved, it is likely that the VAP/FXa protease also cleaves a monobasic arginine-phenylalanine cleavage site (Ogasawara et al, 1992). Tryptase Clara is a calcium independent, serine protease, that is found in rat lung and will also process the XXXR-F, as well as the KXXR-G, cleavage sites (Tashiro et al, 1992). The results presented here indicated that furin does not recognize the dibasic cleavage site if a leucine is at the C-terminal side of the cleavage site. Neither does this ubiquitous enzyme efficiently recognize a single dibasic site (XXKR) nor the RXXR site with two single basic residues (Creemers et al, 1993). Therefore, two amino acid substitutions would have to occur for the virulent phenotype, that recognized by furin, to evolve from an avirulent strain. If a single amino acid change from RXXR-L (avirulent site) to either RXXR-F or RXKR-L occurred, the isolate would not be activated by furin and still need to be propagated in the presence of the VAP/FXa, or possibly the tryptase Clara, protease. In addition, a minimum of three point mutations must occur to produce the virulent (RXRR-F) motif from the avirulent (RXXR-L). The codon for the glycine in the avirulent RXGR-L sequence would have to change from GGA (for glycine) to AAA (for lysine) and would require two base changes to code for the amino acid substitution. In addition, a third base change, and second amino acid substitution would be required to create the virulent RXKR-F cleavage site from the avirulent RXXR-L site. Viral replication in the presence of the VAP/FXa protease would be under no selective pressure to evolve either of these genotypes. Therefore, virulent fusion proteins would be unlikely to evolve from avirulent strains.

A recent report describes a substitution of the phenylalanine at the amino terminus of the F₁ with a leucine in the SV5 F glycoprotein that had no effect on fusion
activity or cleavage (Horvath and Lamb, 1992). The cleavage site of the SV5 fusion protein is RRRRRF. As the SV5 protein has arginines in the -2 and -3 positions, unlike the NDV F which has glycine and lysine residues in these respective positions, it is possible that the SV5 fusion protein is cleaved by an enzyme other than the one that cleaves the NDV F protein. The existence of subtilisin-related enzymes with slightly different specificities has been suggested by Kawahara et al (1992), who found a lymphoid cell line which would readily cleave the fusion protein of SV5 and PIV3 but not virulent NDV.

With the widespread use by the U. S. poultry industry of live virus vaccines involving the avirulent NDV strains such as B1, the possible emergence of more pathogenic strains from vaccine strains of low virulence is a concern. However, these vaccines have been used for over three decades and this event has not been documented, suggests that there is not a strong selection pressure for virulence. This pattern lends support to the arguments presented here regarding the evolutionary restrictions placed on the cleavage-activation site of this protein. However, further studies will be necessary to determine cleavage-site specificity and tissue distribution of the poteases responsible for processing the fusion protein precursor.
### Legend to Figure 27 A.

Comparison of amino acid sequences (NDV residues 109-132) among virulent and avirulent NDV isolates (Toyoda et al., 1989). Mutants F117G and F117L are shown for comparison (Morrison et al., 1993). The amino terminus of F1 initiates the fusion peptide sequence and mutations are underlined.

### B.

<table>
<thead>
<tr>
<th>Paramyxovirus</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MuV</td>
<td>RRHKR FAGIAIGIAALGVA</td>
</tr>
<tr>
<td>SV5</td>
<td>RRRRR FAGVVIGLAALGVA</td>
</tr>
<tr>
<td>NDV</td>
<td>RRQKR FIGAIGSVALGVATA</td>
</tr>
<tr>
<td>RV</td>
<td>RRHKR FAGVVLAGAALGVA</td>
</tr>
<tr>
<td>MV</td>
<td>RRHKR FAGVVLAGAALGVA</td>
</tr>
<tr>
<td>SV</td>
<td>APQSR FFGAVGVTIALGVA</td>
</tr>
<tr>
<td>PI3</td>
<td>PRTKR FFGVVTIALGVA</td>
</tr>
<tr>
<td>RSV</td>
<td>KRKRR FLGFLGVGSAIASG</td>
</tr>
</tbody>
</table>

### C.

<table>
<thead>
<tr>
<th>Retrovirus</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>FLGFLGAAGST</td>
</tr>
<tr>
<td>HIV-2</td>
<td>FLGFLATAGSA</td>
</tr>
<tr>
<td>STLV-3</td>
<td>FLGGLATAGSA</td>
</tr>
</tbody>
</table>

Comparison of amino acid sequences of paramyxovirus fusion protein cleavage site sequences (Morrison and Portner, 1991). MuV, mumps; SV5, Simian 5; NDV, Newcastle Disease; RV, Rinderpest; MV, measles; SV, Sendai; PI3, parainfluenza 3; RSV, Respiratory Syncytial virus. C. Partial fusion peptide sequence of representative retrovirus glycoproteins containing the FXG motif (Gallaher, 1987).
Legend to Figure 28. Western analysis of mutant fusion proteins. Proteins present in extracts of cells transfected with various DNAs for 48 hour were electrophoresed in the absence (Panel A) or presence (Panel B) of β-mercaptoethanol on 10% polyacrylamide gels and subjected to western blot analysis as previously described. Blots were probed with an antibody directed against the C-terminus of the NDV F protein. Lanes 1, NDV virions grown in eggs (AV); lanes 2, pSVL-F (F), lanes 3, pSVL-F117G (G); lanes 4, pSVL-F117L (L); lanes 5, pSVL (v).
Membrane insertion. The cell-free translocation system has revealed that the NDV fusion protein behaves like a typical type I integral membrane glycoprotein. Only the hydrophobic amino terminal sequence of the protein is recognized by SRP and this segment targets the precursor to the rough ER vesicle. Translocation of deletion mutants indicated that membrane translocation is arrested by integration of the hydrophobic domain near the C-terminus. Following insertion of the nascent molecule, enzymes within the lumen of the ER vesicles cleave the signal sequence from the protein and add oligosaccharides to the protein.

This translation system also produced other species of the protein. A major species of protein was not targeted to the ER, possibly due to a limiting amount of SRP or vesicles, and remained as full-length unprocessed molecules. In addition, a population of fusion protein molecules were integrated in the membrane by the fusion peptide. These molecules were glycosylated at only one site and were initially undetected as they comigrated with the nascent, untranslocated, protein. Protease digestion of the protein products completely digested the untranslocated protein and revealed the additional population in which a 21 kD portion of the molecule was translocated. In the absence of the fusion peptide, this topology was not observed.

The possibility that a partially anchored fusion protein also occurs during cellular synthesis of the fusion protein has been suggested by several curious
experimental results. A consistent observation is that the amount of the F protein detected in NDV infected cells is unexplainably low. During replication of the virus, the amount of viral mRNAs and their protein products are made in amounts that generally correspond to their position in the viral genome (Peeples, 1988; Kingsbury, 1990). As the gene order is NP-P-M-F-HN-L, the amount of F protein synthesized should occur in an amount less than the M protein but in higher amounts than the HN protein. However, following a short radioactive label of infected cells, electrophoresis of the resulting cell extract revealed the viral proteins and indicated that the 69 kD F0 protein is synthesized in approximately the same amount or less as that of the HN protein (Figure 5). One possible reason for this result is that a population of the fusion protein molecules halt translocation prematurely, as occurs during the cell-free synthesis. These underglycosylated molecules would have an expected molecular weight of ~60 kD and would comigrate with the NP protein and be undetectable. Efforts to immunoprecipitate this potential F species were hampered by a potential contamination of co-immunoprecipitated NP molecules.

The transfection studies allowed examination of the topology of the F protein in the absence of other viral proteins. Following optimization of the transfection protocol, the Cos cells and pSVL expression vector were used to express the cDNA. When F-expressing cells were lysed and the proteins electrophoresed under reducing conditions, an underglycosylated species was expected to comigrate with the F1 subunit (also ~60 kD) and be undetectable. When precursor cleavage was inhibited with the F117L mutant, an underglycosylated species was not detected. This result undeniably indicated that a partially translocated population of molecules is not detected following cellular biosynthesis.

However, further examination is required to determine if a partially translocated species of the fusion protein escapes detection due to aggregation or degradation.
Following anchoring of the protein at the fusion peptide, most of the molecule would remain exposed to the cytoplasm. This abnormally translocated protein may be quickly degraded in the cell (Rechsteiner, 1991; Bradshaw, 1989). In addition, hydrophobic proteins often require the addition of oligosaccharides to remain soluble (Gibson et al., 1979; Vidal et al., 1989), and the unglycosylated portion of the molecule may form an insoluble aggregate. Alternatively, the lysis conditions may cause the protein to aggregate such as occurs with the hydrophobic prion protein on contact with nonionic detergents (Garbizon, 1987).

Another explanation for the lack of a population anchored by the fusion peptide is that the cell-free translocation system does not faithfully reflect the cellular synthesis of this glycoprotein. During cell-free synthesis, translocation may pause at the fusion peptide and allow the untranslocated portion to prematurely fold, thereby inhibiting further translocation. The cell may either not allow translocation to pause, or it may inhibit premature folding. Both scenarios are possible. The cytosol contains numerous components which inhibit premature folding (reviewed in Eilers and Schatz, 1988) but may be lacking in the cell-free system. For example, Chirico and coworkers (1988) have determined that the 70 kD yeast heat shock proteins, SSA1 and SSA2, increased the rate of post-translational translocation in the wheat germ translation system. Also, the yeast SSS1 cytosolic gene product is required for complete translocation of secretory preproteins (Esnault et al., 1993). As antisera raised against this 9 kD protein recognized a small amount of a protein in a commercial microsomal preparation, it is likely that eukaryotic cells may have a similar component. This small protein may be lost during isolation of the ER membranes and supplementing the translocation system with this component may further simulate cellular translocation. It is possible that further optimization of the reaction conditions could also increase translocation of the molecule. For example, the driving force for
translocation or the rate of the synthesis may be inadequate and may allow translocation to pause. In addition, a receptor that recognizes the membrane-spanning segment during translocation has not been identified, and this system may be useful in characterizing this potential component. For example, the fusion peptide may prematurely close the ER transport channel during cell-free synthesis. Fusion peptide mutants could test the specificity of this integration mechanism.

The experimental findings described in this work have led to several predictions that need to be addressed. The cell-free experiments attempted to determine whether the fusion peptide could function as a signal sequence. The lack of translocation of the MF and FSS mutants suggested that the fusion peptide failed to target the mutant proteins to the membrane due to an inability of SRP to recognize this domain. However, mutational analysis of the fusion peptide sequence could further define the amino acid requirements for a hydrophobic domain to interact with SRP and function as a type II signal sequence. In addition, the fusion peptide has been predicted to be on the threshold of hydrophobicity to function as a membrane anchor (Paterson, 1987). This prediction could be tested by increasing the hydrophobicity of the domain. The potential for the fusion peptide to function as a stop-translocation signal in vitro but not during cellular synthesis also needs to be further investigated. It is likely that some required cellular component is altered or missing in the cell-free translocation assay. In order to identify this factor, the reaction conditions could be varied or supplemented with cellular factors.

**Role of conserved motifs.** The pSVL expression system has been useful in characterizing the transport, folding, processing, and biological activity of the wild-type and mutant fusion proteins synthesized in vivo. The system was used to examine the significance of single conserved residues and specific sequence motifs within the fusion protein. When the primary sequence of the protein was compared to that of
other proteins within the paramyxovirus family, a leucine zipper motif was identified immediately upstream from the transmembrane region (Buckland and Wild, 1989). This region shows high conservation of the leucine residues against a background of nonconserved residues and predicted that the leucines have an important function for the biological activity of the fusion protein. Functional assays indicated that these multiple leucines are required for the fusogenic activity of the protein. Single leucine substitutions and nonconservative changes between the conserved leucines had little affect on the fusion activity. The lack of fusion activity with the multiple leucine substitutions could not be accounted for by any obvious defect in processing, folding or expression of the mutant fusion proteins.

The presence of a leucine zipper motif suggested a function in oligomerization of proteins. This prediction led to an examination of the oligomeric structure of the wild-type and mutant fusion proteins. The wild-type protein was found to sediment in sucrose gradients as a Triton-resistant trimer. The conserved leucine motif was found not to be required for the stability of this oligomer. In addition, removal of 91 residues from the C-terminus of the F protein did not disrupt the oligomeric form indicating that the leucine zipper, the transmembrane domain and cytoplasmic tail are not necessary for oligomer formation. Also, immunoprecipitation of the truncated F protein by a conformation-specific anti-F monoclonal antibody indicated that these domains were not necessary for the transport or conformation of the oligomer. These results were consistant with similar examinations of the influenza HA and VSV G fusogenic proteins (Singh et al, 1990; Crise et al, 1989).

The domains of the protein responsible for the oligomeric structure of the protein should be further elucidated. It is possible that an alpha helix immediately downstream from the fusion peptide is responsible for this structure. Several other viral fusogenic proteins contain a long heptad repeat within a potential alpha helix
immediately downstream from the fusion peptide (Chambers et al, 1990). Of these viral fusion proteins, the structure of the influenza HA molecule is the best characterized due to X-ray crystallography data (see Wiley and Skehel, 1987). A model proposed by Carr and Kim (1993) suggests that, following activation, this potential alpha helix forms a three-stranded coiled coil and increases the length of the helix extending from the membrane, interwound with two other HA2 polypeptides as a three-stranded coiled coil. This long helical structure appears to assist in the stability of the trimer of the fusogenic form of the protein (Carr and Kim, 1993).

The NDV fusion protein shares similarities with the fusogenic influenza HA protein. Both are type I glycoproteins which are proteolytically processed and exist as disulfide-linked heterodimers. The membrane-anchored subunits retain the fusion peptide at the newly formed amino terminus. By analogy, it seems possible that the comparably placed helix in the NDV fusion protein may also function in trimerization. This prediction was tested with two mutants that had alterations adjacent to, and in, the predicted alpha helical region adjacent to the fusion peptide. Although those substitutions did not result in detection of a monomer following centrifugation through sucrose gradients, it is still possible that this helix is essential in the noncovalent oligomerization of the molecule. Neither of the mutations involved the hydrophobic face of the helix. In addition, the characteristic 4-3 hydrophobic repeat involves over 8 turns of the helix, and it is likely that single substitutions will not completely disrupt this potential structure. Alternatively, a protein that is unable to oligomerize, or remains in monomeric form, may be held in the ER by host-cell proteins (Doms et al, 1993). Therefore, the block in cellular transport of the K155L mutant (Sergel et al 1994) may be due to faulty oligomerization.

Investigations with the available analytical tools were unable to show a correlation of an absence of fusogenic activity of the triple leucine mutant with an
alteration in conformation. However, multiple leucines in the zipper regions are required for the biological activity of the protein. There were several possible explanations for this result. The leucine motif is recognized as a structure that allows coil-coiled oligomer formation (O'Shea et al, 1988). Since this motif was not noticeably required for the F oligomeric structure it was possible that it functioned in the required interaction with HN. Due to an inability to detect an interaction between the wild-type proteins, this possibility cannot be ruled out.

It appears likely that the fusogenic NDV protein has adopted the leucine motif for an unexpected function. The subtlety of the substitutions and the requirement for multiple leucines led to a re-evaluation of the potential function of this region and to the proposed model for the conserved leucines to interact with the bilayer. As previously described, there are several structural features that support this model and, in addition, it is in full agreement with the experimental results. According to the model, single leucine to alanine substitution will not be expected to disrupt the association with the membrane. Multiple substitutions should severely reduce the propensity to interact with the bilayer.

The model predicts that the charge distribution on the cell surface along with the fluidity of the bilayer will allow for the nonconservative changes on the polar sides of the potential helix. The fusogenic ability of the E482K and N485K mutants support this prediction. In addition, the nonpolar face of the predicted helix is adjacent to a face rich in basic lysine residues in position g (Figure 12). This basic strip of the helix may interact with the negatively charged phosphate groups in the bilayer and affect the association of the helix with the membrane. This model predicts that substitutions of the lysine residues should alter the membrane association of with the helix and may inhibit fusion.
The model for the function of the leucine zipper requires further characterization. The absence of fusion with the multiple alanine substitutions was proposed to be due to a decreased interaction with the membrane. A substitution with another hydrophobic residue such as isoleucine should maintain the interaction while the smaller valine residue may not. It would also be interesting to increase the potential membrane interaction by adding additional hydrophobic residues to the hydrophobic face of the helix. Such mutations may cause the helices to embed deeper into the membrane. These mutations may increase the disruption of the membrane and may increase fusion. The sequence between the transmembrane and leucine zipper is rich in glycine and serine residues. These amino acids are often found at \(\beta\)-turn regions within proteins and are predicted to allow a bend between the two domains. Removal of these residues would test this prediction and may inhibit the ability of the helix to interact with the membrane.

Additional insight into the function of the two amphipathic helices of NDV may be obtained by testing for the activities proposed for other natural amphipathic alpha helices such as the apolipoproteins. Plasma lipoproteins are macromolecular particles that transport lipids. They are composed of a monolayer of phospholipids, cholesterol, and apolipoproteins surrounding a core of nonpolar lipids, cholesterol esters and triglycerides (Ponsin, 1986). The helices of the apolipoproteins are proposed to lie parallel to the membrane and activate the plasma lecithin:cholesterol acyltransferase (LCAT) enzyme. This enzyme transfers fatty acids from phosphatidylcholine to cholesterol allowing for the efflux of cholesterol from membranes (Frohlich, 1992). As plasma membranes are approximately 50% cholesterol by weight, local activation of this enzyme by the leucine zipper helices may alter the microenvironment of the membrane in such a way as to facilitate membrane fusion. Alternatively, the fusion protein may have an inherent lipid transport activity.
Demel and coworkers have determined that Sendai virions mediate phosphatidylcholine transport and that the F protein shares some homology with a phosphatidylcholine-specific transport protein (1987). This prediction could be tested by the addition of transport inhibitors to the fusion assay.

Another activity found with amphipathic helices such as melittin is the stimulation of phospholipases C (Okano et al, 1985) and A2 (Sharma, 1993) which in turn stimulate membrane fusion (Nieva, 1993). Curiously, lysophosphatidylcholine inhibits both phospholipase C-induced and Sendai virus-induced membrane fusion (Yeagle, 1994), as well as NDV-induced syncytia formation (Kohn, 1965), by a mechanism which is believed to inhibit membrane destabilization (Yeagle, 1994). These results suggest that the leucine zipper motif may potentially promote fusion by stimulating phospholipase activity and ultimately bilayer disruption. Using phospholipase inhibitors during fusion assays could test these predictions.

A phenomenon observed during this study is that membrane association appears to correlate with decreased cellular expression. Expression of the multiple leucine mutant cDNAs was more efficient than with the wild-type protein. Similarly, the dC91 (Figure 5) and dC49 mutants had increased expression. It is possible that extensive membrane association hampers transport from the ER. Curiously, work in this laboratory has indicated that mutations that decrease the hydrophobicity of the fusion peptide, along with propensity to act as a membrane anchor, also show significantly increased expression (Sergel et al, 1994). This potential correlation deserves characterization.

Precursor cleavage. A common theme among many, though not all, fusogenic proteins is the requirement for precursor cleavage (White, 1990). This cleavage often occurs following a dibasic or monobasic processing site and is dependent on specific
cell-derived proteases (Barr, 1991). Virions with uncleaved fusogenic proteins are unable to infect a target cell. A leucine on the C-terminal side of the NDV F processing site blocks cleavage by the Cos cell protease and, in effect, results in the avirulent phenotype. Trypsin digestion of the cell surface leucine mutant indicated that the leucine substitution does not affect cleavage by this enzyme or the subsequent fusion event. Furthermore, a glycine at this position allowed recognition by the cell associated protease and allowed syncytium formation. Thus, the phenylalanine residue at the amino terminus of F1 is not conserved for the fusogenic activity of the protein.

The cellular protease in Cos cells does not recognize the RXXR-L site of the avirulent strains such as B1 (unpublished observation). However, propagation in fertilized eggs or avian respiratory tract cells produces the cleaved F protein of avirulent strains (Ogasaware et al, 1992). Thus, there are at least two different natural proteases that exist and separately process either the virulent or avirulent fusion proteins. It is likely that more cell-type specific proteases will be identified that cleave viral fusion proteins. For instance, the LCMV GP precursor contains a cleavage site RR-L that is cleaved in BHK cells (Southern et al, 1991). However, BHK cells do not cleave an NDV fusion protein with an RXRR-L cleavage site, although MDBK cells will process this site (Pritzer et al, 1990).

Similarly, the virulence of avian influenza A viruses depends on the cleavability of the fusogenic HA precursor. All avian avirulent HA precursors contain only a single basic amino acid at the cleavage site (Donis et al, 1989) and cause a mild or inapparent disease. The first residue following the cleavage site is a conserved glycine. However, a glycine to alanine substitution suggested that the glycine is not conserved for cleavability but because it promotes the fusion activity (Walker, 1993). Kawaoka and Webster have proposed that a virulent influenza A virus arose from the
repeated replication of an avirulent virus in chickens (1985). Although a similar increase in virulence of the nonpathogenic strains of NDV is possible, such an occurrence has yet to be documented. A thorough mutational examination of the cleavage site recognized by purified proteases may reveal additional information as to the evolutionary pressures placed on the virulent and avirulent NDV strains.
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