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Viral ultrastructure and sequencing of the P gene of the porcine paramyxo-like virus, ISU-92

by

Peter Kwame Buor Akoto

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Veterinary Microbiology
Major Professor: Prem S. Paul

Iowa State University
Ames, Iowa
1998
This is to certify that the Master's thesis of

Peter Kwame Buor Akoto

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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LIST OF ABBREVIATIONS

BME – basal medium Eagle
BPIV 3 – bovine parainfluenza virus type 3
cDNA – complementary DNA
CNS – central nervous system
CPE – cytopathic effect
dATP – deoxyadenosine 5’ triphosphate
dCTP – deoxycytidine 5’ triphosphate
dGTP – deoxyguanosine 5’ triphosphate
dTTP – deoxythymidine 5’ triphosphate
DMSO – dimethyl sulfoxide
F – fusion (protein)
FBS – fetal bovine serum
H – hemagglutinin (protein)
HA – hemagglutinin
HPIV 1 – human parainfluenza virus type 1
HPIV 3 – human parainfluenza virus type 3
HPIV 4B – human parainfluenza virus type 4B
L – large (protein)
M – matrix (protein)
M2 - variant of matrix protein
MEM – minimum essential medium
mRNA – messenger RNA
N – nucleoprotein
NA - neuraminidase
NP – nucleocapsid protein
NZY-agar – arginine, glutamine and tyrosine containing agar
OD$_{260}$ – optical density at 260 nm wavelength
Oligo d(T) – small sequence (piece) of deoxythymidine triphosphate
ORF – open reading frame
PFU – plaque forming units
PIV 3 – parainfluenza virus type 3
PPMV – porcine paramyxovirus
RNA – ribonucleic acid
RNase - ribonuclease
SDS – sodium dodecyl sulphate
SM – sodium- magnesium
SPF – specific pathogen free
TAE – Tris-acetate
TEN – Tris-EDTA- Sodium
UV – ultraviolet
VSV – vesicular stomatitis virus
$^{32}$PdCTP – $^{32}$P labeled deoxythymidine 5’ triphosphate
ABSTRACT

Porcine paramyxovirus (PPMV) was isolated from pigs experiencing a neurological and respiratory disease outbreak on a commercial pig farm in Southern Minnesota. The virus has been shown to be infectious and pathogenic to swine. Antigenic studies determining cross reactivity have demonstrated reactivity with antibodies of parainfluenza viruses, PIV-1, PIV-3 and PIV-4-B.

There has not been extensive investigation into the virion morphology and ultrastructure of the PPMV nucleocapsids, proteins, and nucleotide sequences of PPMV. Virions were purified by sucrose-gradient centrifugation. Electron microscopy of the purified virions and nucleocapsids revealed the pleomorphism of the virions and serrated nucleocapsids.

To characterize the genome of PPMV we cloned the total RNA of PPMV-infected cells and generated a cDNA library. The library was screened with a radiolabeled probe prepared from virus infected cells. One virus specific clone was identified and sequenced. Sequence analysis and comparison to previously published sequences of paramyxoviruses revealed that this clone was from the P gene of PPMV and had 98% homology with the P genes of parainfluenza virus 3 (PIV-3) and bovine parainfluenza virus 3 (BPIV 3). The cloned gene will be beneficial in developing diagnostic tests for the detection of PPMV infections.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

An investigation of a respiratory and a central nervous system (CNS) disease outbreak in a farrow-to-finish farm in Minnesota in 1992 led to the isolation of a viral agent that was characterized to be a paramyxo-like virus (Janke et al., 1992). The virus was designated as ISU-92. This porcine paramyxovirus-like infection had an infectious nature. All 350 pigs in the first barn identified with the disease were affected and the infection appeared to gradually spread to the other barns on the site (Janke et al., 1992; Battrell et al., 1993). In addition, this disease presented as a fast spreading disease, within the barn and between barns on the site (Janke et al., 1992; Battrell, 1995). In the initial barn where the infection was first noted, mortality was estimated at approximately 4% and the infection in subsequent barns produced high morbidity but no mortality (Janke et al., 1992; Battrell, 1995). The main clinical manifestations of the infected pigs were dyspnea, harsh barking cough and CNS disorders in the form of rear-end ataxia, intermittent head pressing, and whole body tremors (Janke et al., 1992; Battrell, 1995).

An attempt to reproduce the infection in pigs with lung homogenates (20%w/v) from infected pigs failed. However, inoculation of three-day old piglets intranasally with cell culture propagated porcine paramyxovirus (PPMV) produced lesions of mild interstitial pneumonia and encephalitis characterized by multifocal gliosis and perivascular cuffing of the piglets (Janke et al., 1992). Studies on this virus were continued by Battrell et al. (1993) by using virus suspensions. Two forms
of the PPMV were demonstrated by electron microscopy, a spherical virion with a
diameter of 250 nm and a filamentous form in virion suspensions (Battrell, 1995).

Furthermore, PPMV was adapted for growth in five different cell lines (swine
testis cells, PK-27 cells, porcine kidney cells and Mengeling Vaughn kidney cells)
and plaque purified in PK-27 cells three times (Battrell, 1995). The growth curve of
PPMV was determined in PSP-27 cells and a virus neutralizing test was developed
for detecting the virus neutralizing antibodies based on inhibition of PPMV- induced
cytopathic effect in monolayers of PSP-27. Then the pathogenicity of PPMV for
specific pathogen free (SPF) pigs was demonstrated in one virus infected three-day
old SPF pig (Battrell, 1995).

With the standardized virus-neutralization test, an assessment of the
seroprevalence of PPMV in sera bank from Iowa swine herds was made using
stored sera collected during the period of 1988-1989 (Battrell, 1995). A total of 876
serum samples collected within this period as part of the NAHMS Iowa Pilot project
were screened. Only six (representing five swine herds) out of the 876 samples
tested positive for the PPMV neutralizing antibodies. This indicated a probable low
level of PPMV infection in 1988 and 1989.

**Purpose and Objectives of the Study**

The main purpose of the study was to further the understanding of the biology
of PPMV, help further characterize it and finally lead to the development of a more
sensitive diagnostic assay for detecting PPMV infections. The major objectives were
to study the morphology of purified virions and nucleocapsids and to determine the
sequence information on selected gene for better characterization of PPMV and the development of diagnostic assays.

Thesis Organization

The thesis is organized into the following sections. The general introduction provides a brief introduction to the research and includes a statement of the purpose and objectives of the study, followed by review of the literature on paramyxoviruses. In the second chapter, we present data on the viral ultrastructure of the ISU-92 strain of PPMV, cloning and sequence of one gene of ISU-92. Our studies showed that the PPMV, ISU-92, is closely related to bovine parainfluenza virus type 3 and human parainfluenza virus type 3. The general conclusions are discussed in Chapter 3. A comprehensive list of the literature cited is provided at the end of the thesis.

Literature Review on Paramyxoviruses

The species that belong to the genus paramyxovirus are human parainfluenza virus 1 (HPIV 1), bovine parainfluenzavirus 3, human parainfluenzavirus 3 (HPIV 3), Sendai virus (murine parainfluenzavirus 3), and simian virus 3. The paramyxoviruses belong to the order Mononegavirales. This order is comprised of three families of viruses possessing linear, non-segmented negative sense ssRNA genomes, i.e., the Paramyxoviridae, Rhabdoviridae and Filoviridae (Bishop and Pringle., 1993).

It has been shown that members of the genus paramyxovirus are RNA, enveloped viruses with helical nucleoprotein core (Compans and Choppin, 1967; Duesberg and Robinson, 1965; Hartley and Choppin 1968; Korzan, 1962; Nayak
and Baluda, 1967; Plowright, 1962; Pons, 1967; Sokal et al., 1966). By previous classifications, the general category of RNA viruses with helical symmetry and limiting membrane includes a variety of agents that resemble one another in superficial morphology but which differ markedly in other physical and biological properties (Burton, 1969).

The myxoviruses were the first “natural” family of RNA viruses shown to possess helical ultra structure and an envelope. Other viruses found to share these structural attributes were, therefore, designated myxoviruses or myxovirus-like even though they may have lacked distinguishing properties of the group. The result was that the myxovirus family tended to denote a heterogeneous group of infectious agents, for many of which the family name had no meaning (Burton, 1969).

The word myxovirus is derived from the Greek word (myxa) for nasal mucous and was coined as the group name because of the reaction of influenza and related viruses with mucoproteins (Andrews, 1962). The main characteristic of this group was defined by Andrews et al. (1955) as the affinity for mucoproteins and an enzyme-like reaction with these substances. This two-phase interaction occurs when mucoproteins are in a free state as in serum or when they are integral parts of cells such as erythrocytes (Burton, 1969).

In the case where these mucoproteins are integral part of the cells, for example, erythrocytes, this characteristic activity of myxoviruses results in agglutination of erythrocytes. This is achieved by the attachment of the virus to the mucoprotein receptors on the cell surface and the resulting hemagglutination is followed by spontaneous elution of the virus with destruction of the erythrocyte
receptors due to a neuraminidase enzyme in the virus (Waterson and Almeida, 1966; Burton, 1969). As specimen preparation techniques for electron microscopy improved, more viral agents were determined to possess an ultrastructure similar to some myxoviruses and were classified as members of the myxovirus group even though they lacked neuraminidase and, in some cases, were unable to hemagglutinate; for example, rinderpest, respiratory syncytial and distemper viruses (Waterson and Almeida, 1966).

Due to the ability of some myxoviruses to hemagglutinate non-enzymatically and the lack of interaction by certain myxoviruses with erythrocytes, it was suggested to remove this property from the list of distinguishing characteristics of the group myxovirus (Andrews, cited in Waterson and Almeida, 1966). It was proposed to restrict the use of the name myxovirus to those RNA helical and enveloped viruses which exhibit the property of myxophily, as originally described by Andrews, and those agents which are structurally indistinguishable from the myxophilic viruses (Waterson and Almeida, 1966). The term true myxovirus, therefore, described those agents that demonstrated two-phase interaction with mucoproteins and possessed the ultrastructure of influenza or paramyxoviruses while “pseudomyxovirus” denoted those viruses which have the characteristic fine structure, but lack the two-phase myxophylic activity. Other RNA, helical, enveloped viruses that have neither interaction with mucoproteins nor distinctive myxovirus structure received no special designation (Burton, 1969).

Based on the proposal and terminology of Waterson and Almeida (1966), the RNA, helical, enveloped viruses are classified into three groups: true myxoviruses,
pseudomyxoviruses and other RNA-helical enveloped viruses. The true myxovirus group is comprised of influenza viruses and paramyxoviruses. The pseudomyxoviruses consist of (a) measles, distemper, rinderpest virus group, (b) pneumovirus of mice, and (c) respiratory syncytial virus. The other RNA-helical enveloped virus group includes: (a) infectious bronchitis virus, (b) rabies-like viruses, (c) bovine viral diarrhea/hog cholera viruses, and (d) foamy virus (Burton, 1969).

**Morphology**

The virions of the viruses of the order Mononegavirales are large enveloped structures generally with a prominent fringe of spikes that are 5-10 nm long and spaced 7-10 nm apart. The morphology of the particles is variable but members of the three families can be distinguished from each other. The member viruses of the family Filoviridae are characterized by simple, branched, U-shaped, 6-shaped and circular filaments of uniform diameter. Filamentous, pleomorphic and spherical forms are characteristic of members of the Paramyxoviridae family. The member viruses of the family Rhabdoviridae are characteristically bullet-shaped or bacilliform (Bishop and Pringle, 1996). The helical ribonucleoprotein core has a diameter of 13-20 nm which in filamentous forms and rhabdoviruses is organized into a helical nucleocapsid of about 50 nm diameter (Bishop and Pringle, 1996).

Virions of the family Paramyxoviridae are pleomorphic but usually spherical in shape (Nagai et al., 1989; Rima et al., 1996). The size of the membrane spheres formed from portions of the cell membrane during budding when virions detach from cell membranes and the amount of virus precursors contained within it, in
conjunction with the other forces that act on the virions affect the size and shape of the virus particle. This accounts for the pleomorphism observed in the influenza virus (Stevenson and Biddlem, 1966). Three strains of the rinderpest virus grown in monolayers of tissue culture that were inactivated with 0.4% formaldehyde, concentrated 100-fold by ultra-centrifugation and examined by negative staining revealed the majority of virus particles to be circular and oval, but larger more irregular particles were also observed (Plowright, 1962). Two of the rinderpest virus strains produced filamentous forms of 300 to 500 A in diameter which may be up to 1000A long (Plowright, 1962).

In the family Paramyxoviridae, the virions vary in size from 150-500 nm in diameter (Rima et al., 1996), with the occurrence of larger particles of 500-600 nm in diameter and long filamentous forms (Nagai et al., 1989). The SV5, a simian parainfluenza virus, virions have a diameter of 120-460 nm (Compans and Choppin, 1967).

The virions consist of a lipid bilayer envelope which is derived from the plasma membrane of the host cell plasma membrane (Compan and Choppin, 1967; Lamb and Kolakofsky, 1996, Rima et al., 1996). The envelope is covered with 7-9 nm long spikes projecting from the surface of the membrane (Compans and Choppin, 1967; Cornishark et al., 1962; Hilleman, 1962; Plowright 1962). The virus particles of the rinderpest virus are bound by well-defined membranes, with an outer layer of projections of about 90A in length (Plowright, 1962). Within the envelope is the central core which in the mumps virus, parainfluenza (Sendai) virus, Newcastle disease virus and parainfluenza viruses consists of a coiled hollow helix with the
outer diameter of 17 nm and internal diameter of 4-5 nm (Egelman et al., 1989; Hilleman, 1962). The coiled hollow helix is a single nucleocapsid, consisting of a single strand of RNA associated with a large number of nucleocapsid protein (NP) sub-units in a rod shaped helical structure (Compans and Choppin, 1967; Duesberg and Robinson, 1968; Egelman et al., 1989; Plowright, 1962). The nucleocapsid has helical symmetry and a pitch of 5.3 to 7 nm, and can be up to 1000 nm long (Egelman et al., 1989; Rima et al., 1996).

**Classification**

The genus paramyxovirus belongs to the order *Mononegavirales*. This order consists of three families of viruses possessing linear, non-segmented, negative sense single-stranded RNA genomes, namely *Paramyxoviridae*, *Rhabdoviridae*, and *Filoviridae* (Bishop and Pringle, 1996). For these three families, negative sense RNA, helical nucleocapsid, the initiation of primary transcription by a virion-associated RNA dependent RNA polymerase, a similar gene order and a single 3' promoter are common features (Bishop and Pringle, 1996). Maturation of the virion is by budding from the plasma membrane and rarely from internal membranes as for the rabies virus or from the inner membrane as for many plant rhabdoviruses (Bishop and Pringle, 1996).

The family *Paramyxoviridae* has been reclassified into two sub-families based on morphological criteria, genomic organization, biological activity of the proteins and sequence relationships: the *Paramyxovirinae* and the *Pneumovirinae* (Bishop and Pringle, 1996). The sub-family *Paramyxovirinae* consists of three genera:
Paramyxovirus, Rubulavirus and Morbillivirus, while the sub-family *Pneumovirinae* contains only one genus, the Pneumovirus.

The morphological distinguishing features of the viruses grouped in the sub-family *Paramyxovirinae* are the size and shape of the nucleocapsids, with a diameter of 18 nm, a length of 1 µm and a pitch of 5.5 nm (Egelman et al., 1989; Lamb and Kolakofsky, 1996). *Within the genus*, members exhibit antigenic cross-reactivity. Neuraminidase activity is present in Parainfluenza virus genus but is absent in morbillivirus. The P proteins coded for by the P gene differ between the genera and in the rubulavirus genus, an extra gene SH is present (Bellini et al., 1985; Lamb and Kolakofsky, 1996).

Pneumoviruses differ from the paramyxoviruses by their narrower nucleocapsids, the number of encoded proteins and an attachment protein that is different from that of the paramyxovirus (Lamb and Kolakofsky, 1996). The distinguishing characteristics of each are shown in Table 1.

**Genome structure**

The genomic RNA of the family *Paramyxoviridae* has been shown to be of a high molecular weight with a sedimentation coefficient of 50S in sucrose gradients (Duesberg et al., 1965; Kingsbury, 1966; Kolakofsky et al., 1974; Lamb and Kolakofsky, 1996). The genome is a non-segmented single stranded molecule of RNA consisting of approximately 15,500 nucleotides (Blumberg et al., 1981; Elango et al., 1988; Griffith et al., 1992; Lamb and Kolakofsky, 1996; Nagai et al., 1989; Rima et al., 1996).
Table 1. Distinguishing characteristics between pneumoviruses and paramyxoviruses.

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<tr>
<th>Characteristic</th>
<th>Paramyxoviruses</th>
<th>Pneumovirus</th>
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<tr>
<td>Genes</td>
<td>6 – 7</td>
<td>10</td>
</tr>
<tr>
<td>Gene size</td>
<td>Larger</td>
<td>Smaller</td>
</tr>
<tr>
<td>M2 protein</td>
<td>M2 absent</td>
<td>M2 present</td>
</tr>
<tr>
<td>Extensive O-linked glycosylation of G protein</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Proteins encoded by P locus</td>
<td>&gt; One</td>
<td>One</td>
</tr>
<tr>
<td>Nucleocapsid diameter</td>
<td>18 nm</td>
<td>13 – 14 nm</td>
</tr>
<tr>
<td>Nucleocapsid pitch</td>
<td>5.5 nm</td>
<td>7 nm</td>
</tr>
<tr>
<td>Length of glycoprotein spike</td>
<td>8 nm</td>
<td>10 – 12 nm</td>
</tr>
<tr>
<td>Hemagglutinin</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>
In the sub-family *Paramyxovirinae*, the genome contains 6-7 genes that encode 10-12 proteins (Rima et al., 1996). Nearly all of the proteins are encoded by unique RNAs except for the P, C and V mRNAs which are synthesized by a mechanism involving the site-specific stuttering (editing) on the same template (Linne et al., 1992; Nagai et al., 1989; Rima et al., 1996). A characteristic feature of the paramyxovirus P gene is that it is transcribed to a single mRNA which is read in two independently initiated overlapping reading frames coding for the P and C proteins (Berg et al., 1992; Georgi et al., 1983; Shioda et al., 1983 and 1986). The P gene of the La Piedad Michoacan virus (LPMV) has been found to have two ORFs of about equal length which overlap in the middle region and a third ORF encoding 126 amino acids (Berg et al., 1992).

The gene order of the six genes common to all the genera of the sub-family *Paramyxovirinae* has been demonstrated to be: 3´ leader, NP or N, P, M, F, HN, L, 5´ trailer (Chambers et al., 1986; Elango et al., 1988; Linne et al., 1992; Lamb and Kolakofsky, 1996; Nagai et al., 1989; Rima et al., 1996; Shioda et al., 1986). Variations in gene order occur in certain genera or species when F₀ and HN₀ occur as precursors for F and HN proteins as in Newcastle disease virus (Nagai et al., 1989) or by differences in the coding potential of the P gene to give P/V or P/C order of genes (Berg et al., 1992; Lamb and Kolakofsky, 1996; Rima et al., 1996). In the genus Pneumovirus the 1C, 1B proteins in that order precede the N protein, 1A and G are located between M and F proteins with 22K located between F and L proteins (Lamb and Kolakofsky, 1996).
There are conserved transcriptional and control sequences that are copied into mRNA at the beginning and at the end of each gene. Intragenic regions exist between genes which are three nucleotides long for the parainfluenza viruses and morbilliviruses but are of variable length for rubulaviruses (Nagai et al., 1989; Rima et al., 1996). All the LPMV genes are terminated by the conserved sequence (in the positive sense) of UUUAGAAAAAA (Berg et al., 1992).

Proteins

The viruses grouped in the subfamily Paramyxovirinae contain six to seven genes that encode 10-12 proteins (Orvell, 1978; Rima et al., 1996; Sanchez and Banerjee, 1985; Sundqvist et al., 1990). The phosphoprotein (P) gene has been shown to code for 4 or 5 (or more) of these proteins from 2-3 overlapping ORFs (Berg et al., 1992; Ohgimoto et al., 1990). Virion proteins common to all genera include the three nucleocapsid associated proteins (an RNA-binding protein NP, a phosphoprotein P and a large polymerase protein L), three membrane associated proteins (an unglycosylated envelope protein M and two glycosylated envelope proteins, fusion protein F and an attachment protein G or H or HN (Rima et al., 1996).

For the LPMV at least six $^{35}$S-methionine-labeled proteins could be resolved by SDS-PAGE and five of them were clearly immunoprecipitated (Sundqvist et al., 1990; Linne et al., 1992). The proteins that vary for the genera are non-structural proteins (C, 1C or NS1), a cystein-rich protein V, a small integral membrane protein
SH or 1A and a second inner envelope unglycosylated protein M2 (Lamb and Kolakofsky., 1996).

**Nucleocapsid Protein (NP)**

The structure-function relationships of the NP of the *Paramyxoviridae* have been deduced based on the studies of NP of various member viruses (Lamb and Kolakofsky, 1996). A large number of (26,000) sub-units of NP is associated with the single stranded RNA genome of the paramyxoviruses (Heggeness et al., 1980; Egelman et al., 1989). The arrangement of the NP sub-units on the RNA serves to confer helical symmetry on the ribonucleoprotein by producing a hollow core which forms an internal helical groove (Egelman et al., 1989; Heggenes et al., 1980; Nagai et al., 1989). NP is considered to be part of the template for RNA synthesis (Hamaguchi et al., 1983). During replication the NP assembles the nascent antigenome (Blumberg et al., 1981). The RNA within the nucleocapsid is resistant to RNase at any salt concentration because it is protected by the surrounding NP (Kingsbury and Darlington, 1968; Heggeness et al., 1981).

From the comparisons of sequences of many paramyxoviruses coupled with protease studies, two domains have been suggested for the NP, the N-terminal 80% of the protein which is relatively conserved among related viruses and the C-terminal 20% which is poorly conserved (Rozenblatt et al., 1985). Curran et al. (1993) found for the Sendai virus that the hypervariable C-terminal tail of NP is required for *genome synthesis in vivo but not in vitro*. 
A comparison of the amino acid sequence of the NP of ten paramyxoviruses indicates a region of high sequence identity near the middle of the protein and a C-terminal region which is enriched in negatively charged residues (Parks et al., 1992). The molecular weight of the NP varies within the paramyxoviruses, 56,534 for simian virus 5; 62,000 for sendai virus; and 68,000 for LPMV (Curran et al., 1993; Parks et al., 1992; Sundqvist et al., 1990).

**Phosphoprotein P**

The P proteins of the paramyxovirus family are highly phosphorylated (Chambers and Samson, 1980; Sundqvist et al., 1990) and they vary in length amongst the virus members of the family. The P protein of the parainfluenzaviruses and morbiliviruses are 507 to 603 amino acids long, that for the rubulaviruses are 245 to 397 residues long while that for the pneumoviruses are 241 residues long (Bellini et al., 1985; Lamb and Kolakofsky, 1996). The P protein is important for RNA synthesis (Deshpande et al., 1985; Hamaguchi et al., 1983; Horikami et al., 1992). The P protein is also needed for the formation of the NP-P complex required for the replication of both defective interfering particles and the formation of intracellular nucleocapsids (Horikami et al., 1992).

Both P and L proteins are required to form a fully active transcription complex with a functional template (Hamaguchi et al., 1983). In addition, P and L must be co-expressed for biological activity. Two protein complexes NP-P and P-L are required for nucleocapsid RNA replication. The P-L complex serves as the RNA polymerase and the NP-P is required for the encapsidation of the newly synthesized RNA.
(Curran et al., 1994; Hamaguchi et al., 1983; Horikami et al., 1992). The vesicular stomatitis virus (VSV) phosphorylated protein NS which is the possible analogue of P protein regulates the direct role that the N protein plays in VSV genome replication (Masters and Banerjee, 1988). This is done by formation of the N-NS complex which specifically binds the nascent VSV genome or anti-genome (Masters and Banerjee, 1988). Similarly, the P protein is thought to form the NP-P complex to prevent NP from binding RNA non-specifically (Nagai et al., 1989; Lamb and Kolakofsky, 1996).

The paramyxovirus P gene is transcribed into a single mRNA that is read in two or three independently initiated reading frames, one coding for the P protein, the second for the smaller C protein (Bellini et al., 1985), and the third for the V protein which is present only in the LPMV (Berg et al., 1992; Linne et al., 1992; Patterson and Lamb, 1990). Curran et al. (1993) noted that for the Sendai virus and vesicular stomatitis virus, the P gene appears to be a modular protein which has a template binding domain with the C-terminal amino acids 345-412 and 479-568 (Ryan and Portner, 1990) and a separate independently acting acidic "activation" domain with residues 1-77 of the N-terminus. The large regions of the protein between the two proteins can be deleted with only moderate loss of activity. The phosphorylated sites of P are concentrated in the N-terminal half (Curran and Kolakofsky, 1990).

**C Protein**

The C protein is a nonstructural protein that is detected only in infected cells and its function is unknown (Lamb and Choppin, 1977a). The C protein of the
Sendai virus has been shown to be synthesized in several different types of cells infected with the same strain of Sendai virus but not in uninfected cells (Lamb et al., 1976; Lamb & Choppin, 1977b). In support of this, Etkind et al. (1980) found strain specific differences in electrophoretic mobility of the C polypeptide synthesized in the same host cells infected with different strains of Sendai virus. An additional polypeptide C\textsuperscript{1} was found to be another form of C by peptide mapping (Etkind et al., 1980). Polypeptides analogous to the C protein of Sendai virus have been found in several paramyxoviruses.

The use of a variable number of in-phase start codons, including ACG and AUG codons to initiate this protein gives rise to the diversity of the C polypeptide (Boeck et al., 1992). The number of C related proteins expressed from the P genes varies for each virus: four for Sendai virus (C, C\textsubscript{1}, Y\textsubscript{1} and Y\textsubscript{2}), two or three for the human parainfluenza virus-1 (HPIV-1), and one for human PIV-3 (Lamb and Kolakofsky, 1996).

**V Protein**

The P gene mRNAs, except for those of the pneumovirus, are co-transcriptionally edited to form the V protein (Lamb and Kolakofsky, 1996). The mRNAs for the V protein contain a single additional G residue which creates an additional open reading frame (Wardrob and Bridis, 1991). For the measles virus, the V gene was shown to bind zinc (Liston and Bridis, 1994), however, the function of V is not clear (Lamb and Kolakofsky, 1996). V protein, like the P protein, was
found to be highly phosphorylated and it was not found in virions (Curran et al., 1991).

**M Protein**

The matrix protein (M) is the most abundant protein in the paramyxovirus virion (Lamb and Kolakofsky, 1996). Studies have shown that the Sendai virus M protein exists in sub-units of 6 nm in diameter that are able to form filamentous aggregates which wind around one another to form a helical structure. It has been suggested that these filaments may be a form of the protein in virions (Hewit and Nermut, 1973). The M protein is important in viral assembly. There is the need for the M protein to be recruited at the perinuclear membranes by the nucleocapsid to participate in viral assembly and budding (Stricker et al., 1994). The M protein is thought to mediate the recognition between the nucleocapsid and the envelope glycoproteins (Portner and Murti, 1986). This M and glycoprotein interaction is important for virus assembly (Yoshida et al., 1986a). The M protein is phosphorylated and incorporated only into plasma membranes that already contain viral glycoproteins (Nagai et al., 1976; Sanderson et al., 1993). The M proteins are basic proteins with net charge at neutral pH of +14 to +17 and they are slightly hydrophobic (Lamb and Kolakofsky, 1996).

**Fusion (F) Glycoprotein**

The major function of the fusion (F) protein of paramyxoviruses is the fusion of viral envelope with host cell plasma membrane to allow the penetration of the virus into the host cell (Lamb and Kolakofsky, 1996). In the naturally synthesized
form, the fusion protein occurs as an inactive precursor $F_0$. It is cleaved by host cell proteases to form the active $F_1$ and $F_2$ proteins. The measles virus precursor was experimentally expressed and found to be cleaved into $F_1$ and $F_2$ and was able to induce syncytia formation and hemolysis of monkey erythrocytes (Alkhatib et al., 1990). The cleavage of $F_0$ creates a hydrophobic N-terminus in $F_1$ that is conserved among paramyxoviruses. Novick and Hoestra, (1988) used hydrophobic photoaffinity labeling to label Sendai virus proteins during fusion with liposomes and demonstrated that hydrophobic interaction of the fusion protein with the target bilayer is an essential event in the fusion mechanism of viral membranes (Hsu et al., 1983). Glycosylation is suggested to play an important role in the cleavage-dependent activation of the precursor $F_0$ protein or in its transport to subcellular regions where proteolytic cleavage occurs (Alkhatib et al., 1990). The N-terminal 20 residues of the $F_1$ proteins are extensively hydrophobic and highly conserved among $F$ proteins of paramyxoviruses (Lamb and Kolakofsky, 1996). By use of co-expression of recombinant fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins of paramyxovirus simian virus 5, it was found that syncytia formation is enhanced when F is co-expressed with homotypic HN protein (Horvath et al., 1992). The $F$ protein gene of the porcine rubulavirus LPMV is similar to the $F$ proteins of other paramyxoviruses but lacks the small hydrophobic protein sequence between the $F$ and HN genes found in SV5 and mumps viruses (Berg et al., 1997)
Hemagglutinin-Neuraminidase (HN) Protein

The HN protein is responsible for the attachment of the virus to sialic acid which is the paramyxovirus receptor and it also mediates the enzymatic cleavage of sialic acid from the surface of virions and the surface of infected cells (Lamb and Kolakofsky, 1996). The HN of members of the genus paramyxovirus have both hemagglutinin (HA) and neuraminidase (NA) activities, the genus morbilliviruses lacks NA and the pneumoviruses lack both HA and NA (Rima et al., 1996). The HN of many paramyxoviruses has fusion-promoting activity and in simian virus 5 there is enhancement of fusion if F and HN proteins are co-expressed (Horvath et al., 1992). Proteolytic processing of HN is not common but some strains of Newcastle disease virus have been shown to have a larger precursor HN₀ which is cleaved to biologically active HN (Nagai et al., 1989).

L Protein

The genome of all the non-segmented negative strand viruses contains an L gene encoding a large polypeptide of ~ 200 KD (Nagai et al., 1989). The L protein is the least abundant of the structural proteins. Both L and P proteins are required for the paramyxovirus RNA synthesis (Hamaguchi et al., 1983; Portner et al., 1988). L and P proteins associate with the nucleocapsid to form an active transcriptive complex (Hamaguchi et al., 1983; Portner et al., 1988). Roux and Kolakofsky (1974) found a protein kinase activity associated with the Sendai virus virions. The L protein of Sendai virus was shown to possess this protein kinase activity that catalyzes the phosphorylation of indigenous polypeptides (Lamb, 1975).
Paramyxovirus replication

The members of Paramyxoviridae can replicate in enucleated cells and also their mRNA synthesis is insensitive to actinomycin D (Lamb and Kolakofsky, 1996). The entry of enveloped viruses into cells occurs by at least two general routes: the recognition of specific cell surface receptors and membrane fusion. Gangliosides and asialoglycoproteins serve as receptors for paramyxoviruses and rubulaviruses (Markwell et al., 1985 and 1986). The cell surface protein CD46 serves as the receptor for the morbillivirus and measles virus. No cellular receptor has been identified for the pneumoviruses. The adsorption of the virus to the cellular receptor is followed by the fusion of the viral membrane with the cellular plasma membrane at the neutral pH at the cell surface resulting in the release of the helical nucleocapsid into the cytoplasm.

Transcription and genome replication

Transcription is the first process in the replication of any negative strand RNA virus and it results in the synthesis of RNA. As the eukaryotes do not contain RNA-dependent RNA polymerases, the virus codes for its own polymerase which is packaged into the virions (Nagai et al., 1989). The Sendai virus, Newcastle disease virus and vesicular stomatitis virus particles have been shown to contain an enzyme which catalyzes the incorporation of ribonucleotides into ribonucleic acid (RNA) (Stone et al., 1971; Huang et al., 1971). This enzyme is a single-stranded RNA which is complementary in base sequence to virion RNA (Stone et al., 1971). After fusion of the viral envelope with the host cell plasma membrane, the nucleocapsids
enter the cytoplasm carrying multiple copies of the P/L polymerase. The polymerases bind the (-) genome template only at its 3' end (Lamb and Kolakofsky, 1996). P molecules occur in 4 to 10 discrete clusters at varying locations along the length of the nucleocapsid in the cytosol while P molecules are uniformly distributed over the entire length of the nucleocapsid in the virion. This indicates the dependence of the distribution of P on the functional state of the nucleocapsid (Portner and Murti, 1986). RNA synthesis starts as soon as the genome encounters the ribonucleoside triphosphate in the cytoplasm with the synthesis of the (+) leader RNA. Leader sequences are important in control of viral genome expression. They are about 50 nucleotides long with the first 12 nucleotides identical with the viruses of each genus and are well conserved across the sub-family Paramyxovirinae. The remainder of the leader sequence is U and A rich (Lamb and Kolakofsky, 1996).

The virus promoters in the Paramyxoviridae family are always turned “on” and the viral polymerases are specific to virus templates. During transcription, the polymerase terminates at the end of the (+) leader region and reinitiates at the beginning of the first gene. Re-initiation defines the polymerase that transcribes the template to make mRNA as opposed to the one that replicates the genome (Iverson and Rose, 1981; Castaneda and Wong, 1990). Re-initiation leads to a transcript which is capped at the 5' end and whose internal sequence can be co-transcriptionally edited by G insertions at specific sites and at specific frequencies. The mRNAs are polyadenylated at the 3' end. The polyadenylation of these viral RNAs is suggested to be due to reiterative copying of the stretch of four to seven U
residues on the template (Gupta and Kingsbury, 1982; Iverson and Rose, 1981; Robertson et al., 1981).

The length of the poly (A) tail (~200 residues) is thought to be limited by chain termination. The polymerase skips the intergenic region, which is exactly 3 nucleotides for paramyxovirus and morbillivirus and 1-56 nucleotides for the rubulaviruses and pneumovirus, in order to reinitiate mRNA synthesis at the next start sequence until it completes the L mRNA (Lamb and Kolakofsky, 1996). Several mechanisms of attenuation of gene expression at the transcriptional level by which paramyxoviruses can fine-tune the relative amounts of their mRNAs have been noted (Lamb and Kolakofsky, 1996). The frequency with which the polymerase restarts mRNA synthesis at each junction is high but decreases downstream which creates a gradient of mRNA abundance according to the position of the gene relative to the 3' end of the template (Cattaneo et al., 1987).

Hamman et al. (1990) observed a disparity at the M-F and HN-L boundaries for the Sendai virus. Another mechanism involves inefficient transcriptional termination and polyadenylation by which some viruses read through some junctions at higher frequency than usual and produce bicistronic mRNAs as noted for the pneumovirus, and the respiratory syncytial viruses that the start sequence of their L gene is situated upstream within the preceding M gene (Collins et al., 1987). Attenuation of gene expression by biased hypermutation has also been described for the measles virus matrix gene in which U residues were changed to C (Bass and Weintraub, 1988; Cattaneo et al., 1988).
Genome replication

After translation of the primary transcripts and accumulation of viral proteins, anti-genome synthesis begins. The polymerase involved in the mRNA synthesis copies the same template without termination and re-initiation at the junctions. The nucleocapsid protein (NP) has been shown to be responsible for the read through for the vesicular stomatitis virus polymerase (Perault et al., 1983). For VSV, it is proposed that when the newly synthesized N proteins attach to the nascent replicating plus leader RNA and encapsidate it, the RNA polymerase reads through boundaries between the leader and the N genes. In the presence of N, this read-through may occur successively throughout the template until full-length plus sense nucleocapsid is formed. The full length plus strand copy of the genome RNA serves as the template for progeny virus (-) RNA. There are no re-initiation sites on the anti-genome template and so termination of the (-) leader RNA serves only to recycle the polymerase (Lamb and Kolakofsky, 1996).

Calain and Roux (1993) observed that for the Sendai virus RNA to serve as a proper template for efficient replication, the RNA must contain a total number of nucleotides that is a multiple of six. This was interpreted as a requirement for the nucleocapsid protein to contact exactly six nucleotides (Calani and Roux, 1993).

The Sendai virus, which is a parainfluenza virus type 1, contains a RNA genome which is specific to the virus-specific mRNAs found in infected cells (Kingsbury et al., 1978; Kolakofsky and Bruschi, 1975). Defective interfering genomes result from replicative errors of the activities of the polymerase creating internal deletions, or by creation of an inverted repeat (Re et al., 1983). The
defective-interfering genomes are thought to interfere with non-defective helper virus because they have a competitive advantage (Lamb and Kolakofsky, 1996). They have also been implicated in establishing permanent infections (Calain and Roux, 1988).

**Virus assembly**

The cytoplasm is the site of synthesis of the nucleocapsid. The Sendai virus nucleocapsid assembly occurs in two steps. First, the free NP sub-units associate with the viral genome RNA to form the helical RNP structure and, next, the P-L protein complex associates with the viral genome (Kingsbury et al., 1978). The assembly of the envelope takes place at the cell surface. The viral integral membrane proteins are synthesized in the endoplasmic reticulum and undergo a stepwise conformational maturation before transport through the secretory pathway (Lamb and Kolakofsky, 1996). The virus particles are assembled at the plasma membrane (Stricker et al., 1994; Lamb and Kolakofsky, 1996). The M protein is required to interact with the nucleocapsid at the perinuclear membranes to participate in viral assembly and budding (Stricker et al., 1994). Glycoprotein cytoplasmic tails make important contacts with the M protein which, in turn, associates with the nucleocapsid (Lamb and Kolakofsky, 1996).

**Pathogenesis**

Viral pathogenesis involves a complex interaction between the virus and the host. Infection of the host is a prerequisite to disease production. Availability of a large number of strains with variable virulence facilitates understanding of
mechanisms of virulence (Nagai et al., 1989). The Sendai virus has been used as a model to understand mechanism of pathogenicity of paramyxoviruses. Sendai virus infection is localized to the respiratory organs in the natural host. The Sendai virus $H_0$ protein has a single basic residue at the cleavage site and it is activated in limited types of tissues (Homann et al., 1990). The spread of virus in the lungs is due to the presence of an appropriate cleaving enzyme which resembles trypsin.

Limited information is available on the pathogenicity of porcine paramyxovirus (PPMV) isolate ISU-92. Inoculation of six-week old specific pathogen free (SPF) pigs with cell-free cultures of lung homogenates did not produce clinical illness although all the inoculated pigs developed antibody titers to ISU-92 (Janke et al., 1992). In another study, inoculation of three day-old SPF pigs with cell culture propagated PPMV, resulted in clinical illness characterized by slightly elevated body temperature for 24 hours, mild diarrhea and dyspnea. Evidence of pneumonia with ten to fifteen percent lung involvement was detected in inoculated pigs (Battrell, 1995). The alveolar walls were irregularly thickened by reactive hypertrophy and proliferation of alveolar pneumocytes and by infiltration of macrophages. The inflammation varied in severity from lobule to lobule. In some lobules alveolar and bronchiolar lumens contained clusters of sloughed pneumocytes and infiltrating leukocytes. The epithelium that lined terminal bronchioles appeared slightly hyperplastic. In one pig glial nodules were observed in the brain. All the subjects developed antibody titers to the paramyxo-like virus (Janke et al., 1992).

Another paramyxovirus, referred to as LPMV for Le Piedad Michoacan virus, has been isolated in Mexico (Moreno-Lopez et al., 1986; Stephano et al., 1988). The
La Piedad Michoacan virus was isolated from the brain of a piglet with a disease associated with encephalitis, reproductive failure and corneal opacity in 1986 (Stephano et al., 1988). The LPMV is a member of the family Paramyxoviridae isolated in pigs and has been characterized as a Rubulavirus (Moreno-Lopez et al., 1986; Rima et al., 1996). LPMV has been found to grow in many cell cultures producing cytopathic effects in the form of syncytia formation and cytopathic inclusion bodies. Hemagglutination, neuraminidase and hemolytic activities have also been observed for the LPMV (Moreno-Lopez et al., 1986). The natural infection of LPMV in pigs is presumed to be acquired by inhalation. The clinical signs of the disease caused by LPMV vary according to the age of the pig. Piglets 2-15 days old are the most susceptible and show a sudden onset of clinical signs. In the 2-15 days old group, the clinical signs include prostration, depression, and nervous signs. There occurs also fever, a staring coat and an arched back sometimes accompanied by constipation or diarrhea, followed by progressive nervous signs (ataxia, weakness, rigidity mainly of the hind legs, muscle tremors and abnormal posture). Other signs include anorexia only in the immobilized piglets, conjunctivitis with swollen eyes and lacrymation, unilateral or bilateral corneal opacity in 1-10% of infected piglets. Death of piglets occurs 48 hours after the appearance of clinical signs in the first cases observed and 4-6 days in later cases (Stephano et al., 1988).

Infectivity is 20-65% in litters farrowed during outbreaks and the morbidity of infected litters is 20-50% with a mortality of 87-90%. Piglet mortality lasts from 2-9 weeks. Most sows from affected litters appear clinically normal. Moderate anorexia in sows one or two days before the appearance of clinical signs in piglets may be
observed. Corneal opacity occurs in some sows in the farrowing houses during outbreaks. Abortions in some dams have been observed and number of stillborn and mummified fetuses increases during outbreaks (Stephano et al., 1988).

In weaned piglets, older than 30 days, moderate and transient clinical signs (anorexia, fever, sneezing and coughing) occur. Nervous signs are rare and include depression, ataxia, circling and swaying of the head. In this group, corneal opacity and conjunctivitis are observed without other signs and continue to appear for another month (Stephano et al., 1988). Clinical signs and lesions similar to those observed in natural infections have been reproduced in one-day old piglets by experimental intracerebral, intratracheal and intranasal inoculation with isolated LPMV (Stephano et al., 1988). Experimental inoculation of boars with LPMV has demonstrated that severe epididymo-orchitis and reduced semen quality in sexually matured boars can result from LPMV infections (Ramirez-Mendoza et al., 1997). The disease has also been reproduced by contact with experimentally infected animals (Stephano, 1988).

The nasal mucosa has been suggested as the initial site of replication of the LPMV from where it spreads early in the infection to the brain and lungs. The interstitial pneumonia observed suggests dissemination through the blood. Corneal opacity which usually occurs late in the course of the disease is thought to be due to an immunologic reaction similar to that produced by canine adenovirus. There are also indications that the virus replicates in the cornea (Stephano, 1992). The virus reaches the uterus through the blood resulting in embryonic mortality, stillbirths and mummified fetuses in pregnant sows (Stephano, 1992).
An apparently new paramyxovirus was isolated from pigs in Australia and it was found to infect humans (Philbey et al., 1998). It was suggested that bats may be involved in the ecology of this Australian porcine paramyxovirus (Chant et al., 1998).
CHAPTER 2. VIRAL ULTRASTRUCTURE AND SEQUENCING OF THE P GENE OF THE PORCINE PARAMYXO-LIKE VIRUS, ISU-92

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Abstract

The ISU-92 strain of porcine paramyxovirus isolated from pigs with neurological and respiratory disease was morphologically and ultrastructurally characterized. Electron microscopic examination of gradient purified virus and nucleocapsids revealed variable forms of the pleomorphic PPMV virions with a diameter of 150-400 nm for spherical virions and the serrated nature of the nucleocapsids. Comparison of the morphological and antigenic data with those of other paramyxoviruses suggested that ISU-92 is a true and unique paramyxovirus.

A random primed cDNA library was prepared in lambda vector from polyadenylated mRNA from vero cells infected with porcine paramyxovirus strain ISU-92. A virus-specific probe prepared by subtractive hybridization of $^{32}$P labeled cDNA from virus infected vero cells was used to screen the cDNA library. One virus-specific clone containing an insert of 1962 base pairs was sequenced and shown to contain the reverse transcript of the mRNA coding for the P gene. The coding sequence (ORF) started at position 52 (ATG included) and ended at position 1839.
(stop codon not included) of this clone. The mRNA was 1962 nucleotides long followed by a poly-A tail. The total length of the gene was 1788 nucleotides and it encoded a protein of 596 amino acids. The sequence of the mRNA was compared with the genome of BPIV-3 (15,548 nucleotides and corresponds to the region of BPIV-3 genome from 1732 to 3699 nucleotides with an overall homology of 98%. This study shows that PPMV, ISU-92, is closely related to bovine parainfluenza virus 3 and human parainfluenza virus 3.

Introduction

In recent years, several paramyxoviruses have been reported in different animal species (Creig, 1971; Lipkind 1986; Moreno-Lopez 1986; Philbey et al., 1998; Richter et al., 1996). A viral agent was isolated in 1992 in the United States (Minnesota) from a disease outbreak in pigs characterized by central nervous system (CNS) and respiratory disorders (Janke et al., 1992). This, previously uncharacterized virus was designated as ISU-92 and called porcine paramyxo-like virus based on preliminary antigenic studies (Janke et al., 1992). Battrell et al., (1993) further characterized the virus and termed it porcine paramyxovirus (PPMV). Previous studies of this virus have shown that it cross-reacts antigenically with antibodies against PIV-1, PIV-3 and PIV-4B but not with antibodies against La Piedad Michoacan virus (LPMV) (Janke et al., 1992). The evidence suggested that the virus belongs to the Family Paramyxoviridae (Janke et al., 1992; Battrell, 1995) but detailed characterization needs to be done.
Few cases of paramyxoviruses in pigs have been reported (Brun et al., 1992; Chang-GuoQuan and Chang, 1996; Creig et al., 1971; Lipkind et al., 1986; Moreno-Lopez et al., 1986; Philbey et al., 1998). A fatal disease outbreak of pigs was first reported in Mexico in 1981. The La Piedad Michoacan virus (LPMV) was isolated from pigs with a disease which was characterized by encephalitis, reproductive failure and corneal opacity (Moreno-Lopez et al., 1986; Stephano et al., 1988). Another porcine paramyxovirus was isolated from the lungs of a pig in Northrhine Westphalia in Germany by Brun et al., (1992). This German strain of porcine paramyxovirus showed no antigenic cross-reactivity with the Mexican LPMV (Groschup et al., 1993). An apparently new paramyxovirus was isolated from pigs in Australia and it was found to infect humans (Philbey et al., 1998). It was suggested that bats may be involved in the ecology of this Australian porcine paramyxovirus (Chant et al., 1998).

The virions of the paramyxoviruses are mostly spherical with other pleomorphic forms with a diameter of 150-500nm (Plowright, 1962, cited in Lamb and Kolakofsky, 1996). The genomes of the paramyxoviruses are single-stranded, non-segmented RNA of negative polarity (Hilleman et al., 1962). The six genes of the paramyxoviruses code for structural proteins (Sakai et al. 1987) and are ordered as 3’ leader, NP (or N), P, M, F, HN, L, 5’ (Elango et al., 1988; Linne et al., 1992; Sakai et al., 1987; Sanchez and Banerjee, 1985). The paramyxovirus virion contains a nucleocapsid which is made up of genomic RNA. Three proteins NP, P and L are associated with the nucleocapsids and contribute to the characteristic helical structure of the nucleocapsids (Oglesbee et al., 1989).
There is limited information available on the biology and molecular biology of PPMV. In this study, we report the results of the electronmicroscopic examination of purified virions and nucleocapsids and the cloning and sequencing of the P gene of the PPMV.

**Materials and Methods**

**Virus and cells**

Vero cells grown in minimal essential medium (MEM) (Gibco) containing 10% fetal bovine serum (FBS) and antibiotics (500U/ml penicillin, 500 U/ml streptomycin and 1.25 mg/ml of amphotericin B) were used for the cultivation of the virus. The virus used for the study was the porcine paramyxo-like virus, ISU-92, isolated from pigs with naturally occurring neurological and respiratory disease (Janke et al., 1992). The virus had been plaque purified three times and was stored at −80°C.

The growth medium was removed from four-day old monolayers of vero cells grown in 75 cm² tissue culture flasks. Cells were inoculated with PPMV at a multiplicity of infection (m.o.i.) of 0.5 PFU/cell. The cells were incubated at 37°C for 2 hours and 7 ml of MEM containing 2% FBS and antibiotics were added. The cells were further incubated until 75% of cells showed cytopathic effect (CPE).

The flasks of cells were frozen-thawed three times, and the contents pooled and clarified at 1,000xg for 10 minutes. This supernatant was stored at −80°C as the virus stock and titrated by plaque assay. A serial dilution (10⁻¹ to 10⁻¹⁰) of the virus was made in MEM. Four-day old monolayers in six-well plates were inoculated with 0.2 ml/well of each of the serial dilutions of the virus. The cells were incubated
for 1 hour and the inoculum was removed and 2 ml of agar overlay (50:50 BME and 2% sea plaque agar containing phenol red and 7.5% NaHCO₃) was added per well. The cells were incubated for 5 days and a plaque count was made to calculate the titer.

**Virus propagation and purification**

The virus was propagated in large quantity and the supernatants were collected at 30-48 hours post infection and frozen at −80°C. Three to four day-old vero cells were infected with PPMV at a m.o.i of 0.5 PFU/cell prepared in MEM without FBS in a final of 3 ml per flask. The flasks were incubated at 37°C for 1 hour with the tilting of the flasks every 15 minutes to distribute the inoculum over the cell layers. After 1 hour incubation, 10 ml of MEM containing 2% FBS, antibiotics and an antimycotic solution were added to each flask. The flasks were incubated at 37°C until extensive fusion of cells and syncytia formation (about 80%) was observed. The supernatants were then collected, frozen and thawed once at −80°C. The supernatants were clarified at 1000xg for 10 minutes or 10,000xg for 10 minutes and layered onto a 20% sucrose cushion. The virus was concentrated into pellets at 100,000xg for 4 hours in SW28 rotor (Beckman). The virus was purified by sucrose gradient centrifugation. The virus pellets were re-suspended in TEN (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl) buffer (pH 8.0) and layered onto a 10-60% sucrose gradient prepared in water and centrifuged at 170,000xg for 2 hours in a SW41 rotor (Beckman). The virus containing bands were collected and diluted in TEN buffer (pH 8.0). The partially purified virus was pelleted at 100,000xg for 4 hours. The
pellets were re-suspended in TEN buffer (pH 8.0) and stored at -80°C or re-suspended in water for immediate use. When necessary, two more sucrose gradient centrifugations were performed to further purify the virus.

**Isolation of nucleocapsids (NC)**

Nucleocapsids were isolated by the method described by Kolakofsky (1976). Four day-old monolayers of vero cells were infected at a high m.o.i. of 0.5 PFU/cell as described earlier. At 24-30 hours post infection, when the CPE was about 60-80%, the media was removed from the cells and the monolayers washed twice with 5 ml of normal saline (4°C). The cells were lysed as described by Maniatis et al. (1989) by addition of 2 ml of 0.6% NP40 lysis buffer, per 75 cm³ tissue culture flask of infected cells. The flasks were incubated on a flat bed of ice for 20 minutes. A plastic cell scraper was used to scrape the lysed cells and the cell lysate was pipetted into a 35 ml centrifuge tube. The cell lysate was mixed vigorously on a stirrer and put on ice for 5 minutes. Cellular nuclei and membranes were removed by centrifugation at 10,000xg (Kolakofsky, 1976). A discontinuous cesium chloride gradient was prepared in 14x89 mm ultracentrifuge tubes from 3.0 ml of 5% (1.176 g/ml) sucrose, 2.6 ml of 25% (1.2255 g/ml) CsCl₂, 2.6 ml of 30% (1.2858 g/ml) CsCl₂ and 1.4 ml of 40% (1.4196 g/ml) CsCl₂. A 0.5-0.7 ml of cell lysate was layered onto the gradients and centrifuged using a SW41 rotor (Beckman) at 170,000xg for 2 hours at 4°C. The distinct bands found at the 30/40% interphase were collected. The collected bands were diluted 4-5 times in TEN buffer (pH 8.0) and centrifuged to pellet the nucleocapsids using a SW28 rotor at 100,000xg for 4 hours at 4°C. The
pellets were washed three times with TEN buffer and re-suspended in TEN buffer. The purified nucleocapsids were stored at 4°C until used.

**Electron microscopy of virions and nucleocapsids**

Virions partially purified by sucrose gradient centrifugation and nucleocapsids purified by CsCl₂ gradient centrifugation were prepared for electron microscopy in two ways. For each sample, 15 drops of distilled water were put in a well. Then two drops of 4% phosphotungstic acid (PTA), 1 drop of 0.5% bovine serum albumin (BSA) and 1 drop of each sample were added in that order to each well. The contents of each well were mixed gently with a Pasteur pipette. The mixture was applied to a carbon coated 200-mesh-grid with a nebulizer (Tel. Pella Inc., Tustin CA). The grids were then examined under the microscope (Hitachi H500) at 75KV. Alternatively, 5 µl of the samples were spotted onto the center of the carbon coated 200-mesh grid and allowed to adsorb for 7-10 minutes. Excess sample was removed with a piece of filter paper. Next, 5 µl of the 3% PTA were spotted onto the grid and the excess removed after 30 seconds. The grids were air-dried for 1 minute and examined under the electron microscope (Hitachi H500) at 75KV.

**Isolation of total RNA from vero cells infected with virus**

Total RNA was isolated using an RNA isolation kit and the procedure outlined for adherent cells grown in cell culture as indicated by the manufacturer (Stratagene, Loyola, CA). Four-day old vero cells in 75cm² tissue culture flasks (x4) were infected at approximately 1.0 m.o.i/cell as described above and incubated for 15 hours. The medium was replaced with 10.0 ml of MEM containing 2% FBS and 2
µg/ml of Actinomycin D. The cells were further incubated for 14 hours till CPE was extensive (80-100%).

The medium was removed from the cells and 4.0 ml of lysis solution D (100 µl β-mercaptoethanol in 14 ml of denaturing solution or 1% SDS) were added. The flask was swirled for 30 seconds and the cell lysate was transferred to the next flask and the procedure continued till the last flask. The pooled cell lysate was collected into a centrifuge tube. Then 0.4 ml of 2M sodium acetate and 4.0 ml of phenol saturated with water were added in order with gentle mixing. A 0.8 ml solution of chloroform:isoamyl alcohol was added and the contents of the tube were vigorously mixed for 10 seconds and placed on ice for 15 minutes. The mixture was centrifuged at 10,000xg for 5 minutes and the upper aqueous layer was transferred into new microcentrifuge tubes in aliquots of 500 µl. Equal volumes of isopropanol were added to the tubes, mixed and then put on dry ice for 10 minutes. The tubes were stored at −20°C overnight to precipitate the RNA. The RNA was pelleted at 10,000xg for 15 minutes and washed with 70% ethanol: 25% diethyl pyrocarbonate (DEPC)-treated water. The pellets were vacuum-dried for 15 minutes and then re-suspended in 20 µl DEPC-treated water. A 1:60 dilution of the RNA was made in 300 µl of water and the RNA concentration determined by spectrophotometry at OD_{260}. 
cDNA synthesis

The synthesis of the first and second strands of cDNA was performed with the cDNA synthesis kit according to the manufacturer's instructions (Uni-Zap, Stratagene, Loyola, CA) except for a few modifications. First strand synthesis was performed by reverse transcription of mRNA with a 50-base oligonucleotide linker primer (Zap-cDNA Synthesis Kit, Stratagene). All of the non-enzymatic reagents of first strand synthesis were thawed, mixed, centrifuged and put on ice. The first strand reaction was set up by combining 5 µl of 10X first strand buffer, 3 µl of first strand 5' methyl nucleotide mix (normal dATP, dGTP, dTTP and 5-methyl dCTP), 2 µl of linker primer (1.4 µg/ml), 35.5 µl of DEPC-treated water, 1.0 µl of ribonuclease inhibitor and 2 µl (~18.78 ng) of total RNA in the order listed and mixed gently. The primer was allowed to anneal to the template for 10 minutes at room temperature. The 1.5 µl of MMLV-RT (50 U/µl) was added to the first strand reaction to obtain a final reaction volume of 50 µl. The reaction sample was mixed gently, briefly centrifuged and incubated at 37°C for 1 hour.

The second strand was synthesized by nicking the hybrid RNA bound to the first strand cDNA to produce a multitude of fragments which served as primers for DNA polymerase I to nick translate these RNA fragments into second strand cDNA. To ensure that the restriction sites in the linker primer remained susceptible to restriction enzyme digestion, the second strand nucleotide mixture was supplemented with dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second strand. All of the non-enzymatic components of the
second strand were thawed, vigorously mixed, centrifuged and put on ice. The second strand components which included 20 µl of 10X second strand buffer, 6 µl of second strand dNTP mix (normal dATP, dGTP, dTTP and dCTP ) and 116 µl of sterile water were added to the 50 µl of the first strand synthesis reaction on ice and mixed. In the order listed, 2 µl of RNase H (1.5 U/µl) and 11 µl of DNA polymerase I (9.0 U/µl) were added to the reaction. The contents of the tube were mixed and centrifuged in a microcentrifuge tube and incubated at 16°C for 2.5 hours after which they were immediately put on ice.

Construction of plasmids

A cDNA library was constructed in bacteriophage lambda Uni-Zap XR vector (Stratagene, Loyola, CA) using total mRNA from vero cells infected with the porcine paramyxo-like virus, ISU-92. The uneven termini of the double stranded cDNA were filled in with cloned pfu DNA polymerase and EcoRI adapters were ligated to the blunt ends. The second strand reaction was kept on ice and 23 µl of blunting dNTP mix and 2 µl of cloned pfu DNA polymerase (2.5 U/µl) were added. The contents of the tube were quickly mixed, and incubated at 72°C for exactly 30 minutes. The tube was removed from 72°C to room temperature and 200 µl of phenol:chloroform [1:1(v/v)] pH 7.8 was added and the contents mixed by agitation. The tubes were centrifuged at a 10,000xg for 2 minutes at room temperature and the upper aqueous layer containing the cDNA was transferred to a new tube. An equal volume of chloroform was added and mixed.
The reaction tube was centrifuged for 10,000xg at room temperature and the upper aqueous layer containing the cDNA was transferred to a new tube. The cDNA was precipitated overnight after adding 20 µl of 3M sodium acetate and 400 µl of 100% (v/v) ethanol. The tubes were agitated and stored at -20°C overnight. The cDNA was pelleted in a microcentrifuge at 10,000xg for 60 minutes at 4°C. The supernatant was carefully discarded and the pellet washed by adding 500 µl of 75% (v/v) ethanol: 25% DEPC-treated water and centrifuged at maximum speed at room temperature for 2 minutes. The ethanol was pipetted out and the pellet was vacuum-dried for approximately 15 minutes until dry. The pellet was re-suspended in 9.0 µl of EcoRI adapters and incubated at 4°C for 30 minutes to allow the cDNA to re-suspend and the tube was put on ice. The EcoRI adapters of the sequence

\[
5' - \text{OH-AATTCCGACGAG} 3' \\
3' - \text{GCCGTGCTC} 5'
\]

were ligated to the termini-blunted cDNA. The tube containing the blunted cDNA and the EcoRI adapters were kept on ice and 1.0 µl of DNA 10X ligase buffer, 1.0 µl of 10 mM of rATP and 1.0 µl of T4 DNA ligase (4 U/µl) were added. The tube was briefly centrifuged in a microcentrifuge and incubated at 8°C overnight. The T4 DNA ligase was inactivated by heating at 70°C for 20 minutes. The reaction mix was briefly centrifuged for 2 seconds and cooled to room temperature for 5 minutes.

The EcoRI ends were phosphorylated by adding 1.0 µl of 10X ligase buffer, 2 µl of 10 mM rATP, 6 µl of sterile water, and 1 µl of T4 polynucleotide kinase (10 U/µl). The reaction was incubated for 30 minutes at 37°C and the kinase was heat
inactivated at 70°C for 30 minutes. The reaction was briefly centrifuged and allowed to equilibrate to room temperature for 5 minutes. Restriction digestion of cDNA with Xhol was performed to release the EcoRI adapter and the residual linker primer from the 3' end of the cDNA. A 28 µl of Xhol buffer supplement and 3 µl of Xhol (40 U/µl) were added to the phosphorylated reaction. The reaction was incubated for 1.5 hours at 37°C and 5 µl of 10X STE buffer and 125 µl of 100%(v/v) ethanol were added. The cDNA was precipitated overnight at -20°C and pelleted in a microcentrifuge at 10,000xg for 60 minutes at 4°C. The pellet was vacuum dried, re-suspended in 14 µl of 1X STE buffer and put on ice.

After Xhol digestion the cDNA was size fractionated to select the size of DNA for cloning by running through a strip column containing Sepharose CL-2B gel filtration medium (Stratagene, Layola, CA) according to the manufacturer's instructions.

Twelve fractions of three drops each were collected into separate 1 ml tubes. Starting from the first fraction, each of the three successive fractions were pooled together and cDNA extracted with phenol:chloroform and precipitated with ethanol. To each sample, 300 µl of phenol (pH 7.8): chloroform was added. The contents in the tubes were vigorously mixed and centrifuged in a microcentrifuge at 10,000xg for 2 minutes.

The upper aqueous layers were transferred to new tubes and an equal volume of chloroform added. The tubes were agitated vigorously and centrifuged at 10,000xg for 2 minutes. The upper aqueous layers were transferred to new tubes
and 500 µl of 100% ethanol added. The tubes were stored at −20°C to precipitate the DNA. The cDNA was pelleted at 10,000xg in a microcentrifuge for 60 minutes, washed with 75% ethanol, vacuum dried 15 minutes and resuspended in 3.0 ml of sterile water.

The DNA in the cDNA fractions was quantitated by the ethidium bromide assay. A 0.8% (w/v) agarose plate containing 0.001 µg/ml of ethidium bromide was prepared using Tris-Acetate-EDTA (TAE) buffer. A standard DNA sample of known concentration was serially diluted to obtain concentrations of 200 to 10 ng/ml and 0.5 µl of each dilution was spotted onto the ethidium bromide plate. Then 0.5 µl of the DNA samples were spotted onto the plate adjacent to the standards. After 15 minutes of adsorption of the spots, the plate was photographed using UV light.

The spotted samples of unknown DNA concentration were compared with the standards. The cDNA fraction corresponding to the largest size DNA with a concentration of ~37.5 ng/ml was ligated into the Uni Zap XR vector (Stratagene). Ligation was performed in a 5 µl volume reaction by adding together, 2 µl of resuspended cDNA (~100 ng), 0.5 µl of ligase buffer, 0.5 µl of 10 mM rATP (pH 7.5), 1.0 µl of the Uni-Zap XR vector (1 µg/µl), 0.5 µl of sterile water and 0.5 µl of T4 DNA ligase (4 U/µl). The reaction was incubated at 12°C for 24 hours.

The recombinant lambda phage was used directly from the ligation reaction for packaging using the Gigapack II Gold 7 packaging extract (Stratagene, Loyola CA). The packaged lambda was briefly centrifuged and stored at 4°C until titration of the cDNA library.
Plating and titration of the cDNA library

The cDNA library was plated in XL1-Blue MRF' cells to determine the titer and the ratio of recombinant plaques to the non-recombinant plaques by blue white color screening. The host bacteria, XL1-Blue MRF' glycerol stock was streaked onto a pre-incubated LB-tetracycline agar plate and incubated overnight at 37°C. One colony was inoculated into 3 ml of LB broth supplemented with 10 mM of MgSO₄ and 0.2% maltose and incubated overnight with agitation. The overnight culture (30 µl) was inoculated into 30 ml LB broth supplemented as above and incubated for 3 hours with continuous agitation to obtain an OD₆₀₀ ~1.0.

The bacterial cells were pelleted at 500xg for 10 minutes, resuspended in 1.2 ml of 10 mM MgSO₄ and placed at 4°C until used. The ligation product was plated by combining 1 µl of packaged reaction (cDNA library) with 199 µl of XL1-Blue MRF' cells (OD₆₀₀ ~ 1.0). Four serial dilutions of the mixture were made by the transfer of 20 µl mixture into 180 µl of XL1-Blue MRF' cells. The tubes were incubated at 37°C to allow the phage to attach to the bacterial cells. Then 3 ml of top agar (melted and cooled to 50°C) was added to each dilution of the phage and plated immediately on NZY agar plates. After 10 minutes the plates were incubated at 37°C for 8 hours.

Color selection with IPTG and X-gal was performed. A dilution of 1 µl of cDNA library in 4 µl SM buffer was made. From the 1:5 dilution of the phage, 1 µl was added to the 200 µl XL1-Blue MRF' cells and incubated for 10 minutes at 37°C. The cells were mixed with 3 ml NZY top-agar (~50°C) to which 10 µl of 0.839 M
IPTG and 50 µl of X-gal (250 mg/ml) had been added. The top agar was plated immediately on NZY agar plate and incubated at 37°C for overnight.

**Amplification of the Uni-Zap XR – cDNA library**

A single amplification of the cDNA library prepared in lambda Uni-Zap XR vector was performed. Four plating reactions were set in NZY agar plates (150 mm). For each plate, 50 µl (~5x10⁴ PFU) of the lambda library was mixed with 600 µl of XL1-Blue MRF’ cells (OD₆₀₀ ~1.0) and incubated at 37°C for 8 hours. Then 10 ml of SM buffer was added to each plate and the plates incubated at 4°C overnight with continuous agitation. The bacteriophage suspension was recovered and the plates were rinsed with an additional 2 ml of SM buffer. Chloroform was added to a final concentration of 5% (v/v) and the tubes were incubated for 15 minutes at 37°C. Cellular debris was removed by centrifugation at 500xg for 10 minutes and the clarified supernatant was divided into two aliquots. One portion of 22.5 ml was treated with 0.068 µl chloroform (final concentration 0.35%) and stored at 4°C. The second portion was aliquoted into 10 ml and 0.753 µl DMSO (final concentration 0.7%) was added and stored at –80°C. The titer was determined as described earlier.

**Hybridization and screening**

Plaque lift was performed according to standard procedure by plating 10-15,000 PFU of lambda phage per 150 mm plate. NZY agar plates were prepared in 150 mm petri dishes and stored at 4°C for at least 2 days. A 10 µl aliquot of the
library (~10-15,000 PFU) was mixed with 600 µl of XL1-Blue MRF- cells (OD$_{600}$~0.5) and plated on the NZY agar plates after 15 minutes incubation at 37°C. The plates were incubated at 37°C for 8 hours and placed at 4°C until plaques were lifted. Nitrocellulose membrane (Magna-nylon, Bio-Rad) was placed on the agar plates for 2 minutes to allow the phage to transfer to the membrane and the orientation of the plates was marked by pricking through the agar with a needle. The nitrocellulose membranes were lifted and denatured by placing them on filter paper moistened in 1.5 M NaOH denaturing solution for 2 minutes. The agar plates were returned to 4°C. The membranes were neutralized for 5 minutes by transferring them to filter paper moistened in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) neutralizing solution. Membranes were transferred to filter paper moistened in 0.2 M Tris-HCl (pH 7.5) and 2XSSC buffer rinsing solution for 30 seconds. The rinsed nitrocellulose membranes were blotted and air-dried on Whatman 3MM paper and baked for 15-30 minutes at 80°C.

A probe was prepared from viral mRNA and used to hybridize to plasmid DNA on membranes. Three-day-old monolayers of vero cells grown in 75 cm$^2$ tissue culture flasks were infected at a m.o.i. of 1.0 PFU/cell as described earlier. At 15 hours post infection, 20 µl of Actinomycin D was added to two flasks (final concentration 2 µg/ml). At 26 hours post infection when CPE was approximately 100%, total RNA was isolated as described earlier and stored in ethanol at -20°C. Total RNA was similarly isolated from four 75 cm$^2$ tissue culture flasks of uninfected two-day old monolayers of vero cells. The total RNA from the virus infected cells was reverse transcribed with AMV-RTase and randomly labeled with $^{32}$P in the
second strand synthesis reaction. Total RNA resuspended in 20 µl of DEPC-treated water was aliquoted into two 10 µl portions. To each 10 µl of RNA, 1.5 µl of DEPC-treated water and 1.0 µl of oligo d(T) primer (Stratagene cDNA synthesis kit) were added and heated at 65°C for 10 minutes. The tubes were incubated at room temperature for 2 minutes. The reverse transcription reaction components, 1 µl RNase inhibitor, 4 µl of 5X reverse transcription buffer, 1 µl of 100 mM dNTPs, 1 µl of 80 mM sodium pyrophosphate and 0.5 µl of AMV-RTase were added in the order listed. The reaction was incubated at 42°C for 60 minutes and denatured at 95°C for 2 minutes. Phenol:chloroform extraction of cDNA was made. The reaction was treated in the order listed with 1 µl of 0.5M EDTA and 20 µl of phenol:chloroform. The tubes were vigorously agitated and centrifuged at 10,000xg for 3 minutes. The upper aqueous layer containing the cDNA was transferred to a new tube and 22 µl of ammonium acetate and 88 µl of 100% ethanol were added. The components were mixed and the tube placed at −80°C for 10 minutes and then transferred to −20°C to precipitate the cDNA overnight. The cDNA was pelleted at 10,000xg, vacuum dried, resuspended in 20 µl of water and used in random labeling reaction according to the multiprime DNA labeling system protocol (Amersham). Half of the cDNA resulting from one reverse transcription reaction was used for labeling. The 10 µl of cDNA was denatured at 95°C for 3 minutes and immediately placed on ice. In the listed order, 10 µl of labeling buffer I, 5 µl random primers, 2.5 µl of α-32PdCTP, 20.5 µl of water and 2 µl of enzyme (Klenow polymerase I) were added. The contents of the tube were gently mixed and incubated at 37°C for 30-60 minutes. The labeled cDNA
was denatured at 95°C for 3 minutes and immediately transferred onto ice. The cellular DNA in the labeled reaction was removed in a subtractive hybridization reaction to make the probe virus specific as described by Rozenblatt et al. (1985).

Total RNA from uninfected and virus-infected vero cells was pelleted and resuspended in 20 µl of DEPC-treated water. These total RNAs were heated at 65°C for 10 minutes and 2 µl of the virus infected and the uninfected cell RNAs were spotted per spot onto the nitrocellulose membrane (Zeta probe, Bio-rad) and baked for 15 minutes at 80°C for use as the control membrane for hybridization. The subtractive hybridization reaction was carried out by combining 50 µl labeled probe reaction, 125 µl prehybridization solution, 25 µl of RNA from uninfected cells and 37 µl of denatured and sonicated salmon sperm DNA (final concentration, 150 µg/ml). The reaction was incubated at 42°C for 4 hours. One hour prior to the completion of the subtractive hybridization reaction, the prehybridization of the spotted membrane and the plaque lift membranes was started. Approximately 100 µl of prehybridization solution (50% formamide, 2X SSC buffer, 5X Denhardt, 0.1% SDS and 100 µl denatured salmon sperm DNA) was added to the membranes and the reaction was incubated at 42°C for 60 minutes.

Hybridization of the probe to the nucleic acids on membranes was performed by adding the subtractive hybridization reaction to the prehybridization reaction and hybridizing at 42°C for overnight (~12-16 hours). The membranes were rinsed twice with washing solution I (2XSSC and 0.1% SDS). The cold wash solution I was replaced with 50 ml of washing solution I preheated to 60°C and the tubes were
incubated for 30 minutes at 60°C with continuous rotation. The first washing solution was replaced with washing solution II preheated to 60°C and membranes were similarly incubated at 60°C for 30 minutes.

The membranes were prepared keeping them moist and exposed to X-ray films (X-Omat, Eastman KODAK, Rochester, NY) in separate cassettes. The cassettes were placed at −80°C for 48 hours and the films developed as per manufacturer’s instructions. The X-ray film was aligned with the marks on the agar plates and the virus specific plaques were picked. Each positive plaque was placed into a mixture of 500 μl SM buffer and 20 μl chloroform. The tubes were vigorously mixed and incubated at room temperature for 2 hours and then placed at 4°C. A secondary screening of the picked plaques was performed as described above.

**In vivo single clone excision of pBluescript from the Uni-Zap XR vector**

The pBluescript (SK−) phagemid containing the inserts was subcloned from the Uni-Zap vector by an in vivo excision assay using the ExAssist helper phage as outlined by the manufacturer (Stratagene, Loyola, CA) with some modification. Single colonies of the XL1-Blue MRF− and SOLR bacteria were separately inoculated into 3 ml of LB broth supplemented with 0.2% maltose and 10 mM MgSO₄. The tubes were incubated overnight with continuous shaking in separate tubes and 300 μl of the overnight cultures were inoculated into 30 ml of LB broth (1:100) and incubated for 3 hours with continuous agitation. The bacterial cells were pelleted at 1,000xg for 10 minutes and resuspended in 1.2 ml of 10 mM MgSO₄ to obtain an OD₆₀₀ ~1.0. The cells were placed at 4°C until used. For the in vivo
excision reaction, 100 µl XL-Blue MRF (OD₅₀₀ ~1), 100 µl phage stock and 1 µl of the ExAssist helper phage (>1x10⁶ PFU/µl) were combined in Falcon 2059 tubes and incubated at 37°C for 15 minutes. A 3 ml of LB-broth was added to each tube and incubated at 37°C for 3 hours with continuous agitation. The tubes were heated in a water bath at 65-70°C for 20 minutes and then centrifuged at 1,000xg for 15 minutes.

The supernatants containing the excised pBluscript phagemid packaged as filamentous phage particles were transferred to new tubes and stored at 4°C. The excised phagemid was mixed with SOLR cells and plated on LB-ampicillin agar plates. In a 1.5 ml microcentrifuge tube, 1.0 µl of excised phage was added to 99 µl of SOLR cells and two more 1:10 serial dilutions were made in 90 µl of SOLR cells. The tubes were incubated for 15 minutes at 37°C and the cells were plated on LB-ampicillin agar plates. The plates were incubated at 37°C for overnight and then stored at 4°C.

**Isolation of plasmid DNA**

Plasmid DNA was isolated by alkaline lysis of bacteria using the Qiaprep spin miniprep kit as outlined by the manufacturer (Qiaprep, Qiagen Inc., USA). The excised single-stranded pBluescript phagemid (SK-) was rescued into double-stranded pBluescript phagemid in SOLR cells plated on LB-ampicillin selecting agar plates and stored at 4°C. Such single bacterial colonies obtained from the listed picked plaques, 714P6A, 714P6B, 714P6C, 714P6D, 714P2A, 714P2B, pPSP714P2C, 714P3A, 714P3B, 714P4A, 714P4B, and 714P5A were separately
inoculated into 3 ml of LB-ampicillin broth and incubated at 37°C for overnight with continuous agitation.

The bacterial cells were pelleted at 1,000xg for 10 minutes and resuspended in 250 µl of P1 buffer. The suspension was transferred to a new tube. The cells were lysed by adding 250 µl of alkaline lysis buffer P2. The tubes were gently inverted 4-6 times to mix and 350 µl of neutralizing buffer N3 was added. The tubes were immediately inverted 4-6 times. Centrifugation at 10,000xg was performed for 10 minutes.

The supernatants were decanted into Qiaprep spin columns (Qiagen) placed in 2 ml collecting tubes and centrifuged for 30-60 seconds. The flow-through was discarded and 0.5 ml of buffer PB was added to the column. Centrifugation at 10,000xg was performed for 30-60 seconds to wash the columns. The flow-through was discarded and 0.75 ml of buffer PE was added. The tubes were centrifuged at 10,000xg for 30-60 seconds. The flow-through was discarded and the tubes were centrifuged for another 60 seconds to remove residual washing buffer.

The Qiaprep columns were transferred into 1.5 ml microcentrifuge tubes and 50 µl of elution buffer EP (10mM Tris-HCl, pH-8.5) was added to the center of the columns. The columns were placed at room temperature for 5 minutes and then centrifuged for 1 minute to collect the DNA.
Sequencing and nucleotide sequence analysis

Several clones selected by hybridization with the virus specific probe were sequenced using universal (or oligo d(T)) and reverse primers by the Sanger dideoxy chain termination method using an automated fluorescent sequencer (Applied Biotechnologies, Foster City, CA). For the clone pPSP714P2C, further sequencing was done with primers designed from the sequence determined using reverse and universal primers. The walking of the insert was continued until the complete sequence was obtained. Two more internal primers derived from the sequences obtained were synthesized and used to further sequence the insert of clone pPSP714P2C. The sequencing strategy is shown in Figure 1.

Figure 1. Sequencing strategy: pSK-L and pSK-R are the left and right arms, respectively, of the circular pbluescript (pSK) phagemid flanking the double-stranded insert pPSP714P2C derived from the viral cDNA. Reverse, universal and oligo d(T) are primers specific to the pbluescript phagemid (pSK). L-1 is a lower primer derived from the sequence obtained with the universal primer and L-2 is a lower primer derived from the sequence obtained with L-1. The U-1 upper primer was derived from the sequence obtained with the reverse primer and U-2 upper primer was derived from the sequence obtained with U-1. A primer walking was done to complete the sequencing of the insert.
Sequence analysis was performed using MacVector (International Biotechnologies, New Haven, CT) and GeneWorks programs. Nucleotide sequence of the PK insert was used to search the database using the BLAST program.

Results

Electron microscopy of virions

The electron micrographs of the purified virions obtained are shown in Figures 2a-e. Some of the various shapes of the PPMV virions observed are represented in the figures. The spherical virions with diameter of 150-400 nm were dominant in the samples (Figure 2d, 2e). Some filamentous forms were also observed (Figure 2b). Virions with several projections were observed (Figure 2c). Nucleocapsids were also seen protruding from a disrupted virion envelopes (Figure 2a) in a sample prepared from virion pellets resuspended in water. A relatively smaller number of disrupted virions was seen in samples from pellets re-suspended in TEN buffer (pH 8.0). In general, the electron micrographs showed the varying shapes of the virions. The diameter of the virions measured from 150-400 nm. The length of the filamentous forms was up to 0.7µm. In all virions, projections of apparent uniform length (not measured) surrounding the virion were present. These projections extended from a denser demarcation that represents the envelope of the virus.
Figure 2. Electronmicrographs of PPMV virions: (a) two disrupted virions with protruding nucleocapsids; (b) two branching filamentous virions; (c) an irregularly shaped virion; (d) a spherical virion; and (e) differently shaped virions shown together. Each bar is 100 nm.
Electron microscopy of nucleocapcids

An electron micrograph of nucleocapcids that banded in the layer of 30% CsCl₂ of the gradient is shown in figure 3. More than 25 long nucleocapcids were measured and the average length was 0.71 µm. It was not possible to tell whether the nucleocapcids measured represented full length nucleocapcids.

Figure 3. Electronmicrograph showing negatively stained nucleocapcids
Shorter strands of the nucleocapsids were abundant in the samples possibly representing fragments from full-length nucleocapsids or defective strands (Fig. 2).

**Cloning of cDNA**

A recombinant cDNA library was constructed from the reverse transcription product of RNA isolated from PPMV-infected vero cells followed by double-stranded cDNA synthesis. Approximately 1:10 ratio of blue colonies with no insert to white colonies with insert was obtained indicating successful cloning. A single amplification of the cDNA library was performed.

**Hybridization and screening**

The cDNA library was screened for virus specific clones by hybridization with a random primed $^{32}$P-labeled cDNA probe prepared from virus-infected vero cells. The probe was specific as the $^{32}$P-labeled probe hybridized only to the virus infected cell RNA spotted on nitrocellulose membrane and not unto infected cell RNA.

Secondary screening was performed on the twenty positive clones and sub-clones were selected from each positive clone. Double stranded DNA was isolated from the sets of clones and analyzed. One of these clones, pPSP714P2C had an insert of about 1.9 Kb and was further characterized.

**Nucleotide sequencing of cDNA clones and analysis**

DNA from several clones representing similar size of DNA insert was sequenced. The partial sequence obtained from one clone, pPSP714P2C, using universal and reverse primers showed homology with that of bovine parainfluenza
virus type 3 P gene in the blast search. The blast search with the complete sequence of pPSP714P2 indicated some homology of this clone to the P genes of human parainfluenza virus type 1, human parainfluenza virus type 3 and bovine parainfluenza virus 3, Sendai virus (HPIV 1), ovine parainfluenza virus and human parainfluenza virus type 4B. The blast search gave the highest homology score for the BPIV-3/PIV-3 P genes. The homology of pPSP714P2C was 98% with BPIV-3 (BY00114), 98% with PIV-3 (HD84095), 39% with HPIV-1 (M74082), and 49% with Sendai virus (X17008).

The nucleotide sequence of the mRNA representing the P gene identified by the sequence of clone pPSP714P2C is shown in the positive sense in Figure 4. The coding sequence starts from position 52 and ends at position 1839. The mRNA is 1962 nucleotides long followed by a poly-A tail and contains 3 open reading frames (ORFs). The largest ORF encoding 596 amino acids starts from position 52 and ends at position 1839. This largest ORF is 1788 nucleotides long and it encodes 596 amino acids and has an estimated molecular mass (Mr) of 66,241 daltons. The second ORF is 603 nucleotides long encoding 201 amino acids and it starts from position 62 and ends at 664 with a calculated Mr 23,585 daltons. This ORF has 98% homology with the non-structural C protein of human parainfluenza virus type 3, strains Y00114, D84095, MNNZB3, 75% with strain U51116, 71% with bovine parainfluenza (AF035681) virus C protein and 40% with C protein of human parainfluenza (M74081) virus 1. A third ORF encoding 140 amino acids was present but showed no homology with any other proteins. The sequence of the cloned ISU-
Figure 4. The nucleotide sequence of the mRNA representing the P gene of PPMV (ISU-92) identified by the sequence of clone pPSP714P2C.

ATG and TAA (in bold) represent start and stop codons, respectively.
92 mRNA was compared to the genome of the BPIV-3 (15,548 nucleotides) and it was found to correspond to the region between nucleotides 1,732 and 3,699. This region of the BPIV-3 includes the P gene coding sequence. A comparison of the coding sequences revealed that both are 1791 nucleotides long with a homology of 98% between the ISU-92 and BPIV-3 P genes. The overall homology between the P genes of ISU-92 and BPIV-3 was 97% and the non-coding regions had 83% homology. There were 58 nucleotide changes in the mRNA of PPMV representing 3% of the total number of nucleotides. In the coding sequence there were a total of 48 nucleotide changes amounting to 2% of the total number of nucleotides in the coding region. The alignments of the PPMV and BPIV3 mRNAs are shown in Figure 5.
Figure 5. The alignments of the sequence obtained from clone pPSP714P2C representing the mRNA of the PPMV P gene with that of the BPIV 3 P gene. Paired dots show homology and blank spaces show mismatches.

**Discussion**

We have characterized the ultrastructure of the ISU-92 strain of PPMV which confirms previous studies that ISU-92 is a paramyxovirus. The pleomorphic nature
Filamentous forms of the virions have also been reported for the rinderpest virus (Plowright, 1962). Surface projections representing the spikes observed were a common feature of the virions and have been observed in other viruses (Creig, 1971). The diameter of virions observed in our study ranged between 150-400 nm which is similar to 150-500 nm reported for paramyxoviruses (Nagai et al., 1989, cited in Lamb, 1996). The serrated nucleocapsids are similar to the serration demonstrated for the LPMV, mumps, Sendai and Newcastle disease viruses (Kolakofsky, 1974; Heggeness et al., 1980; Nagai et al., 1989; Sundqvist et al., 1990).

The variable forms of the PPMV virion demonstrated by electron microscopy and the morphology of the nucleocapsids observed strongly suggest that ISU-92 is a paramyxovirus. In conjunction with the antigenic reactivity with the BPIV-3 and HPIV-3, (Janke et al., 1992), it is suggested that ISU-92 is a PPMV and a member of the genus paramyxovirus. However additional studies are warranted to conclusively demonstrate its relationship to other paramyxoviruses.

The P protein of paramyxoviruses is required for the formation of a fully active transcriptive complex during replication (Hamaguchi et al., 1983). It is, therefore, expected that the mRNA representing the P gene should be found in relatively high abundance during the replication of the paramyxoviruses. Since the cDNA we used for cloning was obtained from infected cells in which virus replication was actively taking place, it is no surprise that the first positive clone we obtained corresponded to the P gene mRNA.
The number of amino acids coded for by the P genes of the paramyxoviruses and the morbilliviruses range from 507 to 603 (Lamb and Kolakofsky, 1996). Analysis of the ISU-92 P gene showed that it encodes 596 amino acids. There are 51 untranslated nucleotides before the start (AUG) of the largest ORF. However, there is no evidence at this time to conclude that the non-coding sequence of the P gene mRNA is only 51 nucleotides long. The high nucleotide homology of 98% between the ISU-92 and BPIV-3 suggests a close relationship of ISU-92 with BPIV-3. Lower homology was also detected with the P proteins of other paramyxoviruses. The RNA dependent RNA polymerases of single-stranded (+) RNA viruses have conserved motifs but these polymerases have been shown to share homology with other distantly related viruses (Koonin and Dolja, 1993). Therefore, there is the need to sequence other genes of the porcine paramyxovirus for its classification.

However, based on the high nucleotide homology of 98% between ISU-92 and BPIV 3, it is clear that the ISU-92 is closely related to the BPIV-3 group of viruses. It is our opinion that the ISU-92 should be classified in the family Paramyxoviridae, sub-family Paramyxovirinae and the genus paramyxovirus. It is possible that the BPIV-3 through mutation has become adapted to a new host. The plasmid pPSP714P2C containing the P gene should be beneficial in development of an in situ hybridization test for the detection of PPMV in tissues.
References


CHAPTER 3. GENERAL CONCLUSIONS

The porcine paramyxovirus, ISU-92 was isolated from pigs in 1992. The disease outbreak from which the virus was isolated was characterized by respiratory and CNS disorders with the tendency to spread rapidly within the population. The disease has been reproduced using cell culture propagated ISU-92 by intranasal inoculation of 3 day-old piglets resulting in mild pneumonia. A virus neutralization test that was developed and used in screening serum for the presence of antibodies to PPMV detected only a low level of PPMV infection in the population studied. The PPMV was also shown to be antigenically related to PIV-3, BPIV3 and PIV-4B, although reactivity with PIV-4B was low.

In this study, we continued the characterization of ISU-92 and started the molecular characterization of ISU-92. The virions of the porcine paramyxovirus were found to show variability in their morphology. This is in agreement with the pleomorphism that has been established as a characteristic of paramyxoviruses. The virion diameter of 150-400 nm observed falls within the range of diameter of paramyxoviruses. These characteristics of ISU-92 strongly support our prediction that ISU-92 is a paramyxovirus.

We have successfully cloned the total mRNA of vero cells infected with ISU-92 into the lambda derived cloning vector Uni-Zap XR. An efficient probe developed from infected cells was used to select a virus specific clone, pPSP714P2C from the cDNA library. The sequence of this clone gave the first information about the genome of ISU-92. By sequence comparison, we identified the sequence to be
representative of the mRNA for the P gene. The ISU-92 P gene had 98% nucleotide homology with BPIV-3 and HPIV-3. In a comparison of this sequence to the P gene of the LPMV only 13% homology was observed. This observation is consistent with lack of biological reactivity with antiserum to LPMV. The high homology with the PIV-3 and BPIV-3 was not expected and raises several questions. Is the ISU-92 a true porcine paramyxovirus? If so, how and where has it evolved from? Did this originate from BPIV 3? Which species are susceptible to this virus?

In order to answer these questions additional studies need to be conducted. First the whole genome of ISU-92 needs to be sequenced. It is our opinion that the development of more sensitive tests, than those currently available, will clarify and help understand the epidemiology of this virus.
LITERATURE CITED


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