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Molecular and Biological Aspects of Porcine Rubulavirus (LPMV) Lytic and Persistent Infections

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Abstract

This thesis summarises and discusses results of studies on the porcine rubulavirus (LPMV). LPMV is the causative agent of blue eye disease of pigs. The specific goals of these studies have been to establish the relatedness of LPMV to other members of the family paramyxoviridae and to describe different aspects of persistent infections in both cell cultures and pigs.

Study I is an analysis of the phosphoprotein (P) gene, which is an essential component of the viral polymerase. LPMV was found to have the P gene organised in a way similar to other members of the rubulavirus genus including the human pathogen mumps virus. Rubulaviruses commonly have an open reading frame which does not encode the P protein but instead encodes a protein designated V. The P protein is expressed by an mRNA in which two additional nontemplated G nucleotides are inserted by the viral polymerase (editing). The editing event occurs at a frequency of ~50% for LPMV.

Studies II and III describes the establishment and molecular characterisation of a persistent infection in porcine kidney cells *in vitro*. The cells showed reduced amounts of the L protein and to a certain extent the P protein, which was reflected in low mRNA levels and a shift in the editing frequency, respectively. Several subgenomic RNAs were also identified. Any of these changes could theoretically have the capacity to modulate an infection, and could ultimately lead to a persistent state.

Study IV addresses the possibility of whether porcine rubulavirus could remain in acute infected pigs after full recovery from the disease. Virus could only be detected in organ samples from pigs which had recovered from acute LPMV infection by RT-PCR. This was especially evident after pigs were immunosuppressed, enabling detection of LPMV RNA in the lung. Furthermore, evidence that LPMV was still transcriptionally active was also obtained. The persistence of LPMV in tissues could be of clinical importance, and the risk of reactivation and shedding of virus in situations of immunosuppression could have profound effects on the epidemiology of the disease.

Key words: porcine rubulavirus (LPMV), persistent infection, L protein, P protein, transcription, editing, subgenomic RNA

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To my parents

Abstract

Hjertner, B. 1997. *Molecular and biological aspects of porcine rubulavirus (LPMV) lytic and persistent infections*. Doctor's dissertation.

This thesis summarises and discusses results of studies on the porcine rubulavirus (LPMV). LPMV is the causative agent of blue eye disease of pigs. The specific goals of these studies have been to establish the relatedness of LPMV to other members of the family paramyxoviridae and to describe different aspects of persistent infections in both cell cultures and pigs. Study I is an analysis of the phosphoprotein (P) gene, which is an essential component of the viral polymerase. LPMV was found to have the P gene organised in a way similar to other members of the rubulavirus genus including the human pathogen mumps virus. Rubulaviruses commonly have an open reading frame which does not encode the P protein but instead encodes a protein designated V. The P protein is expressed by an mRNA in which two additional nontemplated G nucleotides are inserted by the viral polymerase (editing). The editing event occurs at a frequency of ~50% for LPMV. Studies II and III describes the establishment and molecular characterisation of a persistent infection in porcine kidney cells *in vitro*. The cells showed reduced amounts of the L protein and to a certain extent the P protein, which was reflected in low mRNA levels and a shift in the editing frequency, respectively. Several subgenomic RNAs were also identified. Any of these changes could theoretically have the capacity to modulate an infection, and could ultimately lead to a persistent state. Study IV addresses the possibility of whether porcine rubulavirus could remain in acute infected pigs after full recovery from the disease. Virus could only be detected in organ samples from pigs which had recovered from acute LPMV infection by RT-PCR. This was especially evident after pigs were immunosuppressed, enabling detection of LPMV RNA in the lung. Furthermore, evidence that LPMV was still transcriptionally active was also obtained. The persistence of LPMV in tissues could be of clinical importance, and the risk of reactivation and shedding of virus in situations of immunosuppression could have profound effects on the epidemiology of the disease.

Key words: porcine rubulavirus (LPMV), persistent infection, L protein, P protein, transcription, editing, subgenomic RNA

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals I-IV.

I. Berg, M., **Hjertner, B.**, Moreno-López, J., Linné, T. (1992). The P gene of the porcine paramyxovirus LPMV encodes three possible polypeptides P, V and C: the P protein mRNA is edited. *Journal of General Virology*: 73, 1195-1200.

II. **Hjertner, B.**, Linné, T., Moreno-López, J. (1997). Establishment and characterisation of a porcine rubulavirus (LPMV) persistent infection in porcine kidney cells. *Acta Veterinaria Scandinavica.* (in press)

III. **Hjertner, B.**, Bergvall, A.-C., Svenda, M., Berg, M., Moreno-López, J. and Linné, T.: Multiple factors including subgenomic RNAs and reduced viral protein expression are associated with a persistent infection by porcine rubulavirus (LPMV). (submitted)

IV. Bergvall, A.-C., **Hjertner, B.**, Linné, T., Allan, G., McNeilly, F., Moreno-López, J., Berg, M.: Porcine rubulavirus LPMV RNA persists in pigs recovered from virus infection. (manuscript)

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Abbreviations

BPIV-3	Bovine parainfluenza virus type 3
CDV	Canine distemper virus
CNS	Central nervous system
CPA	Cyclophosphamide
CPE	Cytopathic effect
DI	Defective interfering
DNA	Deoxyribonucleic acid
F	Fusion protein
HN	Haemagglutinin-neuraminidase protein
HPIV-1-4	Human parainfluenza virus types 1-4
HRSV	Human respiratory syncytial virus
IFN	Interferon
kb	Kilobases
kD	Kilodalton
L	Large protein
LPMV	La Piedad-Michoacan Mexico virus
m.o.i.	Multiplicity of infection
M	Matrix protein
MeV	Measles virus
mRNA	Messenger-RNA
NDV	Newcastle disease virus
nm	Nanometer
ORF	Open reading frame
NP	Nucleoprotein
P	Phosphoprotein
PCR	Polymerase chain reaction
PI	Persistent infection
PK-15	Porcine kidney cells
RNA	Ribonucleic acid
RPV	Rinderpest virus
RT	Reverse transcribed
SDS-PAGE	Sodiumdodecyl sulphate - polyacrylamid gel electrophoresis
SH	Small hydrophobic
SSPE	Subacute sclerosing panencephalitis
SV-5	Simian virus type 5
SV-41	Simian virus type 41

Introduction

Prologue

A new disease of swine called blue eye disease emerged in Mexico in 1980. The first outbreak was registered in a commercial farm housing 2500 sows in La Piedad, Michoacan State. The disease was characterised by pneumonia, encephalitis, corneal opacity (blue eye) and neurological disorders. Piglets were most susceptible, showing high morbidity and mortality. In the following years numerous new outbreaks occurred in the neighbouring states, constituting a serious economical problem in the swine industry. The disease has so far remained endemic in Mexico.

In 1984 a haemagglutinating virus was isolated from a piglet showing neurological disorders, pneumonia and corneal opacity, and it was shown to belong to the paramyxoviridae family. The virus was named "La Piedad Michoacan Mexico virus (LPMV)" (Moreno-López et al., 1986), and has recently been renamed porcine rubulavirus (LPMV) and classified into the rubulavirus genus (Rima et al., 1995). This thesis is based on studies on porcine rubulavirus genes and their expression in lytic and persistent infections, *in vitro* as well as *in vivo*, with special emphasis on the expression of the P-gene.

Paramyxoviridae

The order mononegavirales is composed of the families paramyxoviridae, rhabdoviridae and filoviridae. All viruses in this order contain genomes consisting of a single-stranded non-segmented RNA of negative polarity. The family paramyxoviridae is divided into two subfamilies, paramyxovirinae and pneumovirinae. The subfamily paramyxovirinae, which is by far the largest, is further divided into three genera: Paramyxovirus, morbillivirus and rubulavirus with type species being human parainfluenza virus type 1 (HPIV-1), measles virus (MeV) and mumps virus, respectively. The subfamily pneumovirinae has only one genus, the pneumovirus genus, with human respiratory syncytial virus (HRSV) as type species (Rima et al., 1995).

Genus rubulavirus

Being the most clinically significant virus within this genus, mumps virus serves as type species. Other human pathogens include the respiratory viruses human parainfluenza viruses type 2, 4a and 4b, but these are of much less importance. Several avian paramyxoviruses, of which Newcastle disease virus (avian paramyxovirus 1) is the most noteworthy, are also grouped in this genus. The Simian parainfluenza viruses, SV-5 and SV-41, also belong to this genus. SV-5 is closely related to mumps virus, a feature it shares with the LPMV, which also has been grouped in the genus rubulavirus. Features

common for the members of the rubulavirus genus are the existence of both haemagglutinating and neuraminidase activities, the need for editing in order to express the P protein and the lack of C proteins. Viruses of the genus paramyxovirus have haemagglutinating and neuraminidase activities, express C proteins and encode the P protein from a faithful mRNA. Morbilliviruses express the P protein in a similar way to paramyxoviruses, contain C- and haemagglutinin proteins, but lack neuraminidase activity (Rima et al., 1995).

Virion structure

Rubulaviruses are fairly large enveloped particles, their diameter being 150 nm or more. They are usually spherical in shape, but other forms have been shown to occur. The virion is composed of an internal nucleocapsid containing the single stranded viral genome and associated proteins, and an outer lipoprotein envelope containing viral peplomers. The genome is a single stranded linear RNA molecule of negative sense, approximately 15 kb long. Untranslated 3′ and 5′ regions contain promoters (negative and positive strand promoters, respectively) involved in both replication and transcription. The 3′end promoter (negative strand promoter) is active on the negative sense genomic RNA and the 5′end promoter (positive strand promoter) is active on the positive sense antigenome RNA. Six or seven open reading frames (ORFs) are arranged on the genome and code for the following proteins; the nucleoprotein (NP), the phosphoprotein (P), the matrix (M) protein, the fusion (F) protein, the haemagglutinin-neuraminidase (HN) protein and the polymerase or large (L) protein (Rima et al., 1995). All six proteins are structural components of the virion (Fig. 1).

Another open reading frame, coding for a small hydrophobic (SH) protein, has been found exclusively in mumps virus and simian virus 5 (SV5) (Hiebert et al., 1985, Elango et al., 1989).

The nucleoprotein, the most abundant viral protein found during infection of a cell is tightly associated with the genomic RNA, providing protection and stability as well as acting together with the viral polymerase complex during transcription and replication (Morgan, 1991). The P protein forms the polymerase complex together with the L protein (Hamaguchi et al., 1983) and interacts with the nucleoprotein during transcription/replication. The N-terminal region of the phosphorylated P protein of Sendai virus has been shown to bind soluble nucleoprotein (Curran et al., 1995). The C-terminal region is also involved in binding to soluble nucleoprotein (Curran et al., 1995), and also to the L protein (Smallwood et al., 1994) as well as to nucleocapsids (Ryan & Kingsbury, 1988, Ryan & Portner, 1990, Ryan et al., 1991). The M protein is aligned just inside the envelope, interacting with both the envelope and the nucleocapsid. The HN and F proteins are both glycosylated transmembrane proteins involved in attachment of the cells and the fusion of membranes (Morrison & Portner, 1991). The HN protein

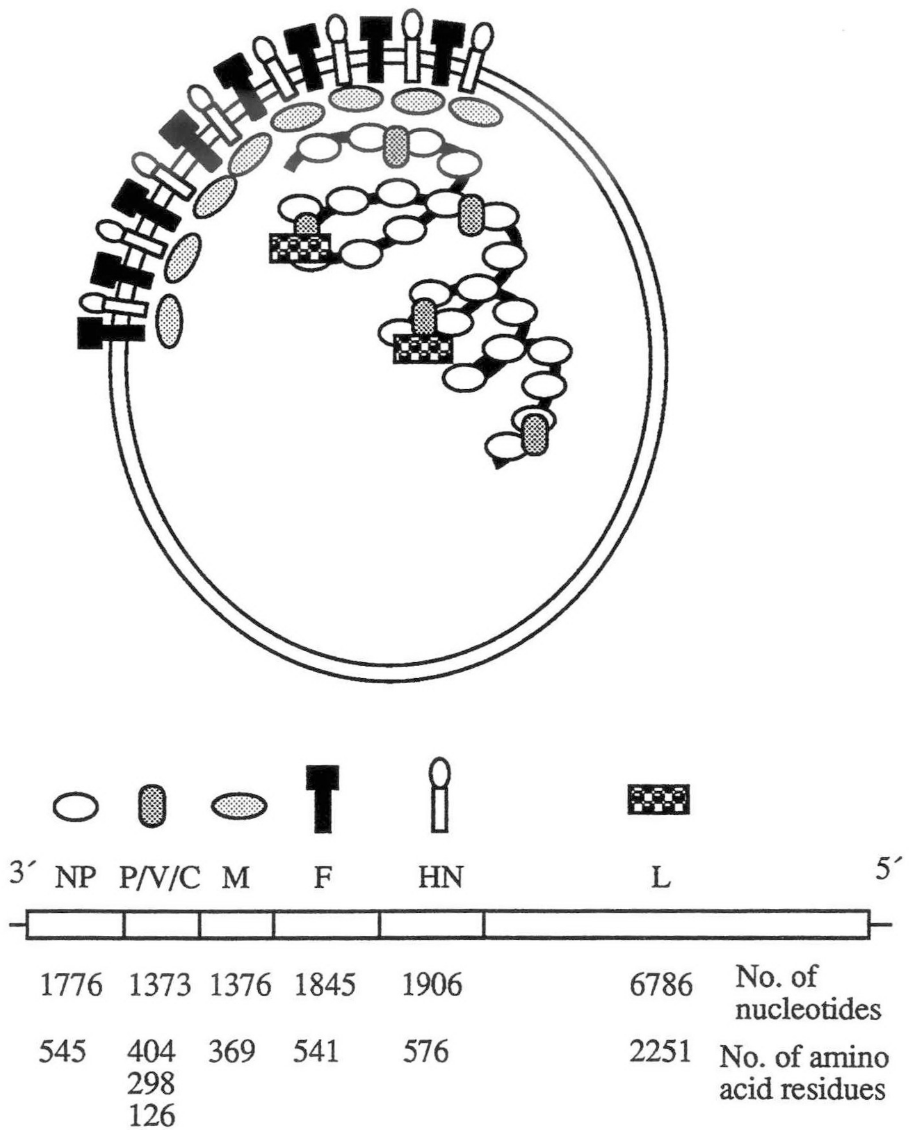

Fig. 1. Schematic presentation of a typical rubulavirus particle. The complete genome of LPMV is depicted under the virus particle (Paper I, Berg et al., 1991, 1997, Sundqvist et al., 1992, Svenda et al., 1997 and personal communication). Indicated is the length of each gene and the amino acid residues of each protein. The 3´and 5´nontranslated ends have not been sequenced.

confers the haemagglutinating and neuraminidase activities characteristic of the paramyxovirus and rubulavirus genera.

A polypeptide originating from the P gene, designated V, is present in all members of the paramyxoviridae family except for human parainfluenza virus types 1 and 3 (Matsuoka et al., 1991, Galinski et al., 1992). It has been found in in mumps virus and SV-5 virions (Takeuchi et al., 1990, Paterson, et al., 1995), but only intracellularly in a Sendai virus infection (Curran et al., 1991a).

The genera paramyxovirus and morbillivirus also encode the intracellular C proteins from the P gene (Lamb & Paterson, 1991).

Replication cycle

The first step of the viral infection cycle involves the attachment of the viral particle to the membrane of a susceptible host cell. This is mediated by the HN protein which has an affinity for cell surface receptors. Specific cellular receptors have not yet been identified for the various members of the paramyxoviridae, with the exception of the human CD46 molecule to which the measles virus H protein binds (Dörig et al., 1993, Naniche et al., 1993). CD46 is widely distributed throughout human tissues and is involved in the inactivation of complement factors C3b and C4b (Liszewski et al., 1991). Moesin, the human membrane organising extension spike protein, is also proposed to be involved in the binding of measles virus to host membranes (Dunster et al., 1994).

After viral attachment to the host cell, the F protein fuses the envelope surrounding the viral particle with the cell membrane at neutral pH, mediating a direct release of the viral nucleocapsid into the cytoplasm of the cell.

Within the cytoplasm, the viral polymerase complex (P and L proteins) initiates mRNA synthesis at the promoter in the 3' untranslated region. This is the only promoter which has been found on the viral genome and the polymerase is thought to reinitiate at every intergenic sequence. Capped and polyadenylated transcripts are formed sequentially according to their localisation on the genome. A gradient of different transcripts from the NP to the L transcripts is formed, as some polymerases detach at each intergenic region. The genome also encodes a short leader RNA upstream of the NP gene which is uncapped and lacks a poly (A) tail. The function of the leader RNA is not known, but it has been found to be localised in the nucleus (Ray et al., 1991)

Every mRNA is monocistronic, but the P gene is capable of producing different transcripts encoding either the P or the V protein. This is accomplished by the insertion of nontemplated guanosine nucleotide(s) at the editing site, a specific stretch of G nucleotides at which the viral polymerase stutters, thus changing the reading frame (Vidal et al., 1990b). Members of the genera paramyxovirus and morbillivirus have unedited transcripts encoding the P protein, but the opposite is true for all members of the genus rubulavirus except for NDV (Steward et al., 1995), implying a need for RNA editing in

order to produce the essential P protein The C proteins of genera paramyxovirus and morbillivirus is encoded by another ORF on the P or V transcripts, accessed via initiation of protein translation at alternate start AUG codons (Dethlefsen & Kolakofsky, 1983, Giorgi et al., 1983).

Once the intracellular levels of viral messengers and their corresponding proteins have accumulated, the pool of NP in the cell switches transcription to synthesis of the antigenome (positive sense) by encapsidating the synthesised RNA, thus abolishing termination at each intergenic site (Vidal & Kolakofsky, 1989, Kolakofsky et al., 1991). The antigenome contains a promoter at it's 3'end which is used in the synthesis of new genomes of negative sense.

The M protein interacts with the nucleocapsid, as well as the intracellular parts of the viral glycoproteins, and this leads to the assembly of all viral components at the cell membrane (Peeples, 1991). Finally, a new viral particle is produced by budding from the cell.

Clinical significance of the family paramyxoviridae

The genus morbillivirus contains the most clinically important viruses. In humans, measles virus causes an acute, infectious respiratory disease. Rarely, the chronic complications subacute sclerosing panencephalitis (SSPE) or measles inclusion body encephalitis (MIBE) develop after the acute phase (Ohuchi et al., 1987, Roos et al., 1981, ter Meulen, 1983). SSPE generally develops 5-10 years after primary infection, and inevitably leads to death. Rinderpest virus (RPV), which is thought to be the archaevirus of morbilliviruses, causes a lethal infection of cattle and other large ruminants (Scott, 1990a). Peste de petit ruminants virus (PPRV) causes goat plague, a disease of small ruminants which frequently leads to death (Scott 1990b). Canine distemper virus (CDV) causes respiratory and intestinal disease in dogs, with occasional CNS involvement. Old dog encephalitis is a late complication with similarities to SSPE in humans (Appel, 1987). A disease of seals characterised by pneumonia, and infection of the CNS, caused by a recently identified morbillivirus (Phocine distemper virus, PDV), appeared in 1988 in the Baltic and North sea (Osterhaus & Vedder 1988). Most recently, a morbillivirus affecting equines was identified in Australia. This virus is capable of spreading to humans, most frequently causing an influenza-like disease, but one case leading to death has been reported (Murray et al., 1995).

Viruses in the genus paramyxovirus include bovine parainfluenza virus type 3 (BPIV-3), human parainfluenza virus types 1 and 3 (HPIV1 and 3) and Sendai virus (murine parainfluenza virus type 1). All of the viruses cause respiratory diseases (Bryson, 1990, Collins et al., 1996).

Within the genus rubulavirus, the avian paramyxovirus type 1 (PMV-1), better known as Newcastle disease virus (NDV), causes a severe systemic disease in poultry (Alexander, 1991). In humans, mumps virus is of considerable importance, mainly as a cause of parotitis, but also as a cause of orchitis and meningitis (Wolinsky, 1996). Parainfluenza virus types 2, 4a and

4b (HPIV-2, 4a, 4b) cause respiratory diseases in humans as does simian virus 5 (SV-5) in dogs (Collins et al., 1996).

There are only two clinically important viruses in the subfamily pneumovirinae, human and bovine respiratory syncytial viruses, which cause respiratory diseases in humans and cattle, respectively (Scott & Taylor, 1984).

Epidemiology and immunity

Members of the paramyxoviridae are sensitive to various kinds of environmental exposure because of the enveloped structure, and are spread by direct contact and aerosol. They have no known mechanism by which they can cause latent infections with subsequent reactivation as has been shown for DNA viruses (for example herpesviridae) and RNA viruses with DNA intermediates (i.e. retroviruses) (Ahmed et al., 1996). Their constant need of circulation necessitates a large population for survival of the virus. Upon infection of a small population with a non-lethal, strictly lytical and antigenically stable virus, all individuals would soon become infected and generate immunity. This "heard immunity" would make continued circulation of the virus impossible, and the infection would die out. Following generations would be fully susceptible to the infection, and another epidemic would arise if the virus was reintroduced into the population (Black, 1991). Mumps and measles virus cause epidemiologically important diseases of humans. Up to 90% of unvaccinated individuals normally have acquired immunity to mumps by the age of fifteen. Of these individuals, more than 70% develop symptoms, and almost all recover without complications (Wolinsky, 1996).

During infection by members of the paramyxoviridae family, antibodies are produced against both internal and external proteins. However, only antibodies to the HN and F proteins are important in eliciting a virus neutralising response. The HN (or H) protein induces the most efficient neutralising antibodies, although the best protective response is achieved if antibodies toward both proteins are produced (Norrby et al., 1975, Giraudon & Wild, 1985, Paterson et al., 1987, Spriggs et al., 1988).

The F proteins of morbilliviruses have been shown to be highly conserved, to a degree that cross-neutralisation between different morbilliviruses can be seen (Appel et al., 1984, Visser et al., 1989).

Today, vaccines are available to most viruses that cause systemic paramyxovirus diseases i.e. MeV, RPV, CDV and mumps virus, providing life long protection.

Persistent infections

Persistent infection of a host can be defined as the prolonged existence of the virus in the body compared to what is normally expected after an acute infection. Members of the family paramyxoviridae have the ability to cause persistent infections, both *in vitro* and *in vivo*. Persistent infections are also commonly caused by the viruses in the related family rhabdoviridae, and findings from studies of these infections have been used in the study of persistent infections by members of the family paramyxoviridae. Persistent infections by certain members of the family paramyxoviridae have been circumstantially implicated in several human diseases (Randall & Russel, 1991).

In vitro

Studies of persistent infection *in vitro* have mainly focused on the existence of subgenomic viral genomes (reviewed by Re, 1991), but temperature sensitive mutants and the inhibition of viral expression by interferon have also been described (Friedman & Ramseur, 1979). More recently, mutations affecting the expression or function of individual proteins have been described (Roux & Waldvogel, 1982, Jordan et al., 1989, Celma & Fernandez-Muñoz, 1992, Hummel et al., 1994)

Since the subgenomic particles lack segments of the complete genome they need the presence of wild type virus for their propagation. They have an inhibitory effect on virus replication because they compete for the viral RNA synthesis machinery, hence their name defective interfering (DI) particles. They can exist in two basic forms: the copy back and the internally deleted RNA (Re, 1991). The copy back RNA is believed to be generated during the synthesis of the negative strand genome. The polymerase is thought to terminate prematurely within the L gene and reinitiate RNA synthesis backward on the nascent strand or on an identical one. As a consequence only the positive strand promoter is preserved in the RNA. Internally deleted DI RNAs consist of genomes with one or several internal deletions but retaining both 3' and 5' promoters. The copy back RNAs are generally more common, and smaller than the internally deleted RNAs. Both types of RNA have the ability to interfere with wild type virus replication because their smaller size gives them a selective advantage. In competition experiments performed with different DI particles the following traits confer a selective advantage; a smaller RNA always eliminates a larger one down to the lower limit of what can be packaged in a viral particle (1.6 kb); copy backs eliminate internally deleted RNAs because the 5' positive strand promoter is considered stronger than the 3' negative strand promoter; internally deleted RNAs that are transcriptionally active, this being to their disadvantage, are also eliminated by copy backs (Re, 1991).

In vitro generated DI particles have been described for Sendai virus (Kingsbury & Portner, 1970), human parainfluenza virus type 3 (Murphy et al., 1987), mumps virus (McCarthy et al., 1981, Andzhaparidze et al., 1982,

1983), simian virus 5 (Azadova & Zhdanov, 1982), Newcastle disease virus (Maeda et al., 1978) and measles virus (Rima et al., 1979).

In vivo

Establishment of persistence *in vivo* could concievably be due to many different mechanisms. A slow infection mediated by the interfering properties of DI particles on wild type infection might be sufficient. Initially, persistence requires that the virus changes to a non-lytic phenotype or infects cells that do not support a lytic infection cycle. Persistence would necessitate the continuous replication of the viral genome, in contrast to latent infection, as caused by certain DNA viruses, in which viral expression is tightly controlled by viral genes, and the genome replicated only at times of cell division, either by transiently expressed viral proteins or by the cellular machinery (Ahmed et al., 1996). The persistence of non replicating genomes would require infection of very long-lived cells (for example nerve cells). In the host the immune surveillance must also be avoided. This can be accomplished by the infection of cells which are not exposed to immune surveillance. The selection of viruses that do not efficiently express viral proteins important in mediating antibody binding to the surface of infected cells could be important at sites not easily accessible to cytotoxic T cells. Cytotoxic T cells are generally considered to be the most important immune effectors in the clearing of viral infections. In tissues which are inaccessible to these cells, antibody mediated cell lysis is an important substitue for this function. Cellular immune responses in the CNS probably cause exessive tisssue destruction and it has been proposed that the local cytokine response in the CNS is geared towards the humoral but not the cellular response (Messelingh and Griffin, 1994).

The immune response might sometimes promote the persistent state. It has been argued that antibody binding to virus proteins at the cell surface can increase the turnover rate of these proteins from the cell surface (antibody-induced antigenic modulation), or reduce the levels of other intracellular viral proteins, thereby increasing the chance of establishment of persistent infection (Joseph & Oldstone, 1975, Oldstone & Tischon, 1978, Oldstone & Fujinami, 1982, Fujinami & Oldstone, 1979, 1980). It is important to note that members of the paramyxoviridae have not been shown to encode proteins specialised in obstruction of the immune response. Such proteins have been shown to be produced by adeno-, pox- and herpesviruses and mediate their effects through interactions with proteins which are essential for the immune response (Smith, 1994, Marrack & Kappler, 1994). Nevertheless, infection by measles virus, a member of the genus morbillivirus, can lead to a downregulation of the expression of MHC class I and II molecules (Rager-Zisman et al., 1981, Leopardi et al., 1993).

The main epidemiological consequence of a persistent infection is the increased time an individual remains infectious and thus able to infect other individuals. The possibility also exists, for viruses that do not provoke long lasting immunity, that the immune response will be prolonged in a persistent

infection, thus rendering the individual less susceptible to subsequent infections (Randall & Russel, 1991). Human parainfluenza virus type 3 (HPIV-3) and respiratory syncytial virus (HRSV) are ubiquitous viruses, infecting children early in life and causing a transient illness with recovery after 2 to 8 days. The protective immunity is short lived and individuals can be repeatedly infected (Knight, 1973, Wright, 1984, Belshe et al., 1984). It has been shown that these viruses can persist in some individuals and can be shed during long periods (Muchmore et al., 1981).

The most important chronic disease clearly associated with a paramyxovirus is subacute sclerosing panencephalitis, SSPE, caused by measles virus. It is usually manifested 5-10 years after the primary infection, is characterised by extensive inflammation and limited demyelination of the brain, and always leads to death (ter Meulen et al. 1987). The matrix protein has been shown to be missing or functionally altered in viruses isolated from SSPE cases, but functional alterations are also frequently noted in the haemagglutinin and fusion proteins (Billeter & Cattaneo 1991). Other chronic diseases which are suspected to be associated with paramyxovirus infections are Paget's bone disease, autoimmune chronic active hepatitis and multiple sclerosis (Randall & Russel 1991). Mumps virus commonly infects the CNS and causes meningitis quite frequently (Wolinsky, 1996). Persistent infection can be established in neuronal cell culture (Löve et al., 1987) and mumps virus may, on rare occasions, persist within the brains of humans, causing progressive CNS disease (Vaheri et al., 1982).

There are some aspects worth considering concerning the role of DI particles in persistence *in vivo*. It has been shown that DI particles of Sendai virus contribute to an increased instability of the matrix protein (Tuffereau & Roux, 1988), a feature which has been observed in virus isolated from some SSPE cases. Furthermore, DI particles of Sendai virus also promote an increased turnover from the cell surface of the haemagglutinin-neuraminidase protein (Roux & Waldvogel, 1983), the major viral antigen involved in eliciting the humoral immune response. Recently, DI particles have been shown to be present in brains from SSPE cases (Sidhu et al., 1994).

The possible involvement of the V protein in persistent infection *in vivo* has also been addressed, and editing and presence of the V protein in SSPE cases have been shown (Gombart et al., 1992).

Previous investigations

Epidemiology and clinical features

The first outbreak of blue eye disease in pigs occurred in 1980 in central Mexico and was characterised by encephalitis, pneumonia and corneal opacity. It was observed in a commercial farm housing 2500 sows in La Piedad, Michoacan (Stephano et al., 1988). From 1980 to 1988 numerous outbreaks occurred in Michoacan and the neighbouring states. The main centre of disease prevalence remained in the States of Michoacan, Jalisco and Guanajuato, areas in which the pig population is very dense. Outbreaks still occur, which makes the development of vaccines and improved diagnostics a priority.

The disease has only been confirmed in pigs, but mice succumb to infection following intra cerebral inoculation, exhibiting CNS disorders. Subclinically infected pigs are the main source of the disease, and the virus spreads horizontally by human contacts and fomites. The disease is self limiting in closed herds, and antibody titers established after acute infection generally persist throughout the lifetime of exposed pigs. The clinical signs are variable depending on the age of the pig. Piglet morbidity and mortality is between 20-50% and 87-90%, respectively. Piglets 2 to 15 days of age are most susceptible, and usually succumb to nervous system disorders within 48 hours after onset of clinical signs. In 1 to 10% of infected piglets corneal opacity can be seen, frequently as the only clinical sign. Mortality is low in pigs older than 30 days, and nervous system disorders are scarce, a respiratory illness being more common. Sows may return to oestrus more frequently, or experience abortions, stillbirths or mummified foetuses. Boars can develop orchitis, epidymitis and testicular atrophy (Stephano et al., 1988).

Isolation and characterisation of the porcine rubulavirus

A haemagglutinating virus was isolated from the brain of a suckling pig showing typical clinical symptoms. Necropsy revealed microscopic lesions characteristic of acute encephalomyelitis. The virus was named La Piedad Michoacan Mexico virus (LPMV) after the town and state where it first emerged (Moreno-López et al., 1986). Electron microscopy indicated a resemblance of the virus to paramyxoviridae (Fig. 2), but no serological relatedness was found to other paramyxoviruses (Moreno-López et al., 1986). Transmission studies showed that the virus produced clinical symptoms and pathological changes similar to those seen in natural outbreaks (Stephano et al., 1988).

The virus was shown to readily infect cell cultures of many animal species, and gave rise to high infectious titers. The morphological changes associated with CPE were vacuolation of cells and extensive formation of syncytia

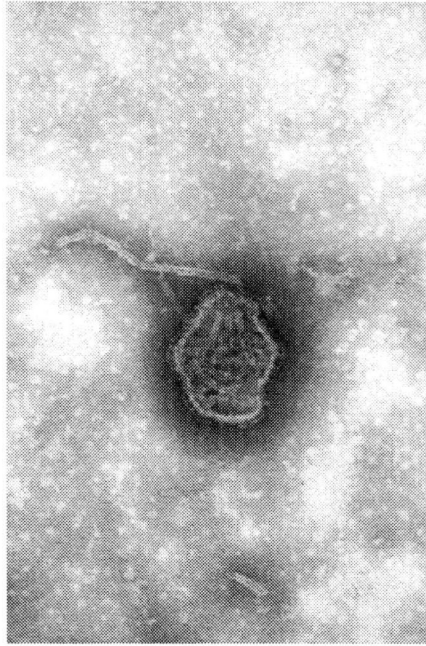

Fig. 2. Electronmicrograph of purified LPMV virion, demonstrating the viral particle with the nucleocapsid structure. From Moreno-López et al., (1986).

(findings from studies performed mainly on PK-15 cells). Haemagglutination could be observed with all types of erythrocytes tested. Haemadsorption was observed in all cell cultures infected (Moreno-López et al., 1986). SDS-PAGE analysis revealed six structural proteins (Sundqvist et al., 1990). Analysis of nucleocapsids isolated from virus suspension revealed one band of 68 kD corresponding to the nucleoprotein. Two glycosylated proteins with molecular weights of 66 and 59 kD were identified as the HN and F proteins, respectively. One protein of 52 kD, corresponding to the phosphoprotein (P) was identified after labelling with [^{32}P] orthophosphate. The remaining two proteins were identified as the L and M proteins. This characterisation was based on their sizes, 200 and 40 kD, respectively (Sundqvist et al., 1990).

Sequence analysis of the genome determined the gene order to be NP-P-M-F-HN-L, similar to other paramyxoviridae. The complete sequences of the M, F and HN genes were obtained, being 1376, 1845 and 1728 nucleotides, respectively (Berg et al., 1991, 1997, Sundqvist et al., 1992). Their ORFs code for polypeptides of 369, 541 and 576 amino acids with predicted molecular weights of 42, 58 and 63 kD, respectively. Estimation of the molecular weights by SDS-PAGE gave 59 kD for the F_1 subunit and 12 kD for the F_2 subunit. This adds up to 71 kD, and leaves a deficite of 13kD when compared with the estimate of 58kD calculated from the primary sequence. This difference can

probably be attributed to the utilisation of the five glycosylation sites, four on F_1 and one on F_2. The difference between empirically determined and predicted size of the HN protein could also be attributed to glycosylation. Amino acid comparisons of the M, F and HN proteins with the corresponding proteins of other members of the paramyxoviridae family revealed that mumps virus and simian virus type 5 were the closest relatives to LPMV, the amino acid identities being 46%, 42% and 41% to mumps and 36%, 45% and 43% to SV-5. An open reading frame corresponding to the small hydrophobic (SH) protein described in both mumps virus and SV-5 (Hiebert et al., 1985, Elango et al., 1989) was demonstrated to be absent in LPMV.

Recently, a sequential study of virus distribution in tissues and body fluids and virus excretion following experimental inoculation of pigs with LPMV by a natural route was performed (Allan et al., 1996). The results confirmed field observations. All pigs infected at three days of age exhibited severe neurological signs prior to death. In, contrast, only 30% of pigs infected at 17 days of age showed mild respiratory and neurological signs, and 30 day old pigs showed no signs at all. The respiratory tract and tonsils were identified as the sites of primary replication and only a low or transient viremia could be shown. The distribution of virus in different tissues was shown to be very localised. Excretion of virus occurred mainly via the respiratory tract and urine. The localisation of virus in the brain indicated an age dependent mode of spread. In 17 day old pigs primary replication was observed in the nasal mucosa with subsequent spread to the olfactory bulb and midbrain via the trigeminal and olfactory nerves. In contrast, 3 day old pigs showed a dual mode of spread to the brain. After primary replication in the respiratory tract and tonsils LPMV spread to all parts of the brain via the trigeminal and olfactory nerves. Viremia leading to passage over immature blood brain barrier was also seen in these animals.

Aims of the study

The aims of this thesis work have been to:

1) further characterise LPMV at the genetical and molecular levels by sequence analysis of the gene encoding the phosphoprotein (P) and evaluation of the relationship of LPMV to other members of the family paramyxoviridae on the basis of the size of the P gene open reading frame and transcriptional properties (editing) of this gene.

2) evaluate whether LPMV can establish persistent infection in cell culture as has been shown for almost all members of the family paramyxoviridae, and to study possible mechanisms involved in persistence at the protein and RNA levels. The existence of defective interfering particles and the editing of the P gene has been specially highlighted.

3) study whether LPMV can persist in pigs for durations longer than would be expected after acute infection and recovery. Immunosuppression as a possibility of reactivation of persistent virus was also evaluated.

Present investigations

The P gene (I)

The P gene encodes a protein which together with the L protein constitues the viral polymerase complex. The P gene utilises a novel and intriguing mechanism in order to code for several polypeptides (Vidal et al., 1990b). Basically, two forms of messenger RNAs have been described. One is a faithful copy of the gene while the other contains additional nontemplated G residues. This addition of extra nucleotides fuses two different reading frames enabling the expression of additional polypeptides. By virtue of this strategy a nonstructural polypeptide designated V can be expressed by a portion of the P transcripts. Members of the different genera differ in the specificities of the ORFs created by editing. All viruses in paramyxovirus and morbillivirus genera encode the P protein from the faithful copy of the gene (Cattaneo et al., 1989, Vidal et al., 1990a, Galinski et al., 1992) whereas the opposite is true for viruses belonging to the genus rubulavirus except for NDV (Thomas et al., 1988, Paterson & Lamb, 1990, Steward et al., 1995). Thus, organisation of the P gene can be used to establish relationships between different viruses of the paramyxoviridae.

The complete P gene of LPMV was sequenced and shown to consist of 1373 nucleotides. The gene is divided in two large open reading frames which overlap in the middle of the gene. The open reading frame starting from the first AUG in the P gene at position 87 corresponds to a protein designated V. Expression of the P protein, which requires an open reading frame spanning most of the P gene, necessitates the addition of two G residues which fuse the two open reading frames, as has been described for members of the rubulavirus genus, including mumps virus and SV5 (Paterson & Lamb 1990, Thomas et al., 1988). The long open reading frame thus created encodes a 404 amino acid long protein with an estimated weight of around 42 kD. The 10kD difference from the empirically determined molecular weight (52kD) can probably be attributed to extensive phosphorylation. The C-terminal region appears to be the most highly conserved part of the protein.

The V protein of LPMV, expressed by unedited mRNA, is 249 amino acids long, 168 which are shared with the P protein at the N-terminus. The C-terminal region of V is significantly conserved within the paramyxoviridae, with an amino acid identity of 60% and 56% on comparison with mumps and SV5, respectively. Within this region eight cystein residues can be found, seven of which are conserved in all V proteins. This region displays similarities to zinc fingers motifs found in DNA or protein interacting regions of other proteins (Klug & Schwabe, 1995), and has also been shown to bind Zinc (Paterson et al., 1995, Liston & Briedis, 1994).

A conserved site, UUUAAGAGGGGGG (in the plus sense), was identified at positions 579 to 591. This corresponds to an editing site. The editing of the

P gene was verified by primer extension analysis of RT-PCR products from P gene specific transcripts. Both unedited (V) and edited (two extra residues corresponding to P) transcripts were found in equimolar amounts at 24 h post infection.

An interesting fact was the observation of an ORF in the 5' end of P or V transcripts corresponding to a C protein, which previously has been described for the genera paramyxovirus and morbillivirus, but not for the genus rubulavirus.

The results of this study confirmed the relationship of LPMV to mumps virus and SV-5, which previously has been shown by sequence homology studies. The organisation of the gene expressing either the P or the V protein offers a fascinating way of modulating viral expression as it has been shown that the V protein can inhibit viral RNA synthesis in Sendai virus infection (Curran et al., 1991b), presumably by inhibiting the recruitment of NP to the nascent strand during RNA synthesis (Horikami et al., 1996). This strategy can have profound effects on many aspects of the replicative cycle, and may be of importance in establishing persistent infections.

Porcine rubulavirus *in vitro* persistence (II and III)

Persistent infections *in vitro* can probably be established by all viruses of the family paramyxoviridae. Many mechanisms have been associated with such infections, the most frequently described involving the presence of defective interfering particles.

An LPMV persistent infection was established in PK-15 by infecting cells at high m.o.i. and subculturing the surviving cells in most cases a subpopulation consisting of less than 5% of the original number of infected cells. The cells were analysed at passage 25, when they had started to grow as a stable monolayer, and at passage 65 (after almost a year in continuous culture). The persistently infected (PI) cells exhibited a reduced growth rate, vacuolisation and syncytium formation, the latter being more pronounced in the early passage. No significant lysis was observed when the cells were superinfected with lytic porcine rubulavirus, but cells were lysed rapidly upon infection with vesicular stomatitis virus (VSV), indicating homotypic interference and no contribution by interferon (IFN) to the persistent state. Measurement of the IFN level in the medium further confirmed it´s absence in PI cells, and demonstrated the presence of a very low concentration in lytically infected cells. Immunoflurescence studies of the expression of NP and HN proteins indicated that all PI cells were infected. The HN immunofluorescence was rather weak and haemadsorption gave different results depending on the extent of confluence, i.e. less than 100% of the cells in a confluent monolayer generally adsorbed erythrocytes. But up to 100% could be shown to adsorb erythrocytes in non-confluent monolayers.

Infectious titers of virus produced by the PI cells were 3 logs lower than those produced after infection of cells by lytic virus. The haemagglutination (HA) titer showed only an 8 fold reduction however, indicating the presence of many non infectious viral particles. Virus purified from the PI cells failed to lyse PK-15 cells when added at an m.o.i. comparable to that at which lytic virus readily lyses these cells. Furthermore, immunofluorescence staining of the infected monolayer (at low m.o.i.), using the NP monoclonal 32.11, showed that spread of PI virus was limited after two days, whereas lytic virus added at the same m.o.i. had spread to all cells within the cell monolayer at this time.

Analysis of viral proteins, both in infected cells and virus released from these, revealed that lower amounts were produced in the PI cells. The NP, P, M, F and HN were detected in both cells and virus particles. However, the L protein was not detected in PI cells at any passage, but was readily detected in lytically infected cells under conditions where NP, M, F and HN were equally labelled in lytically and persistently infected cells. A certain reduction in the relative amount of the P protein was also seen in the PI cells.

Northern blots of the PI RNA identified the NP, P, M, F and HN transcripts, which migrated in the same manner as their lytically produced counterparts. The L transcript of the PI cells was of low abundance, and the amount in relation to the genome was reduced in PI cells compared to lytically infected

cells. Bicistronic messengers were evident in both lytically and persistently infected cells at comparable levels. Neither the HN-L transcript or the P-M transcript was seen in either lytically or persistently infected cells. Further analysis of the transcripts of persistently infected cells showed that the gradient between different transcripts was similar to that seen in lytic infection with the exception of the L mRNA. This mRNA was clearly present in lower amounts relative to the other transcripts in the persistently infected cells.

The PI cells also contained several subgenomic particles (sg). Three of these were identified as putative copy-back RNAs (sg2, a 2 kb RNA in PI cells of passage 65, sg4, a 7.5 kb RNA in PI cells of passage 25 and sg7, an 11 kb RNA in PI cells of passage 65). Sg4 and sg7 are unusually large copy-back RNAs and, since the non translated 3´and 5´ends of the LPMV genome have not been sequenced, no probes can be made against them. This makes a definite characterisation of these sg RNAs impossible. One probable copy back of 1.9 kb length (sg1) is also found in lytically infected cells. Sg1 and sg4 do not seem to accumulate since they are missing from later passages. Subgenomic RNAs that were interpreted as internally deleted RNAs seemed more stable, and accumulated proportionally with the number of passages, as seen with sg5 and sg6 of PI cells of passage 25. These were still seen to be present at passage 65. Furthermore, passage 65 PI cells contained another new internally deleted RNA (sg3).

As northern blotting does not discriminate between P gene transcripts encoding P or V, the frequency of editing of mRNA was investigated by both primer extension analysis and cloning and sequencing of PCR products from mRNA. The mRNA was prepared from persistently or lytically infected cells, or brain cells from an experimentally infected pig. Primer extension analysis confirmed the roughly equal distribution of P and V transcripts previously described in lytically infected cells described before (Berg et al., 1992). In contrast, V transcripts were more frequent than P transcripts (2:1) in PI cells of both passages. A slightly different result was obtained by cloning and sequencing. Now, the ratio V/P transcripts was already 2:1 in lytically infected cells. However, an even greater shift in the ratio was revealed in PI cells (5:1), confirming the increased amount of V mRNA. The distribution of P and V transcripts in the experimentally infected pig, as determined by cloning and sequencing, was identical to that seen in lytically infected cells. Differences between cloning/sequencing and primer extension analysis have been described by Vanchiere et al. (1995), who endorsed the reliability of primer extension as a method to quantify the actual distribution of different transcripts.

This study confirms the ability of LPMV to cause persistent infections *in vitro* and identifies several differences compared to lytic infections. Each of these features unique to the PI state theoretically has the potential of being important for the establishment of persistence. Similar features could prevail in an *in vivo* situation though immune pressure complicates the picture.

Porcine rubulavirus *in vivo* persistence (IV)

Viruses of the paramyxoviridae do not rely on the possibility of remaining in an infected host for a long time before being transmitted to the next individual. These viruses are in constant circulation and require large populations (in the order of 200 000 individuals for measles and mumps viruses) in order to survive (Wolinsky, 1996). However, they still have the ability to remain for long periods in a host after infection. The epidemiological importance of persistent infections is questionable but one well described example is that of an outbreak of an human parainfluenza virus type 3 initiated by an apparently persistently infected individual (Randall & Russel, 1991).

The possibility of LPMV establishing persisting infections *in vivo* was evaluated in three pigs experimentally infected at 17 days of age.

It has been shown that pigs infected at this age show mild clinical symptoms, with virus being detectable in various tissues between 3 and 14 days post infection. After this period, no virus production can be detected and the pigs recover (Allan et al., 1996).

Three pigs were infected, allowed to recover and were sacrificed at 53 days post infection. Two of them were immunosuppressed with cyclophosphamide (CPA) at day 49 post infection. The convalescent immunosuppressed pigs showed signs of active inflammation involving leptomeninges and brain tissues (meningoencephalitis). These pathological lesions were similar to those seen in the control, a pig sacrificed during the acute phase of LPMV infection. Areas of the brain which were seen to be affected included the olfactory system and adjacent frontal lobe, midbrain and pons. Perivenous lymphocytic cuffing, neuronophagia and microglial nodule formation was evident. The inflammatory signs were most prominent in nasal submucosal tissue and in the adjacent olfactory area in the acutely infected pig, whereas brain stem structures were more affected in convalescent immunosuppressed pigs.

Virus isolation and immunofluorescence staining of cryostat sections from a number of different tissues including brain, respiratory, circulatory, reproductive and digestive systems, eye, thymus, spleen, bone marrow, liver, kidney and muscle, failed to indicate the presence of any LPM virus or antigen in the convalescent pigs.

Midbrain, forebrain and lung tissues were chosen for detection of LPMV RNA. The brain tissues were chosen because of the CNS tropism previously shown for LPMV (Allan et al., 1996) and because of properties of central nervous tissue makes it suitable for harboring persistent infections (Wharton & Nash, 1993). Furthermore, diffuse lesions have been observed in these regions (Kennedy S., personal communication). The lung was chosen because it is an organ in which viral replication leads to shedding.

Both NP and P gene specific nested RT-PCR were developed and optimised. The sensitivity of the nested NP RT-PCR was titrated using defined amounts of *in vitro* transcribed NP RNA. It was concluded that the detection limit was somewhere between 100 and 1000 RNA copies. The sensitivity of the

P gene specific RT-PCR was not determined but it was found empirically to be somewhat more sensitive than the NP RT-PCR

Tissues from the acutely infected pig contained both NP and P specific RNAs. The convalescent pig which was not treated with CPA was negative in the PCR specific for the NP gene, but positive in mid brain using the PCR specific for the P gene. Both of the CPA treated pigs were clearly positive by NP- and P RT-PCR in mid brain and fore brain. One of them was also weakly positive in the lung. A differentiation between the genomic RNA and transcripts could be made by the selective usage of primers in the reverse transcriptase step. The primer P347+ selectively binds to the negative sense genome and oligo (dT) binds to the poly (A) tail of the transcripts. The results obtained clearly indicated the presence of both genome and transcripts in all positive tissues, except in samples from the convalescent pig which was not treated with CPA which was not positive for genomic RNA.

Transcription was also analysed in terms of editing. Using the primer extension assay described by Berg et al., (1992) it was shown that the genomic specific PCR product only contained 6G as expected. With oligo (dT) selected material both 6G and 8G containing products corresponding to the V and P transcripts were identified in all tissues and animals except for the convalescent pig not treated with CPA, in which only P transcripts could be seen.

Messenger RNA was isolated from tissues and used in primer extension assays confirming the results obtained with total RNA. This assay, however, only detected V transcripts in the convalescent pig which was not treated with CPA. This finding, and the difference between the sensitivities of the NP and P specific PCR assays, suggests that the target molecules are present at the lower detection limit of these methods.

The results of this study indicate that LPMV RNA can persist in pigs for periods longer than those which could be expected after an acute infection. The choice of midbrain for use in this study was influenced by the fact that this tissue has been shown to produce the highest titers of LPMV virus during the acute phase of infection. From an epidemiological point of view assays of other organs such as tonsils, lungs and kidneys might be more informative. In this respect it is noteworthy that LPMV RNA could be detected in lung tissue after immunosuppression.

Discussion

Since the closest phylogenetic relatives of LPMV have been shown to be mumps virus and SV-5 (Berg et al., 1991, Sundqvist et al., 1992), the P gene of LPMV was presumed to be organised in a similar way to the P gene of these viruses. Mumps virus, SV-5 and human parainfluenza virus types 2, 4a and 4b all have the P gene organised in a similar way. Two ORFs, of almost equal length, overlap in the middle of the gene. The P protein is encoded by a transcript in which the two ORFs are fused via the nontemplated addition of two G nucleotides at a specific site, the editing site (Thomas et al., 1988, Elliot et al., 1990, Kondo et al., 1990, Ohgimoto et al., 1990, Paterson & Lamb, 1990, Southern et al., 1990).

As expected, the P gene of LPMV does have an organisation similar to other rubulaviruses. Two overlapping reading frames of approximately equal length were found, as well as a putative editing site, UUUAAGAGGGGGG. Analysis of the two reading frames indicated that the addition of 2 extra nucleotides would fuse the reading frames. The difference in the estimated and true molecular weigths of the P protein (Sundqvist et al., 1990) is probably due to extensive phosphorylation. The phosphorylation sites of the Sendai virus P protein are located in the N-terminal region (Vidal et al., 1988). This part of the protein is thought to interact with soluble forms of the NP (Curran et al., 1995) but also to contain a region important for RNA synthesis per se (Curran et al., 1994). The less well conserved C-terminal region of the Sendai virus P protein interacts with the L protein (Smallwood et al., 1994), soluble NP (Curran et al., 1995) and nucleocapsids (Ryan and Kingsbury, 1988, Ryan & Portner, 1990, Ryan et al., 1991).

The V protein shares a common N-terminus end with the P protein, and is therefore probably also phosphorylated. It has been shown that the Sendai virus V protein has an inhibitory effect on viral RNA synthesis (Curran et al., 1991b), probably through competion with the P protein for binding to the nucleoprotein (Horikami et al., 1996). Other possible activities of the V protein are currently not well understood. The C-terminal V specific end is well conserved within the rubulaviruses. This region is rich in cystein residues, and is organised similarly to zinc finger motifs of transcription factors (Klug & Schwabe, 1995). It has been shown that the V proteins of SV-5 and measles virus bind two atoms of zinc (Paterson et al., 1995, Liston & Briedis, 1994). The importance of the V protein in paramyxoviridae infections is highlighted by it´s high conservation and by the fact that most paramyxoviridae express it. Only HPIV-1 and HPIV-3 contain dysfunctional V open reading frames, with multiple stop codons abolishing their translation (Matsuoka et al., 1991, Galinski et al., 1992). It has been proposed that the V protein is an accessory protein in cell culture. This conjecture is based upon the findings that neither ablation or overexpression of Sendai or measles V protein have any effect on cultured cells (Delenda et al., 1997, Schneider et al., 1997). The human

parainfluenza type 2 V protein has been shown to be localized in the nucleus, indicating a possible interaction with the expression of host cell genes (Watanabe et al., 1996). Recently, interactions between measles and SV-5 proteins and some unknown cellular proteins of 17, 38 and 60 or 150kD, respectively, was reported (Liston et al., 1995, Precious et al., 1995).

Editing in LPMV was demonstrated by primer extension analyses, as described by Pelet et al. (1991). Both mRNA and RT-PCR amplified DNA extending over the editing site were used as template. The frequency of editing was estimated at approximately 50%. Editing of the P gene is a prerequisite for viral propagation of rubulaviruses since the faithful mRNA copy encodes the V protein, and the P protein, which is essential for virus replication is encoded by the edited transcript. Another ORF, which is absent in other rubulaviruses, is located at the 5' end of the P or V transcript of LPMV. It encodes a putative protein rich in leucines and positively charged amino acids, which resembles the C protein of Sendai virus. C proteins have some similarities to a class of nucleic acid binding proteins known as leucine zippers, which also may mediate protein-protein interactions (Landschultz et al., 1988). The C proteins of Sendai virus have been found to be associated to nucleocapsids (Yamada et al., 1991) and to inhibit viral transcription (Curran et al., 1992) and replication of some viral RNAs (Cadd et al., 1996). The existence of a C protein in LPMV is surprising since no other virus in the genus rubulavirus encode such a protein. All other common characteristics of the genus rubulavirus are however shared by LPMV. The C open reading frame has been confirmed by several unrelated cloning experiments.

A considerable amount of work has been done on the characterisation of persistent infections, both *in vivo* and *in vitro*, but mechanisms leading to the establishment and maintenance of persistence still remain fairly unknown. All members of the paramyxoviridae are probably capable of establishing persistent infections *in vitro*. DI particles, which are subgenomic RNAs that require help from but also compete with the wild type genome, occur in any high titer viral stock due to the high error frequency of the viral RNA polymerase (Re, 1991). The effects of DI particles were first described in 1952 by von Magnus, who showed a cyclic rise and fall in virus titers, when influenza virus was repeatedly passaged by the inoculation of undiluted virus stock in embryonated hens eggs. It is thought that the simple competition between subgenomic and wild type genomes and the subsequent reduction in expression of the virus could be enough to maintain a persistent state.

The persistent LPMV infection induced in PK-15 cells was the result of inoculation of cell cultures with a high multiplicity of infection (m.o.i.) and subcultivation of surviving cells. This process probably involves the selection of cells and/or viruses which support persistent infections. Cells were analysed at two passages. Passage 25 was chosen because it was the first passage at which the cells were seen to grow as a complete monolayer, thus representing the primary establishment of persistent infection. Passage 65 was chosen to exemplify a stably maintained persistent infection

Cells must meet some specific criteria to be defined as persistently infected. The cells resisted challenge with LPMV virus, despite the lack of detectable interferon production. The PI cells were furthermore not cured by growth for a long time in the presence of a neutralising monoclonal antibody against the HN. This rules out the possibility of a low grade lytic infection in a portion of the cells. Furthermore, immunofluorescence and haemadsorption studies indicated that all cells were infected.

The involvement of subgenomic RNAs could be suspected, and these were subsequently identified in both early and late persistently infected cells. Several unusually large subgenomic RNAs were identified. Hybridisation analyses with probes against each viral gene identified both copy-backs and internally deleted RNAs. Competition studies usually results in the internally deleted RNAs being eliminated by the copy-backs (Re, 1991). In this case however, it seems likely that subgenomic RNAs interpreted as being internally deleted RNAs prevail at the expense of the copy-backs. In cases where internally deleted RNAs have been shown to eliminate copy-backs they have been demonstrated to be transcriptionally inert (Re 1991). The study by Garcin et al. (1994), in which a large transcriptionally active internally deleted Sendai virus RNA resulted from an infection with the small copy-back DH4, indicates that this rule does not always apply.

Transcription gradients indicate that the L transcript is present at lower amounts in the persistently infected cells than in lytically infected cells when compared to other viral transcripts within each type of infection. This could argue that the internally deleted RNAs are in fact transcriptionally active since the decrease in L transcripts coincides well with the fact that most deletions seem to have occurred in the L gene. The lack of any abberrant transcripts requires that the deletions must be close to the start of the genes, i.e. the HN and L genes, respectively. If they are not being transcribed then another mechanism has to explain the reduction in numbers of L transcripts. The L transcripts of the PI cells might be more unstable or the polymerase complex might be more prone not to reinitiate at the L gene, either due to alterations in the surrounding sequence or in any of the polymerase components. The reduction in the L transcripts is mirrored in the amount of L protein seen. No L protein could be detected in the PI cells, either intracellularly or in virions, compared to lytically infected cells. Interestingly, a small reduction in the amount of P protein was observed. This was not reflected in the amount of P transcript seen, but in the editing frequency, which was characterised by an increased number of V transcripts. Taken together, both the reduced expression of the L and P proteins and the presence of subgenomic RNAs in the persistently infected cells could have the same result. The introduction of subgenomic RNAs probably interfere with the replication and transcription of the virus. This will be further exacerbated by the relative scarcity of viral polymerase resulting from the reduction in amount of P and L. Finally, although not identified in this study, an increase in the V protein, which is

known to inhibit viral RNA synthesis (Curran et al., 1991b), would also have an inhibitory effect on the propagation.

The possibility of LPMV remaining in convalescent pigs was investigated after experimental infection. Three pigs infected at 17 days of age (an age at which the disease is characterised by mild respiratory distress followed by recovery from the infection) were analysed for the presence of viral particles, antigens and RNA at 53 days post infection. Two of the pigs were immunosuppressed at 49 days post infection. These individuals showed signs of active inflammation in the brain (meningoencephalitis) similar to those seen in an acutely infected pig, indicative of a viral infection. They were shown to harbor LPMV RNA in brain tissue and one of them was also weakly positive for LPMV RNA in the lung. Without immunosuppression, no viral RNA could be detected, except for one pig which tested weakly positive for P gene RNA in the midbrain. No infectious virus could be isolated from any of the convalescent pigs, although it must be said that the duration of immunosuppression could have been too short for any virus to appear extracellularly.

By selective primer usage and primer extension assays (Berg et al., 1992) both P and V specific transcripts were identified, although the limited amount of material made determination of the editing frequency impossible. The presence of V protein could be of importance in viral persistence (Gombart et al., 1992), because of it's negative effect on RNA synthesis (Curran et al., 1991b).

These results clearly demonstrated that LPMV RNA can persist for a relatively long time in a convalescent pig, but it's detection generally requires some stimulation, i.e. immunosuppression. Similar persistence of viral RNA in hosts for a long period after acute infection has recently been shown, both for members of the paramyxoviridae (Mori et al., 1995), and the family rhabdoviridae families (Letchworth et al., 1996, Barrera et al., 1996). This indicates that persistence might be a common feature. This study indicates that the virus may persist in different tissues/cells. In the brain, neurons could be considered a good target for persistent infection since their long life implies that virtually no virus replication is needed and their low expression of MHC class I molecules makes them suitable cells in which to escape immune surveillance. LPMV has been shown to infect neurons (S. Kennedy, personal communication). The detection of LPMV RNA in the lung of one of the immunosuppressed pigs at a time when no infectious virus could be isolated from any other organ or serum indicates that at least the RNA can persist in cells outside the CNS. It is known that older pigs in badly managed herds are affected with graver symptoms upon infection with LPMV than do pigs in well managed herds (Stephano et al., 1988) indicating the importance of the immune system in limiting pathological lesions. It is possible that recovered pigs still harboring the virus could start to shed virus when immunosuppressed.

Concluding remarks

Porcine rubulavirus (LPMV) is the causative agent of "blue eye disease" in pigs. The virus has different effects on pigs depending on their age at the time of infection. Young piglets are most vulnerable, showing signs of pneumonia, encephalitis and nervous disorders. Frequently, corneal opacity is also observed, and is sometimes the only sign of infection. Sows are mainly afflicted with reproductive disorders such as abortions, stillbirths and mummified fetuses. Boars can develop orchitis, epidymitis and testicular atrophy.

LPMV has been classifed as a rubulavirus based on sequence analysis and the existence of both haemagglutinin and neuraminidase activities. Paper I in this thesis further confirms this by analysis of the P gene. Insertion of nontemplated G residues (editing) in a portion of the transcripts changes the reading frame and allows several different N-coterminal polypeptides to be synthesised. The organisation and expression of the P gene differs markedly between the genus rubulavirus and genera paramyxovirus and morbillivirus. The rubulaviruses, including LPMV, encode the P protein on an edited transcript, whereas paramyxo- and morbilliviruses express it from an unedited transcript. A surprising feature of LPMV is the finding that an open reading frame encoding a putative C protein was intact. This has been proved not to be the case for the other rubulaviruses.

The first outbreak of "blue eye disease" occured in 1980. Numerous new outbreaks occured in the follwing years, causing great economical problems to the swine industry. The disease still prevails today, but outbreaks are less common than before. Pigs which are introduced into farms with a prior history of infection sometimes develop the disease, indicating that subclinically or persistently infected pigs might serve as a reservoir for the virus. Orchitis and epidymitis are the most usual signs today (Stephano, personal communication). This observation has led us to investigate whether LPMV is able to establish persistent infections.

By establishing persistent infections in porcine kidney cells it was shown that LPMV could assume a non-lytic mode of infection. The presence of subgenomic RNAs as well as reduced expression of the components of the viral polymerase was noted. Both of these changes could lead to a reduction in viral replication/transcription, and subsequently be of importance to the persistent state.

It was further shown that LPMV RNA can remain in convalescent pigs for a relatively long duration after recovery from the disease. Viral RNA, but not infectious particles, was identified in forebrain, midbrain and lung of pigs that were chemically immunosuppressed for four days prior to sacrifice at 53 days

post infection. These data clearly show that individuals can retain LPMV RNA for a considerable time after infection. Animals such as these might play an epidemiologically important role if they started to shed virus during extended periods of immunosuppression induced by stress. In view of this, further studies of the disribution of LPMV in tissues of the upper-, and lower respiratory tract as well as in the kidney are needed.

Finally, the LPMV-pig system might offer a useful model system for paramyxovirus CNS invasion and tropism. Especially interesting in that respect is the close phylogenetic relationship to mumps virus.

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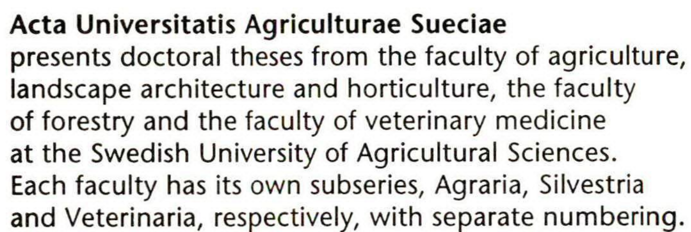

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