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Recombinant Vaccines Against Infectious Pancreatic Necrosis Virus

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, Trinity College, Dublin.

Department of Microbiology.....March 2000

Presented by Bronagh M. McKenna

FOR MY PARENTS

DECLARATION

This thesis represents my own unaided work unless otherwise stated and has not been submitted for a degree at this or any other university. I agree that this thesis may be lent or copied at the discretion of the librarian, Trinity College, Dublin, Ireland.

Signed  Bronagh Mary McKenna

Dated March 2000

Publications and presentations.

Publication:

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Presentations:

Expression of infectious pancreatic necrosis viral antigens using the Semliki Forest virus (SFV) vector system. **McKenna, B.**, Fitzpatrick, R., Vaughan, L. & Atkins G. Moyne Institute of preventive medicine, Trinity College, Dublin. Oral presentation at the Society for General Microbiology meeting, Nottingham, April 1998.

Recombinant vaccines against infectious pancreatic necrosis. **McKenna, B.**, Fitzpatrick, R., Vaughan, L. & Atkins G., Phenix, K., Todd, D., Reitan, L., Ramstad, A., Lillehaug, A. & Liljestrom, P. Poster presentation at the European Association of Fish Pathologists 8th international conference, "Diseases of fish and shellfish", Heriot-Watt University, Edinburgh, September 1997.

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Summary

The rapid expansion of the aquaculture industry in recent years has led to an increase in disease outbreaks. Over the last few years bacterial disease problems have been brought under control, largely due to the use of a new generation of oil-adjuvanted vaccines. Vaccine development for viral diseases such as infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus and infectious haematopoietic virus has proven to be more difficult. IPNV is the most important infectious disease in the Norwegian aquaculture industry. Recent reports estimate that IPNV is responsible for the loss of approximately 5% of the Atlantic salmon smolts transferred to sea. To date vaccine research has focused on the use of inactivated virus, live-attenuated strains, or recombinant sub-unit vaccines. The traditional vaccines based on live IPNV are not deemed acceptable to the aquaculture industry because of their safety, environmental and economic disadvantages. Current methods of vaccine development have turned to recombinant DNA technology. It is hoped that this technology may provide efficient and inexpensive vaccines.

A consortium project was established in 1996 to evaluate novel systems for the expression of IPNV antigens in fish cell culture and to assess the efficacy of any candidate recombinant vaccines produced.

A Norwegian field isolate was chosen for the study. The A-segment of the RNA genome was cloned and sequenced. The isolate was found to be 99% identical to the Sp strain of IPNV, which is reported to be the predominant serotype in Norway.

One of the newest approaches in fish vaccinology is DNA immunisation. Although the technique was developed for mammalian systems it has been shown to be applicable to fish. In this study expression of IPNV antigens from a DNA vector was evaluated. The potential of the Semliki Forest virus (SFV) expression vector as a delivery system in fish was also examined.

The SFV expression system was able to function in mammalian cells at 37°C, producing recombinant proteins which were recognised by conformation-dependent monoclonal antibodies. The expression system also functioned in fish cell lines, again producing a recombinant protein that was correctly folded. However, consortium partners found that the expression in fish cell lines was temperature dependent and no expression could be detected when fish were inoculated intramuscularly with expression constructs. It is therefore unlikely that the SFV system will function as a vaccine to salmonid fish.

The A-segment of the virus was also cloned into the DNA vector, pcDNA3 and into the layered DNA/RNA vector, pBKT-SFV. Both systems express recombinant IPNV A-segment but have not yet been evaluated in fish cell culture or included in a vaccine challenge.

Recombinant SFV particles expressing IPNV A-segment were also produced. Infection of a fish cell line with the recombinant particles resulted in the formation of IPNV virus-like particles (VLP). The VLP formed were similar in size and morphology to IPNV although they did not form characteristic crystalline arrays in the cytoplasm of the cells. Purified VLP may be an alternative vaccination strategy to combat IPNV as they are likely to induce an immune response similar to that of purified virus.

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Abbreviations

AMV - avian myeloblastosis virus

BHK – baby hamster kidney

bp – base pairs

BSA - bovine serum albumin

C - carboxy

CCVD – channel catfish virus disease

CHSE – Chinook salmon embryo

CMV – cytomegalo virus

cpe – cytopathic effect

cDNA – complimentary DNA

ddNTP – dideoxynucleoside triphosphates

DI – defective interfering particles

dNTP – deoxynucleoside triphosphates

dsRNA – double stranded ribonucleic acid

EEV – European eel virus

ELISA – enzyme linked immunosorbent assay

EPC – epithelioma papulosum cyprini

ER – endoplasmic reticulum

FCS – foetal calf serum

FITC – fluorescein isothiocyanate

GCHD – grass carp haemorrhage disease

GMP – guanosine monophosphate

hepes – N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid

HLA – human lymphotrophic antigen

HPLC – high pressure liquid chromatography

HRP – horseradish peroxidase

IBDV – infectious bursal disease virus

Ig – immunoglobin

IHN – infectious haematopoietic necrosis

IHNV – infectious haematopoietic necrosis virus

IPN – infectious pancreatic necrosis

IPNV – infectious pancreatic necrosis virus

IPTG – isopropyl-β-D-galactopyranoside

LPS – lipopolysaccharide

MAb – monoclonal antibody

MCS – multiple cloning site

MHC – major histocompatibility complex

N - amino

NOK – Norwegian krona

NP-40 – Nonidet P-40

NTR – nontranslated region

ORF – open reading frame

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PFU – plaque forming units

PHA - phytohaemagglutinin

PMSF – phenylmethylsulphonyl fluoride

RPS – relative percentage survival

r.p.m. – revolutions per minute

RNase – ribonuclease

RT-PCR – reverse transcriptase-polymerase chain reaction

S – Svedberg unit

SDS- sodium dodecylsulphate

SFV – Semliki Forest virus

SIN – sindbis virus

SIV – simian immunodeficiency virus

VEE – Venezuelan equine encephalitis

VHS – viral haemorrhagic septicaemia

VHSV – viral haemorrhagic septicaemia virus

VLP – virus like particles

X-gal – 5-bromo-4-chloro-3-indolyl-β-galactopyranoside

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Chapter One

Introduction

1.1 The Aquaculture Industry

Much of the expansion in annual global aquatic production is attributable to aquaculture, which is itself one of the fastest growing food producing sectors. In 1995 the total global production of cultured finfish, shellfish and aquatic plants was 27.8 million tonnes, providing US$42.3 billion in revenue. Since the early 1980's the commercial cultivation of fish in Europe has increased dramatically with a total aquaculture production of 1.4 million tonnes. This corresponds to a 51.6% increase by weight since 1984. In 1995 Europe accounted for 65.2% of the total global production of salmonids, the main fin-fish species group cultured in Europe. Salmonids are cultured at high stocking densities in intensive cage-, tank- or pond-based farming systems. The two main cultivated species are Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). The production of Atlantic salmon alone increased by 1,236% from 1984 - 1995 (363,107 tons in Europe: 250,000 tons in Norway) (FAO Fisheries Circular No.886 1997, ODIN Ministry of Fisheries, 1994-1995). In 1998 Norway was the worlds largest exporter of salmon with production exceeding 390,000 tonnes, almost half the global production (Annual report, Institute for Marine Research, Bergen, 1998). It is widely expected that the production in Norway will exceed 1 million tonnes by 2005 (Torrissen, 1995).

1.2 Disease in the Aquaculture industry

The rapid growth in the aquaculture industry has in turn led to outbreaks of infectious disease, both bacterial and viral, which affect production and incur additional treatment costs. Disease prevention is therefore a priority. The major bacterial diseases affecting fish farms are yersiniosis, furunculosis, vibriosis and cold-water vibriosis with the respective causative agents *Yersinia ruckeri, Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio salmonicida.* The major viral diseases of fish are infectious pancreatic necrosis (IPN), infectious haematopoietic necrosis (IHN) and viral haemorrhagic septicaemia (VHS). Until 1987 the method of choice to combat bacterial disease in fish was the use of antibiotics. However, due to the emergence of drug resistant strains, limitations in the prescription of medicine to farmed fish have now been introduced in Europe (ODIN Ministry of Fisheries, 1994-1995). In the late 1980's large outbreaks of cold-water vibriosis and furunculosis occurred, resulting in significant financial loss. The first commercial vaccines became available to the Norwegian market in 1989. They consisted of *Aeromonas salmonicida* bacterins containing aluminium salts as adjuvants. Although the use of these vaccines increased in the period 1989-1992, the level of antibiotic use did not decrease significantly until the availability of oil-adjuvanted vaccines in 1992. By the end of 1993 almost all farmed salmonids were vaccinated against furunculosis using oil-adjuvanted vaccines leading to a considerable reduction in the use of antibiotics. This reduction continued in 1994. (FAO Fisheries Circular, 1997; Markestad and Grave, 1997).

In Europe, commercial vaccines have been available since the mid 1980's to combat the four major bacterial diseases yersiniosis, furunculosis, vibriosis and cold-water vibriosis.

All four vaccines are formalin-inactivated preparations of the causative agents. Vaccines are still not available for another two major bacterial diseases of salmonids: bacterial kidney disease, caused by *Renibacterium salmoninarum* and salmonid rickettsial septicemia, caused by *Piscirickettsia salmonis*. Intervet Norbio has recently developed a recombinant injectable vaccine for immunising post-smolt Atlantic salmon against IPN. The vaccine consists of a recombinant form of the major antigen, VP2, added to a commercial oil/glucan adjuvanted vaccine against furunculosis, vibriosis and cold-water vibriosis.

An economic model has been constructed for calculating costs of vaccinating salmonid fish (Lillehaug, 1989). Important factors that must be taken into consideration include the cost of vaccines, their efficacy and any losses due to side effects. Losses associated with disease must be more than the overall cost of vaccination.

The three principle methods of fish vaccination include injection, immersion and oral delivery. Each have their own inherent advantages and disadvantages (Press and Lillehaug, 1995).

1.2.1 Injection vaccination

Using this method, a dose of 0.1 ml vaccine is injected intraperitoneally (i.p.) into an anaesthetised fish. A doubling dose is often recommended for administering polyvalent vaccines and those containing oil-based adjuvants. The injection method is generally not recommended for small fish under 10 g due to the difficulty in handling fish of this size. Also, small fish may not tolerate the volume of injected vaccine. Superior protective immunity is afforded by this vaccination method, with relative percentage

survival (RPS) in vaccination trials often surpassing 95% (Amend, 1981). Major disadvantages of this method include the stress caused to the fish during administration due to anaesthesia and handling. Additional labour costs are also a major consideration.

1.2.2 Immersion vaccination

Vaccination by immersion has several advantages over injection methods. These include reduced stress, lower labour costs and less time taken to immunise large numbers of fish. This method is also much safer for both the vaccinator and the fish. Several delivery methods are encompassed by the general term of immersion vaccination. These include spraying, direct immersion, hyperosmotic dipping and flush exposure. Hyperosmotic infiltration was first reported in 1976 and involved immersing trout in a hypertonic solution of NaCl with 2% bovine serum albumin (BSA) for three minutes (Amend and Fender, 1976). Alternatively, trout were immersed in a solution of 5.3% NaCl for two minutes followed by immersion in a solution of 2% BSA for three minutes. Although hyperosmotic infiltration was found to be slightly more effective than direct immersion at low bacterin levels, most commercial immersion vaccines are given by direct immersion due to additional stress caused by the hyperosmotic solutions. For direct immersion, 100 kg of fish are immersed in 1000 litres of a 1:10 vaccine solution for 20-30 seconds.

A method that affords the same degree of protection as the dipping method is the spray or shower method (Gould *et al*, 1978). Vaccines can be sprayed or showered onto

fish passing along a conveyor belt. However the specialist equipment required for this process means that this method is less used. Bath immunisation techniques have also been developed, where vaccine is poured directly into tanks holding fish. The water supply is stopped and extra oxygen is supplied. A standard fish density of 100 kg per 1000 litres is used with 1 litre vaccine added per 100 kg fish. Fish are bathed in the vaccine solution for one hour before the water supply is resumed. Bath immersion affords poorer protection than the other methods described and is generally used for fish particularly susceptible to handling.

A number of factors affect uptake of antigen from a vaccine bath. These include the concentration of vaccine, length of immersion, size of the fish, stress encountered, pH and salt concentration of vaccine solution, water temperatures and the physical state of the antigen. In general it has been found that there is less efficacy with higher dilutions and shorter immersions. The minimum size of fish for immersion vaccination is 1 g and it is well documented that immunity is related to the size rather than the age of the fish (Johnson *et al*, 1982; Zapata *et al*, 1997). Solubilised antigens appear to be taken up more readily than whole bacterial preparations with the use of adjuvants or alkaline conditions (pH 9.0) also tending to enhance antigen uptake. Maximum antigen uptake also occurs at 18°C rather than cooler temperatures.

1.2.3 Oral vaccination

In theory oral vaccination appears to be the easiest method to control infectious disease. Incorporating the vaccine into the fish feed saves time, reduces labour costs and minimises stress caused to the fish. This procedure was first used as far back as 1942 (Duff, 1942), whereby a chloroformed preparation of *A. salmonicida* induced protection in orally immunised cut-throat trout. However poor protection is often achieved in comparison to the other methods of vaccination and large quantities of vaccine are also required, proving the method to be very expensive. Methods to improve the level of protection include encapsulation of antigens to prevent gastric enzyme degradation and adjuvant usage to improve antigen uptake.

1.2.4 Time of vaccination

Anadromous fish, such as salmonids, possess a series of distinctive life cycle stages. Eggs are laid in gravel pits and develop into alevins. These small fish retain the yolk sac. The yolk sac is subsequently absorbed as alevins leave the gravel pits and become fry. Small active fry develop into parr and this stage may last from a few months to over a year. These stages of the salmonid life cycle are spent in fresh water. It is after the transformation of parr to smolts that migration to the sea occurs. In the oceans smolts gradually mature and return to their home streams for spawning. In aquaculture

salmonids also spend the first part of their lives in fresh-water before being transferred to sea sites. The transfer to sea sites generally occurs in the Spring after smoltification has occurred. The time spent in fresh-water is an ideal period for vaccination if the fish are to be protected against diseases they will encounter once in sea water. Atlantic salmon may be vaccinated at any time during the freshwater stage with oil-adjuvanted vaccines. Immunity should last throughout the entire sea water period, although it is generally believed that fish should not be vaccinated until they reach a minimum weight of 5g to ensure immune competence.

1.3 Infectious pancreatic necrosis

Infectious pancreatic necrosis was first described in 1941 as acute catarrhal enteritis, a disease affecting brook trout *(Salvelinus fontinalis)* in Canada (McGonigle, 1941). Wolf determined the viral nature of the causative agent in 1960 by feeding four and eight-week old brook trout filtered tissue homogenates from fish moribund from IPN. Homogenates were also used to inoculate tissue-cultured cells. Many of the fish that were fed the filtered homogenates developed IPN and within 18-24 hours post-inoculation tissue cultures showed pronounced cytopathic effect. Up to nine serial passages of the initial inoculum were made in tissue culture and when culture fluids were fed to young brook trout they also developed IPN (Wolf, 1960).

1.3.1 Clinical symptoms.

Generally the first sign of an IPN outbreak is a rapid increase in mortality. Clinical signs include darkened pigmentation, abdominal distention and a 'cork-screwing' and spiralling swimming motion. Internally the liver and spleen are pale and there is a large amount of milky-white mucus found in the stomach and anterior intestine.

1.3.2 Histopathology

The pancreas appears to be the primary tissue involved in IPN although the extent of the damage varies considerably. In acute cases there are large areas were the acinar structure is no longer recognisable and the tissue has degenerated into an amorphous eosinophilic mass. In some areas the acinar structure may still be recognisable although individual cells have rounded up and zymogen granules have been liberated from the affected acini into normal tissue. Nuclei of infected acinar cells are often pyknotic and vary considerably in size. Fish surviving the chronic infection develop fibrous tissue in the acinar area which has been destroyed by virus.

Acute enteritis visible in the mucosa of the pylorus, pyloric caeca and anterior intestine is manifested as complete necrosis and sloughing of the epithelium, contributing to the characteristic catarrhal exudate.

Occassionally, small focal areas of mild degenerative change within the renal haemopoietic tissue are observed (McKnight & Roberts, 1976).

1.3.3 Electron microscopy

Electron microscopic examination of thin sections of infected pancreatic acinar cells reveals crystalline arrays of virus particles (Ball *et al*, 1971). Virus particles can also be detected six hours after infecting tissue-culture cells and again appear as clusters of particles randomly distributed throughout the cytoplasm (Moss and Gravell, 1969). Electron microscopic examination of purified virus particles revealed unenveloped single shelled icosahedrons with isometric hexagonal profiles having a diameter of 60 nm (Cohen *et al*, 1973; Dobos *et al*, 1977; Dobos *et al*, 1979). The various electron microscopic studies showed that the properties of IPNV were different from those of reoviruses in that they only possess a single protein shell. (Figure 1.1)

1.3.4 Disease in Atlantic salmon post-smolts

Initially IPN was thought to be a disease affecting fry but a recent problem for fish farmers has been the high mortality in post-smolts following transfer from the freshwater hatcheries to sea sites (Christie *et al*, 1988; Smail *et al*, 1992; Melby *et al*, 1994;

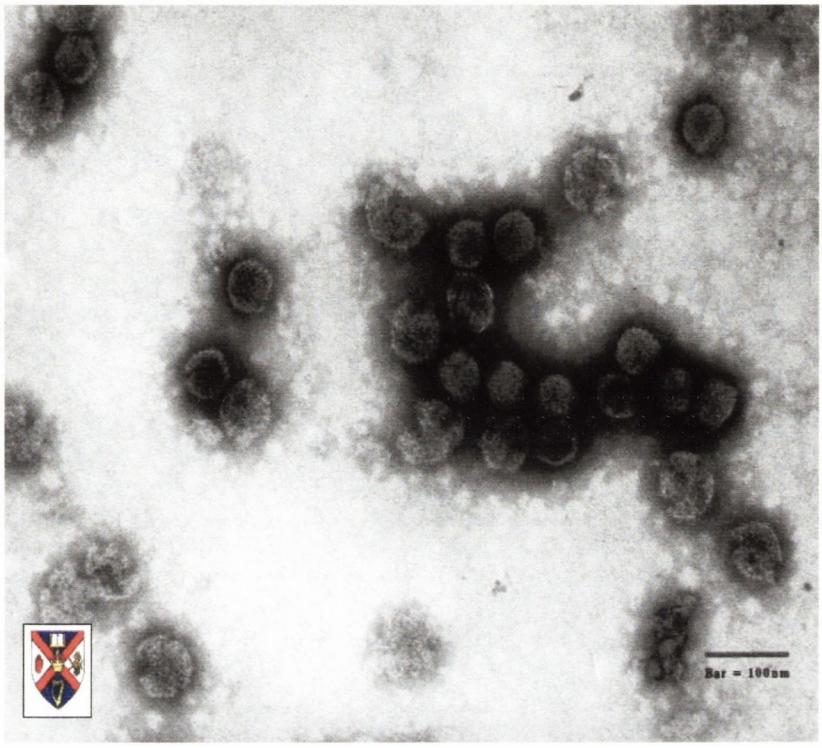

Figure 1.1 - Electron micrograph of purified IPNV. Non-enveloped viral particles with icosahedral symmetry are apparent with a particle diameter of 60 nm. Copyright 1994 Veterinary Sciences Division, Queens University, Belfast.

Ross and Munro, 1995). A recent report estimated that IPN accounted for losses of approximately 5% of the Atlantic salmon smolts transferred to Norwegian sea sites during 1995. This corresponds to a production loss of 12,000 tons (3.5 million smolts) with a replacement value of 40 million NOK (Anon, 1994). The serotype most commonly associated with post-smolt IPN in Norway and Scotland is the Sp(A2) serotype (Smail *et al*, 1992; Melby *et al*, 1994; Ross and Munro, 1995). This serotype was previously reported as causing differing degrees of subclinical pancreas necrosis in Atlantic salmon post-smolts in Scottish farms (Smail and Munro, 1985). In the farms studied there were no unusual mortalities in the early life of the fish, indicating infections were not severe, although 100% of smolts were infected with viral titres peaking in the early summer months. The rise in virus levels corresponded to an increase in water temperature, the development of smolt status and transfer of smolts to sea-water. A survey of the transfer of smolts to Norwegian sea sites in 1991 indicated a number of putative risk factors associated with disease outbreaks. These included the number of hatcheries delivering to each sea site, the age and geographic location of the sea sites and the method of transfer from hatchery to the sea site. Experimentally, IPN can be induced in post-smolts by exposure to environmental stresses such as drainage of tank water (Taksdal *et al*, 1998).

1.3.5 Challenge models

Vaccine efficacy can be defined as the degree to which protective immunity is induced in the target host. In the development of a challenge model, experimental induction of the disease is a pre-requisite. The second stage in developing a challenge model is the vaccination of fish, which are subsequently challenged with virulent virus in the manner designed to induce infection.

1.3.5.1 Brook trout

In natural infection Brook trout are known to be the most susceptible to IPNV infection with almost all survivors becoming carriers (Sadasiv, 1995). While trying to develop a challenge model Bootland *et al,* 1986 discovered that fry, 8.5 week post-hatch and 0.15g, were not protected from IPNV challenge by immersion vaccination with inactivated, purified virus. Indeed, mortalities in the vaccinated groups were higher than for the unvaccinated control group and appeared to be dose dependent. It was believed that vaccinated fry developed immune tolerance rather than immunity, as they were not fully immunocompetent (Bootland *et al,* 1986). In a subsequent study the same authors discovered that fry could be protected against IPNV by direct immersion in inactivated IPNV but only when the fry were vaccinated over a narrow age/weight range (Bootland *et al,* 1990). The highest levels of protection were obtained when the fry were 2-3 weeks post

hatch with corresponding weights of 49-60 mg. The relative percentage survival (RPS) at this age/weight was 45-50% but immunisation did not prevent IPNV infection in immunised fry. The carrier-state has also been induced in yearling Brook trout by i.p. injection of virulent IPNV (Bootland *et al*, 1991). Adult Brook trout immunised with an i.p injection of inactivated IPNV mount a strong humoral immune response, but following challenge, fish still shed virus in their faeces and reproductive products (Bootland *et al*, 1995). Immunisation may have resulted in a less widespread virus infection of the viscera as there was a trend towards fewer infected organs per fish and lower virus titres in most organs.

1.3.5.2 Rainbow trout

In natural infections Rainbow trout are better antibody producers and have a lower and more rapidly declining carrier rate. Rainbow trout fry have been successfully vaccinated by immersion in baths containing bacterial cell lysates expressing recombinant IPNV proteins. Although the VP2 protein alone induced higher levels of protection than negative control groups (32% compared with 9% RPS) the most effective vaccine was the plasmid that expressed VP2, NS and VP3 proteins (60-95% RPS). A control group vaccinated with inactivated virus had a RPS of 50% (Manning and Leong, 1990; Bootland *et al*, 1993).

1.3.5.3 Atlantic salmon

Atlantic salmon seems to be the least sensitive of these three fish species to infection and pathogenesis appears to be more complex. Until quite recently, it proved difficult to experimentally induce IPN in Atlantic salmon. The disease has now been successfully induced in both Atlantic salmon fry and Atlantic salmon post-smolts. Bath challenge of 13 week/0.19g fry resulted in cumulative mortalities of 82%, 69 days post-challenge. The cumulative mortality of unchallenged fry was 15% (Taksdal *et al,* 1997). In post-smolts IPN was induced using a design which combined stress and smolt groups of different origin. Relatively low levels of mortality are induced by i.p. injection (14%). However, outbreaks of longer duration and higher mortalities (30-40%) were observed when uninjected fish were cohabiting with IPNV injected fish (Stangeland *et al,* 1996; Taksdal *et al,* 1998).

1.3.6 IPNV

Cells infected with the causative agent of IPN were found to contain unenveloped, icosahedral viral particles in their cytoplasm. The particles were reovirus-like in size, morphology and ultrastructure (Moss and Gravell 1969). Subsequent electron microscopical studies revealed IPNV did not contain the characteristic inner capsid structure of reoviruses although they were similar in size and shape (Kelly and Loh, 1972).

Purified virus has a buoyant density of 1.33 g/cm^3 in CsCl gradients (Kelly and Loh, 1972; Cohen *et al*, 1973; Dobos, 1976; Macdonald and Yamamoto, 1977) and a sedimentation coefficient of 435 S (Dobos *et al*, 1977).

1.3.6.1 IPNV genome

Studies with metabolic inhibitors indicated that the virus nucleic acid was RNA (Malsberger and Cerini, 1965). However there was considerable controversy for a number of years over the nature of the genome, i.e. was the RNA genome single (Kelly and Loh, 1972; Nicholson, 1971) or double-stranded (Cohen *et al*, 1973) and was it segmented (Cohen *et al*, 1973) or unsegmented (Kelly and Loh, 1972). The issue was finally resolved in 1977 by Dobos (Dobos, 1976) and later confirmed by Macdonald and Yamamoto (Macdonald & Yamamoto, 1977). They found the viral genome to be resistant to ribonuclease (RNase), and sedimented at 14S in sucrose gradients when in its native state. Denaturation resulted in the genome sedimenting at 24S in sucrose gradients. Acrylamide gel electrophoresis of viral RNA resulted in the separation of two sharp bands with molecular weights of 2.3 x 10^6 and 2.5 x 10^6 Da. Similar properties have been reported for the genomes of viruses infecting marine molluscs, chickens and *Drosophila* (Underwood *et al*, 1977; Dobos *et al*, 1979). Their unique properties led to the establishment of a new family of viruses, the *birnaviridae*, with double stranded, bisegmented RNA genomes.

1.3.6.2 Viral polypeptides

The structural proteins encoded by the viral genome fall into three class sizes (Cohen *et al*, 1973; Loh *et al*, 1974; Dobos, 1977). These were initially designated the α, β and γ classes of proteins with the following molecular weights: α_1 100kDa; α_2 90 kDa; β_1 59 kDa; β_2 56 kDa; γ_1 32 kDa; γ_2 30 kDa; γ_3 28 kDa. The higher molecular weight α_1 protein was not always observed in infected cells and was therefore thought to be an artifact. Since the coding capacity of the bisegmented genome is only 266 kDa, a precursor-product relationship must be in operation. Initial studies did not support the synthesis of a precursor polyprotein that would be post translationally cleaved to produce the viral proteins as the polyprotein was never observed in infected cells. Also, proteolytic cleavage could not be arrested using amino acid analogues, protease inhibitors, $ZnCl_2$ or supraoptimal temperatures (Dobos, 1977). Pulse-chase experiments and tryptic peptide mapping did, however, prove that processing of primary gene products was occurring. Of the IPNV proteins detected in infected cells, initially only four were believed to be primary gene products: α_1, β_1, γ_1 and γ_2. The β_1 protein was processed to β_2 and β_3 and the peptide maps of these proteins were nearly identical. The peptide map of γ_1 was different from those of γ_2 and γ_3, although it was processed to γ_{1a}. Peptide maps and pulse chase experiments also confirmed the processing of γ_2 to γ_3. The high molecular weight protein α was not the precursor of any of the smaller proteins. A similar situation was reported for the structural proteins of infectious bursal disease (Muller and Becht, 1982). The larger RNA segment, or A-segment, was found to encode all but the α protein, which was

encoded by the B-segment. This was confirmed by *in vitro* translation (Macdonald and Dobos, 1981; Mertens and Dobos, 1982). The primary gene products have subsequently been renamed: α - VP1, β - preVP2, γ_1 - VP3 and γ_2 - VP4.

1.3.6.2.1 A-segment

Hybrid arrested translation and deletion mapping revealed that the order of the viral genes in the A-segment was 5'- pVP2 - VP4 - VP3 - 3' (Huang *et al*, 1986; Nagy *et al*, 1987). cDNA copies of the A-segments from IPNV and infectious bursal disease virus (IBDV), were sequenced in 1986. The complete nucleotide sequence revealed a single continuous open reading frame (ORF), which in the case for IPNV, was capable of coding for a 108 kDa polyprotein (Hudson *et al*, 1986; Duncan and Dobos, 1986). A small amount of uncleaved polyprotein was successfully isolated in 1987 when run-off transcripts from A-segment cDNA were translated *in vitro* (Duncan *et al*, 1987). The polyprotein migrated above VP1 in acrylamide gels and peptide maps confirmed it was the polyprotein as it contained the same polypeptide patterns as digested VP2 and VP3. The polyprotein was subsequently detected when expressed in insect cells using recombinant baculovirus (Magyar and Dobos, 1994a) and also in IPNV infected Chinook salmon embryo (CHSE) cells (Magyar and Dobos 1994b). The VP4 gene is believed to encode a viral protease (Duncan *et al*, 1987; Azad *et al*, 1987; Jagadish *et al*, 1988; Magyar and Dobos, 1994a).

Processing of the polyprotein requires a functional VP4 gene product as deletion of the C-terminal third of the putative protease gene results in complete loss of proteolytic activity (Duncan *et al*, 1987; Magyar and Dobos, 1994a). The VP4 gene product does not act *in trans* (Magyar and Dobos, 1994a). The genomes of IPNV and IBDV are arranged in a similar manner and processing of the viral proteins appears to occur in both members of the *birnaviridae* family. However, nucleotide sequence comparisons between IPNV and IBDV strains revealed no sequence similarity in the VP4 region of the A-segments (Havarstein *et al*, 1990; Kibenge *et al*, 1990; Bayliss *et al*, 1990; Kibenge *et al*, 1991). The processing of the primary gene products, in particular pVP2-2 to VP2 and VP4 to VP4-1 can be blocked by serine protease inhibitors such as aprotinin at a concentration of 800μg/ml. However, none of the inhibitors tested inhibited processing of the polyprotein to pVP2, VP4 and VP3.

An alternative ORF that partly overlaps the large A-segment ORF was discovered in both IPNV and IBDV isolates (Duncan *et al*, 1987; Azad *et al*, 1985). The small ORF of IPNV N1 strain has the potential to code for a 17 kDa, arginine rich polypeptide of 148 amino acids. SDS-PAGE analysis of virus radiolabelled with [^{35}S] methionine revealed the presence of a 17 kDa protein (Havarstein *et al*, 1990). A protein of the same size has also been observed in infected cells (Magyar and Dobos, 1994b; Heppell *et al*, 1995a). The A-segment of birnaviruses is therefore structurally and functionally bicistronic.

1.3.6.2.2 B-segment

The smaller IPNV genome segment encodes a single polypeptide of 94 kDa, known as VP1. Purified IPNV preparations were shown to possess a virion associated RNA polymerase activity (Cohen, 1975). VP1 is believed to be the RNA-dependent RNA polymerase due to its size, which is similar to other known virion polymerases. It has a low copy number per virion (Dobos *et al*, 1977) and it is an internal protein (Dobos and Rowe, 1977). Sequence analysis of birnavirus VP1 genes revealed the presence of several conserved domains associated with RNA-dependent RNA polymerases of other RNA viruses. Regions of conserved secondary structure were also found, however they lacked the Gly-Asp-Asp motif characteristic of these enzymes (Duncan *et al*, 1991). The protein is present in the virion in two forms: a free polypeptide and a genome-linked protein (VPg) (Personn and Macdonald, 1982; Muller and Nitschke, 1987). In IPNV a serine residue of the VPg is linked to the 5′ terminal G residue of both RNA segments by a phosphodiester bond (Calvert *et al*, 1991). Although IBDV VP1 can be guanylylated *in vitro* the enzyme is not a true guanylyl transferase as this reaction is not reversible. Also the VP1-GMP complex does not transfer the GMP to a common acceptor (Spies and Muller 1990; Dobos, 1993). However during *in vitro* RNA synthesis, VP1 was shown to act as a primer. Labelled VP1-GMP was shown to be "chased" *via* nascent RNA strands and replicative intermediates to a VP1-dsRNA complex. Radiolabelled VP1 can be recovered from these complexes by digestion with a dsRNA specific ribonuclease. Only the plus strands of the genome segments were being synthesised and these remained base-

paired to their templates. Thus, *in vitro* transcription of IPNV RNA is primed by VP1 and proceeds *via* an asymmetric, semi-conservative, strand displacement mechanism. Although genome linked proteins play a fundamental role in replication of viral genomes birnaviruses are the first dsRNA viruses shown to possess VPg's and are also the only dsRNA viruses that exhibit protein primed RNA synthesis. The virus structure is depicted in figure 1.2 and the genome organisation is shown in figure 1.3.

1.3.7 Serotyping of IPNV isolates

1.3.7.1 Polyclonal antisera

Most aquatic birnaviruses cross-react to some degree although significant antigenic differences occur among the numerous isolates. Initial studies suggested that the three isolates VR299, Ab and Sp were antigenically distinct (Underwood *et al*, 1977). When the reciprocal cross neutralisation kinetics of ten IPNV isolates from the United States and Europe were compared three distinct groups of serotypes were established. The first group contained all isolates from the United States with VR299 as the type strain. Three of the European isolates, Sp, Bonnamy and d'Honnincthun fell into the second group with Sp as the type strain. Initially the third group only contained one isolate, the European Ab isolate. However, a study comparing the cross neutralisation kinetics of the same American and European IPNV isolates with an aquatic birnavirus, European eel virus

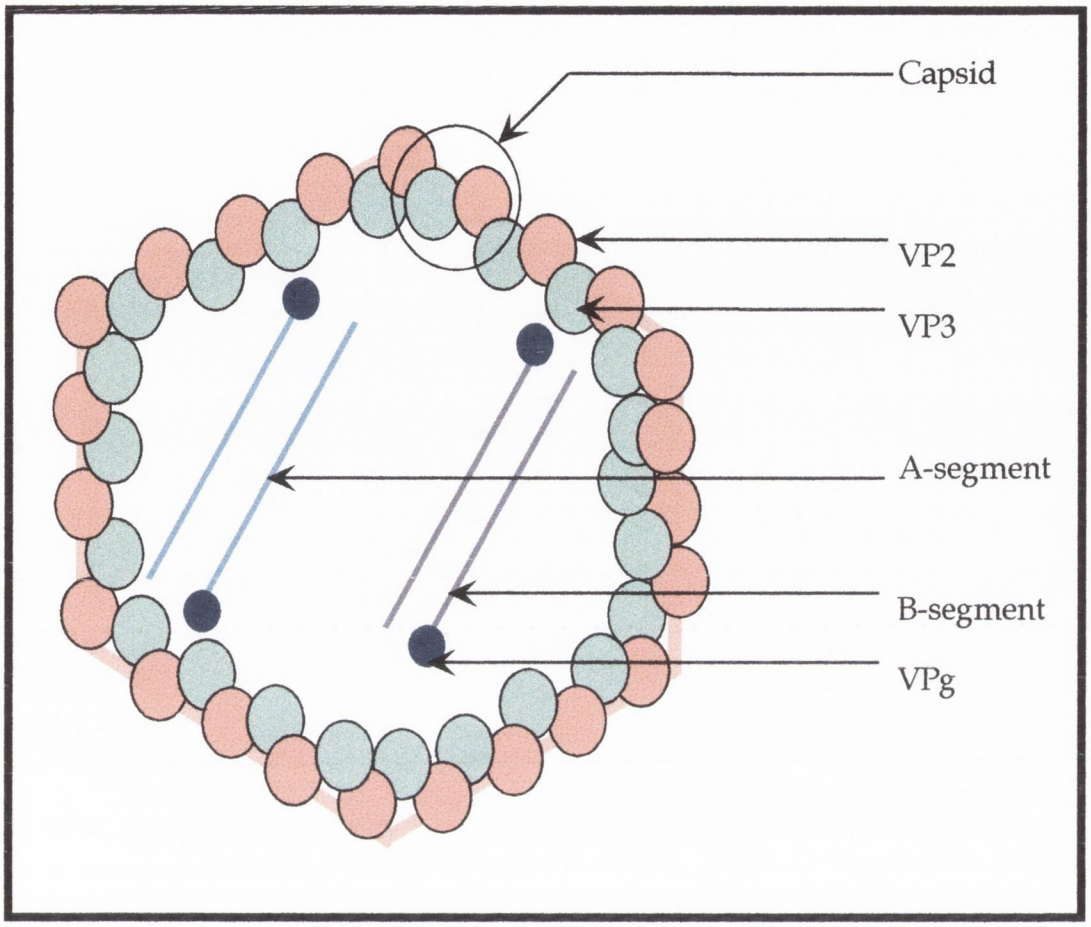

Figure 1.2 – IPNV virion structure. Each segment of the double stranded RNA genome is linked to a form of the viral protein VP1. VP2 is the major structural protein and constitutes the outer capsid layer. The inner capsid is composed of the minor structural protein VP3.

Figure 1.3 – Organisation of coding regions of the IPNV genome. The A-segment is produced as a polyprotein and is post-translationally cleaved to produce the structural proteins VP2 and VP3, and the non-structural protein VP4. In addition to the A-segment polyprotein open reading frame an overlapping open reading frame, encoding a 17 kDa protein of unknown function, is present. Both the A-segment and the B-segment have viral genome proteins (VPg) linked to the 5'end of the RNA.

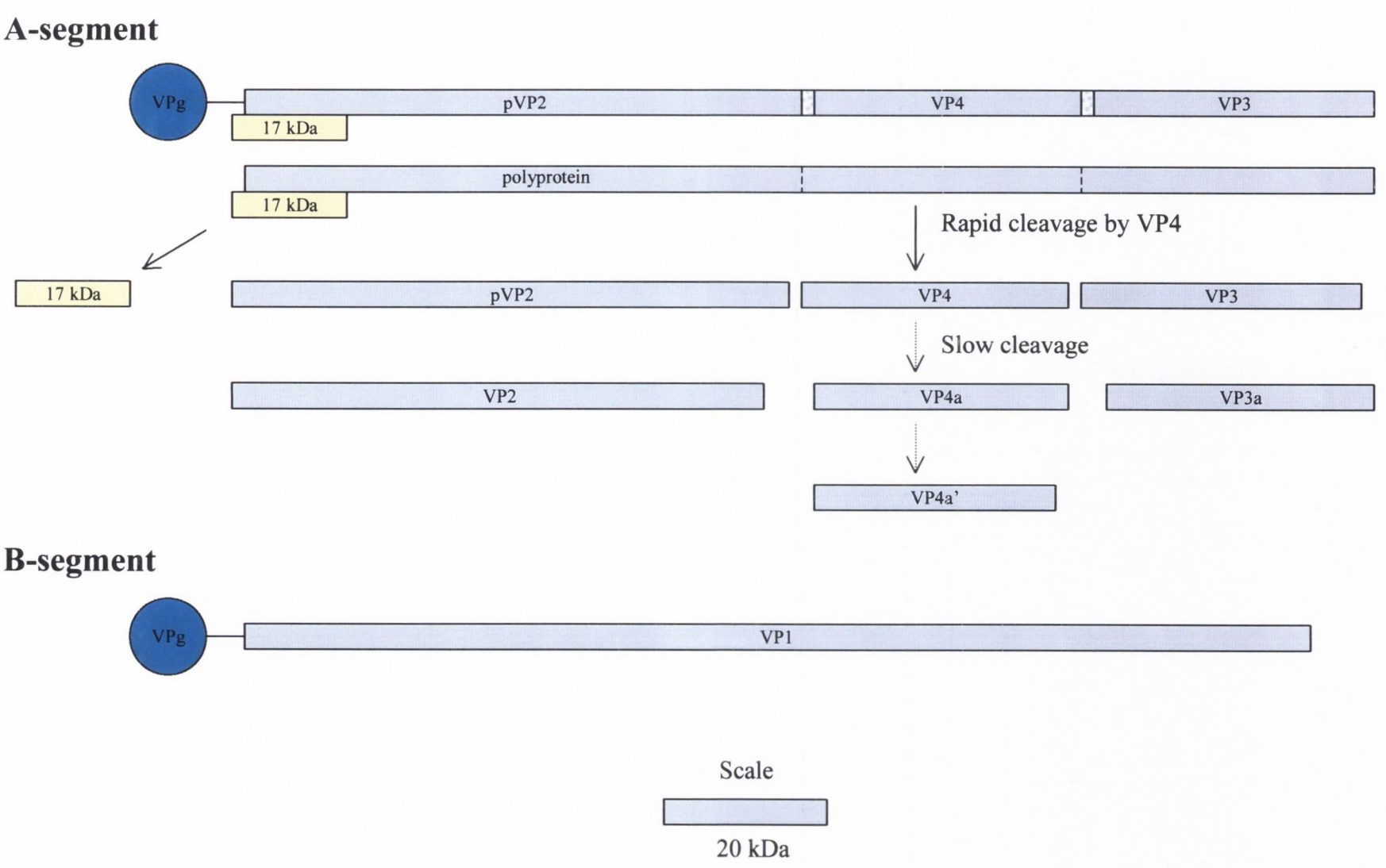

Figure 1.3 - Organisation of the coding region of the IPNV genome.

(EEV), isolated from eels, determined that EEV also belonged to the third group with Ab (Okamoto *et al*, 1983). A more extensive study using almost 200 isolates of IPNV and other aquatic birnaviruses isolated from molluscs and crustaceans, in a standardised cross-neutralisation procedure, was performed by Hill & Way (Hill and Way, 1995). They proposed that all aquatic birnaviruses can be divided into two serogroups. The first group contains nine serotypes while the second currently contains only one serotype. The West Buxton strain was chosen as the type strain for the A1 serotype group in place of VR299. The majority of isolates fell into the three original serotype groups. Other isolates were found to form tight clusters within distinct serotypes with the designated type strains He, Te, Can 1, Can 2, Can 3 and Jasper. Serotype Can 3 contained only one other isolate and the type strains for Can 2 and Jasper serotypes were the only members of their respective groups. The nine serotypes were proposed to form the first serogroup. Only six isolates out of the 196 tested were not neutralised with any of the reference strain antisera. One of these isolates was Tellina virus (TV-1), originally described by Underwood (Underwood *et al*, 1977). Antisera from TV-1 neutralised the remaining isolates and it was therefore proposed that these isolates form the second serogroup with only one serotype member. New strains isolated from IPN outbreaks in post smolt Atlantic salmon in Norway (Christie *et al*, 1988) and Scotland (Ross and Munro, 1995) have been proposed to form the new serotypes N1 and Shetland or SS respectively.

1.3.7.2 Monoclonal antibodies

An alternative to the use of polyclonal antisera for serotyping is the use of a panel of monoclonal antibodies (MAbs) in an immunodot assay for the rapid identification and presumptive serotyping of IPNV. One of the first reports of a panel of MAbs used for presumptive serotyping was in 1986 (Caswell-Reno *et al*, 1986). The MAbs were raised against the West Buxton isolate and were used in enzyme-linked immunosorbent assays (ELISA) and neutralisation studies with 14 isolates from the four serotypes West Buxton, Sp, Ab and Can 1. The 14 isolates were found to show distinct patterns of reactivity even within the same serotype and represented nine antigenically distinct viruses. A larger panel of MAbs was subsequently developed (Caswell-Reno *et al*, 1989) and used for the presumptive serotyping of aquatic birnaviruses. One of these MAbs, MAb AS-1, was found to recognise an epitope found on all serogroup A isolates and will therefore prove useful as a diagnostic tool. The same panel of MAbs have been used to serotype field isolates in Norway (Melby *et al*, 1994) and Scotland (Ross and Munro, 1995). Eighteen Shetland and three Scottish mainland isolates were found to have reaction patterns that shared characteristics of the Sp and N1 strains with the panel of MAbs. In the Norwegian study, 70 field isolates were randomly selected from nine different host groups representing host species, fish age and fish health status. Also included in the study were 11 isolates from an IPN outbreak in Atlantic salmon post smolts. 83% reacted identically to the Sp type strain with a further 14% showing reaction patterns that were Sp related. Although all isolates from IPN infected post-smolts were Sp

serotype, no correlation could be derived between fish species, age or health status and strain variation. The N1 strain, which had been proposed as a separate serotype, (Christie *et al*, 1988) reacted identically to the Sp reference strain. Monoclonal antibodies have also been produced against the N1 strain (Christie *et al*, 1990; Melby and Christie, 1994). The panel of six MAbs against N1 can distinguish between the Sp reference strain and the N1 strain. When the 81 Norwegian field isolates were analysed using these MAbs, none were found to react identically to the Sp strain. However, 77 out of the 78 Sp or Sp related isolates from the earlier study reacted identically with the N1 strain. This shows that the two strains, N1 and Shetland, initially thought to be members of new serotypes are members of the Sp serotype. Some serotypes cannot be distinguished in ELISA using panels of Mabs. The panel of 11 MAbs cannot distinguish between serotypes C2 and C3. N1 MAbs cannot distinguish between VR299 and Ja or between Te and C1. Three panels of MAbs raised against the VR299, Sp and Jasper serotypes could not distinguish between Ab and Te (Tarrab *et al*, 1993). Other strains could be related on the basis of VP2 or VP3 similarities. Considering this the authors suggest the subdivision of serogroups should be re-evaluated.

1.3.8 Genomic grouping

A related birnavirus, IBDV of chickens, can also be divided into two serotypes on the basis of virus neutralisation tests. Two serotypes of IBDV exist; the first contains

isolates of varying degrees of pathogenicity whereas the second serotype only contains non-pathogenic isolates. Isolates from both serotypes have now been cloned and their genomes sequenced in order to identify any sequence differences that may account for different biological properties of the serotypes (Kibenge *et al*, 1990; Bayliss *et al*, 1990; Havarstein *et al*, 1990; Kibenge *et al*, 1991). These sequences have also been compared with sequences from IPNV strains Jasper and N1. Strains from the two serotypes of IBDV are approximately 83% identical at the nucleotide level and 90% identical at the amino acid level. Strains within serotype 1 appear to be even more closely related with between 92.3 and 99.7% conservation at the nucleotide level and 97.3 and 99.5% conservation at the amino acid level. When IBDV and IPNV sequences were compared, the VP2 gene was the most conserved region with 44% identity at the amino acid level. The least conserved area was the VP4 or non-structural protein gene, which was only 20% identical at the amino acid level (Havarstein *et al*, 1990; Kibenge *et al*, 1990; Kibenge *et al*, 1991). Both viruses exhibit most of their amino acid changes within a central region of the VP2 gene, known as the hypervariable region. This area of the A-segment is believed to harbour the conformational epitopes recognised by virus neutralising MAbs (Azad *et al*, 1987; Frost *et al*, 1995). Only the sequences from IPNV strains Jasper and N1 have been compared directly revealing a nucleotide identity of approximately 80% between these two strains with the VP2 gene most identical at 88%. As few strains have been completely sequenced, Heppell attempted to correlate genomic differences with serotype by comparing the restriction fragment profiles of a 359 bp fragment amplified from 37 strains of IPNV. (Heppell *et al*, 1992). The fragment amplified consisted of the C-terminal part of VP2 and

the N-terminal region of VP4. Cluster analysis of the profiles generated by restriction digests with five enzymes revealed the presence of three major groups, termed Genogroups I - III. These groups correspond to the three originally reported seroptyes, LWVRT, Sp and Ab. The six remaining serotypes are not distinct from these three isolates and therefore form the ten sub groups within the three genogroups. A classification of IPNV strains was also established on the basis of the amino acid sequence homology within the same VP2-VP4 junction region (Heppell *et al*, 1993). Again, three major genogroups were formed, each containing distinct subgroups. Serologically related viruses were found to be highly identical (96%) and some strains that were initially reported to be serologically different were almost identical at the genomic level (i.e N1 with Sp and Fr.21 and C1 with C3). This method can also distinguish IPNV isolates from other aquatic birnaviruses (Hosono *et al* 1996).

1.4 Semliki Forest virus

Semliki Forest virus (SFV) belongs to the Alphavirus genus of the *Togaviridae* family. The type-specific member of the Alphavirus genus is Sindbis virus (SIN) and knowledge of the molecular biology of alphaviruses has been based on studies with SFV and SIN. The viruses are arthropod borne, with mosquitoes being the usual vector. Natural vertebrate hosts include avian and mammalian species.

1.4.1 Virion structure.

SFV is a small circular virus with a diameter of approximately 60nm. Viral particles are composed of enveloped nucleocapsid structures. The nucleocapsid is RNase sensitive and has a diameter of 40 nm (Soderlund *et al*, 1979). Two hundred and forty copies of the viral capsid protein surround the RNA genome in an icosahedral T=4 lattice (Choi *et al*, 1991). Two domains are present in the capsid protein. The C-terminal domain is homologous to a chymotrypsin-like serine protease (Choi *et al*, 1991), while the N-terminal domain is believed to interact with the RNA genome and possesses regions important for the specific encapsidation of genomic RNA and nucleocapsid assembly (Owen and Kuhn, 1996). The envelope surrounding the nucleocapsid core consists of a host derived lipid bilayer (Laine *et al*, 1972; Renkonen *et al*, 1971), with virus encoded glycoproteins forming 80 spikes (Garoff *et al*, 1974; Helenius and von Bonsdorff, 1976; Mattila, 1979). The glycoprotein spikes also form an icosahedral surface lattice with a T=4 triangulation number (von Bonsdorff and Harrison, 1975; von Bonsdorff and Harrison, 1978; Cheng et al, 1995). Each spike is composed of three E1/E2/E3 heterotrimers. E1 and E2 glycoproteins span the lipid bilayer but it is only E2 that is believed to interact with the nucleocapsid core *via* its 31 amino acid C-terminal region (Simons and Garoff, 1980; Garoff and Simons, 1974; Ziemiecki *et al*, 1980; Metsikko and Garoff, 1990; Vaux *et al*, 1988). (Figure 1.4)

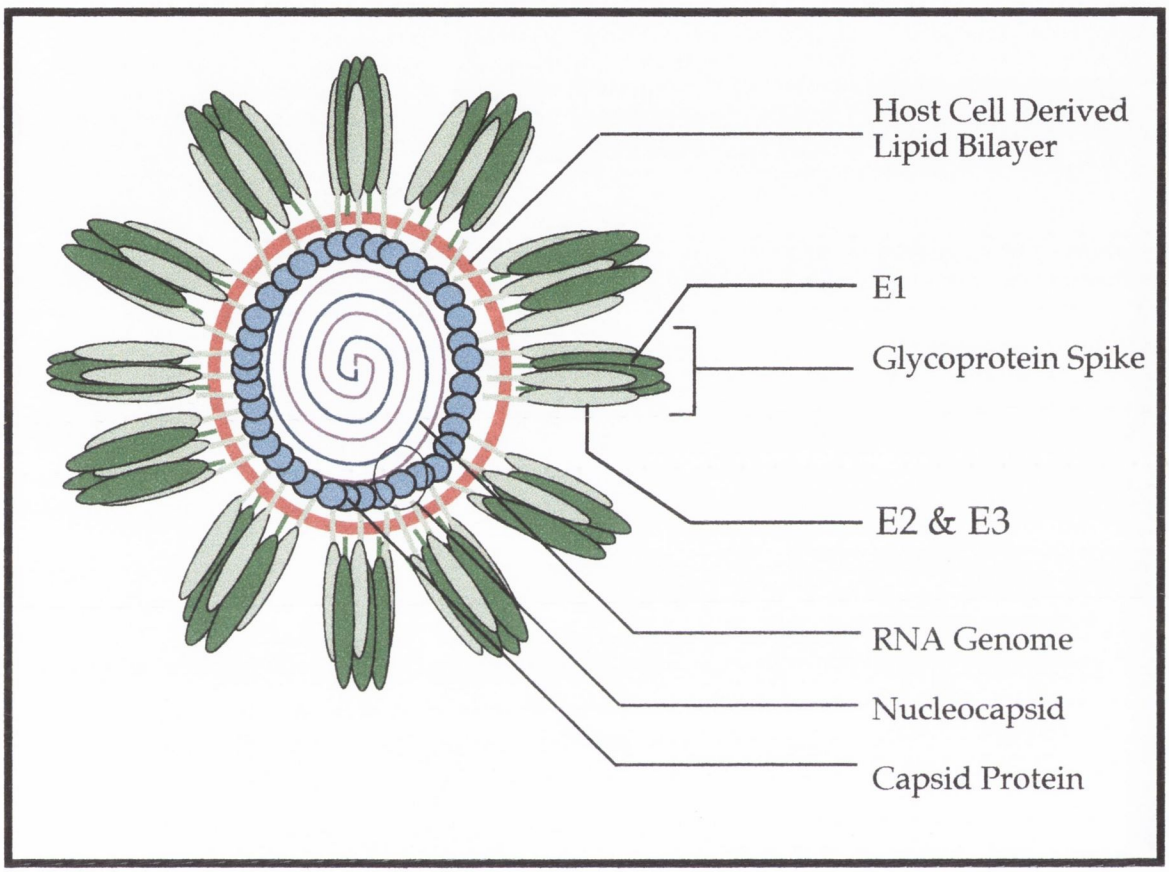

Figure 1.4 – Structure of SFV virion. The viral RNA is encapsidated by 240 molecules of the capsid protein to form the nucleocapsid. The nucleocapsid is surrounded by a lipid bilayer derived from the host cell plasma membrane by virus budding. 80 viral glycoprotein spikes are embedded in the lipid bilayer and are composed of trimers of the E1/E2/E3 viral proteins. Only the C-terminal tail of the E2 glycoprotein extends through the lipid bilayer and is in contact with the nucleocapsid core.

1.4.2 Genomic organisation.

Alphavirus genomes are composed of a single strand of positive polarity RNA that, for SFV, has a sedimentation coefficient of 42S. In infected cells, two main species of viral specific RNA are produced. Both possess a 5′ 7-methylguanosine cap and are polyadenylated at the 3′ end (Sawicki and Gomatos, 1976). These are the 42S genomic RNA and 26S RNA. The 42S genomic RNA is divided into two major regions. The 5′ two-thirds codes for the non-structural proteins that make up the viral replicase:- nsP1, nsP2, nsP3 and nsP4. As there is only one initiation site for protein synthesis on the 42S RNA (Glanville et al, 1976) the non-structural proteins are produced as a polyprotein, which is post-translationally cleaved (Lachmi and Kaariainen, 1976). Shortly after infection the replicase catalyses the formation of full length negative strand RNA (Sawicki and Sawicki, 1980). This serves as a template for the synthesis of full-length genomic positive strand RNA molecules and a subgenomic 26S RNA species which is colinear with the 3′ end of the genomic RNA (Sawicki and Gomatos, 1976; Wengler and Wengler, 1976; Garoff *et al*, 1980a). The 26S RNA is responsible for the production of all SFV structural proteins (Sawicki and Gomatos, 1976; Wengler and Wengler, 1976; Garoff *et al*, 1978, 1980a; Clegg and Kennedy, 1974; Glanville *et al*, 1976). A short junction region separates the non-structural coding region from the structural protein coding region and each is translated in a different ORF (Riedel *et al*, 1982). The structural proteins are also produced as a polyprotein, which is both co-translationally and post-translationally cleaved. The structural genes are arranged in the following order on the 26S mRNA: 5′ Capsid - p62 -

6K - E1 (Keranen and Kaariainen, 1975; Lachmi *et al*, 1975; Lachmi and Kaariainen, 1976; Garoff *et al*, 1978; Kalkinnen *et al*, 1981). The envelope spike proteins E2 and E3 are subsequently cleaved from the p62 precursor. (Figure 1.5)

1.4.3 Viral entry

Alphaviruses enter cells by receptor mediated endocytosis. Briefly this process involves binding of virus particles to the cell surface, internalisation by coated vesicles and transfer to endosomes. A low-pH-induced fusion of the viral envelope with the endosomal membrane finally results in the uncoating of the virus and release of the viral genome. The initial attachment of virus particles to the cell surface involves specific viral proteins interacting with specific receptors on the host cell plasma membrane. Cellular molecules that have been identified as viral receptors include the epidermal growth factor receptor for vaccinia virus (Eppstein *et al*, 1985), and CD4 and CD8 for human immunodeficiency virus (Dalgleish *et al*, 1984; Klatzmann *et al*, 1984; McDougal *et al*, 1986). A number of host cell proteins are implicated as receptors for SFV, including the major histocompatibility surface antigens HLA-A and HLA-B (Helenius *et al*, 1978). However, these antigens were subsequently shown to bind SFV and other viruses nonspecifically (Oldstone *et al*, 1980). Alphaviruses have a wide host range, infecting a number of species from mammals to insects. These viruses can also infect many different cell types. Therefore, it is likely that the viruses can

Figure 1.5 – SFV genome organisation. The 11 kb RNA genome of SFV is illustrated. The non-structural proteins are produced as a polyprotein by translation of the 5′ two-thirds of the genome. The polyprotein is subsequently processed to produce the replicase proteins nsP1, nsP2, nsP3 and nsP4. The replicase produces full length minus-strand RNA which acts as a template for genomic RNA synthesis and synthesis of a 26S subgenomic RNA species that represents the 3′ third of the genome. Structural proteins are translated from the 26S subgenomic RNA as a polyprotein.

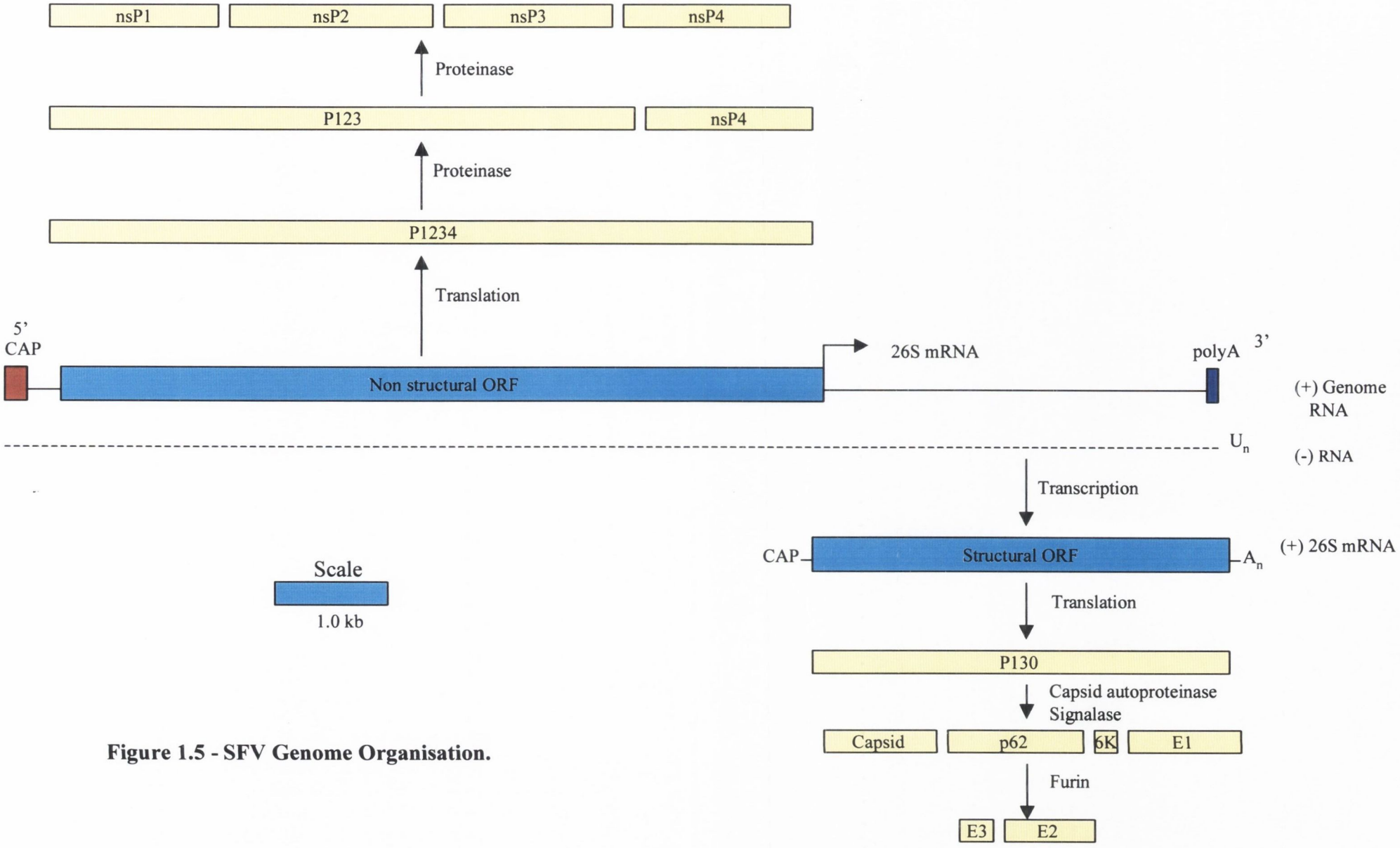

Figure 1.5 - SFV Genome Organisation.

utilise a number of different surface antigens as receptors with varying affinity (Strauss and Strauss, 1994, Ubol and Griffin, 1991). Once internalised by endosomes a series of low pH induced reactions occur. It has been shown that p62 cleavage is required for viral infection (Salminen *et al*, 1992). Cleavage of p62 leads to dissociation of the E2E1 heterodimer (Lobigs *et al*, 1990a, 1990b; Salminen *et al*, 1992; Lobigs and Garoff, 1990) and formation of a stable E1 heterotrimer complex (Wahlberg *et al*, 1992) with the concomitant exposure of a new E1 epitope. The E1 heterotrimer is the fusion active conformation of the spike protein and is only formed after a short pH- and temperature-dependent lag phase (Bron *et al*, 1993). After another short delay, fusion ensues releasing the nucleocapsid into the cytoplasmic compartment, although they remain associated with the lysosomal membranes, (Froshauer *et al*, 1988) anchored by nsP1 (Peranen *et al*, 1995). Binding of ribosomes to the nucleocapsid probably triggers uncoating of the nucleocapsid and release of genomic RNA (Singh and Helenius, 1992).

1.4.4 RNA replication

Once released, the positive polarity genomic RNA functions directly as mRNA and initially translates the 5′ two thirds of the genome corresponding to the non-structural proteins of the virus (Kaariainen *et al*, 1987; Takkinen, 1986). This region is composed of a single ORF, translating the non-structural proteins as a polyprotein precursor that is co- and post-translationally cleaved to yield nsP1, nsP2, nsP3 and nsP4 (Takkinen *et al*, 1991;

Lehtovarra *et al*, 1980). The precursor p1234 can be cleaved in one of two ways. The prevalent pathway involves nsP4 production by nascent cleavage from the growing p1234 polypeptide by its independent protease. The subsequent cleavages between nsP1-nsP2 and nsP2-nsP3 are carried out by the nsP2 protease (Takkinen *et al*, 1991; Hardy and Strauss, 1988; Hardy and Strauss, 1989). Alternatively cleavage can occur between nsP2-nsP3 generating two further precursors, P12 and P34. The nsP2 protease then catalyses the cleavage of P12 and the cleavage of P34 is accomplished by the nsP4 protease.

The genomic mRNA also acts as a template for the synthesis of a full length, negative strand, intermediate RNA species (Sawicki and Sawicki, 1980). The negative strand RNA is responsible for the production of new 42S RNA plus strands and also acts as a template for the synthesis of subgenomic 26S RNA (Sawicki *et al*, 1978). A translational enhancer sequence present in the first 102 nucleotides of the capsid coding region results in the efficient translation of the subgenomic RNA species (Sjoberg *et al*, 1994; Sjoberg and Garoff, 1996). The structural proteins are also produced as a polyprotein in the order Capsid - p62 - 6K - E1 (Welch *et al*, 1981; Welch and Sefton, 1980).

As the capsid protein is translated from the 26S RNA it folds to form a serine protease and is autoprotolytically cleaved from the nascent polyprotein chain (Garoff *et al*, 1978; Melancon and Garoff, 1987). The catalytic triad of the serine protease is His (141), Asp (163), Ser (219) (Melancon and Garoff, 1987; Hahn and Strauss, 1990). Once cleaved, the capsid protein is free to associate in the cytoplasm with viral 42S RNA to form nucleocapsids after a brief association with ribosomes (Soderlund and Ulmanen, 1977). Cleavage of the capsid from the polyprotein precursor exposes the N terminus of p62

which acts as a signal sequence for the transocation of the translation complex to the endoplasmic reticulum (ER) membrane (Garoff et al, 1978). The p62 signal sequence is not cleaved by a signal peptidase but is retained in the ER lumen where it becomes glycosylated. A hydrophobic stretch of amino acids at the C-terminus of p62 acts as a stop transfer signal and anchors the protein in the membrane (Garoff *et al*, 1980b). A second internal signal sequence is present in the C-terminal tail of p62 and is followed by a recognition site for signal peptidase cleavage, releasing p62 from the nascent chain. The internal signal sequence directs the translocation of the 6K protein which in turn contains a hydrophobic region in its C-terminus to direct the translocation of E1 (Liljestrom and Garoff, 1991a). E1 translocation is arrested once anchored in the membrane. Fatty acylation of the trans-membrane proteins occurs shortly after synthesis and may be required for the correct orientation of the membrane (Ivanova and Schlesinger, 1993). A heterodimeric complex is rapidly formed between p62 and E1 and this acid resistant complex is transported through the exocytic pathway of the trans-Golgi network. Proteolytic processing of p62 does not occur until the p62E1 complex has passed through the trans-Golgi network (Ziemiecki *et al*, 1980; de Curtis and Simons, 1988; Wahlberg *et* al, 1989; Sariola *et al*, 1995). The processing of p62 to E2 and E3 is crucial for virus maturation (Keranen and Kaariainen, 1975).

1.4.5 Nucleocapsid formation and virus budding

Packaging signals are present in the genomes of alphaviruses to selectively package only genomic RNA. In Sindbis virus, the signal has been located to a region in the nsP1 gene (Weiss *et al*, 1989; Frolova *et al*, 1997). A similar signal is present in the nsP2 gene of SFV (Liljestrom and Garoff, 1991b, White *et al*, 1998). Assembled nucleocapsids freely diffuse to the plasma membrane where they interact with the viral glycoproteins (Garoff and Simons, 1974). Virus release is strictly dependent on the coexpression of nucleocapsids and spike proteins (Suomalainein *et al*, 1992) and the capsid protein has a specific receptor for the E2 cytoplasmic domain (Vaux *et al*, 1988). The nucleocapsid-E2 interaction appears to require the multimeric form of E2, present in the assembled trimeric spike, for which E1 protein expression is necessary (Metsikko and Garoff, 1990; Barth and Garoff, 1997). The 6K protein is also involved in the budding process (Lowey *et al*, 1995). A sequence-specific interaction between the 6K protein and the E1 and E2 glycoproteins appears to be necessary for virus assembly to occur. It is suggested that the 6K protein promotes the correct folding of the E1 protein present in the heterotrimeric form of E1/E2 (Yao *et al*, 1996). The life cycle of the virus is depicted in figure 1.6

Figure 1.6 - Semliki Forest virus life cycle. After the virus enters the cell by receptor mediated endocytosis, the nucleocapsid is released from modified endosomes and lysomes. The positive stranded viral genome acts directly as mRNA and the viral replicase is produced by translation of the 5′ two thirds of the genome. The replicase is responsible for the production of full length genomic RNA and subgenomic 26S RNA. Translation of the 26S RNA yields the structural region polyprotein. The capsid protein is autoproteolytically cleaved from the nascent polyprotein and interacts with newly trancribed 42S genomic RNA to form nucleocapsids. The nascent structural polyprotein is co-translationally cleaved to p62, 6K and E1 and these proteins are translocated to the endoplasmic reticulum where they become N-terminally glycosylated and dimers are formed between p62 and E1. After transport through the Golgi apparatus p62 is cleaved to E2 and E3 and the three viral proteins are embedded in the host cell plasma membrane. The cytoplasmic tail of E2 interacts with newly formed nucleocapsids to initiate budding from the host cell.

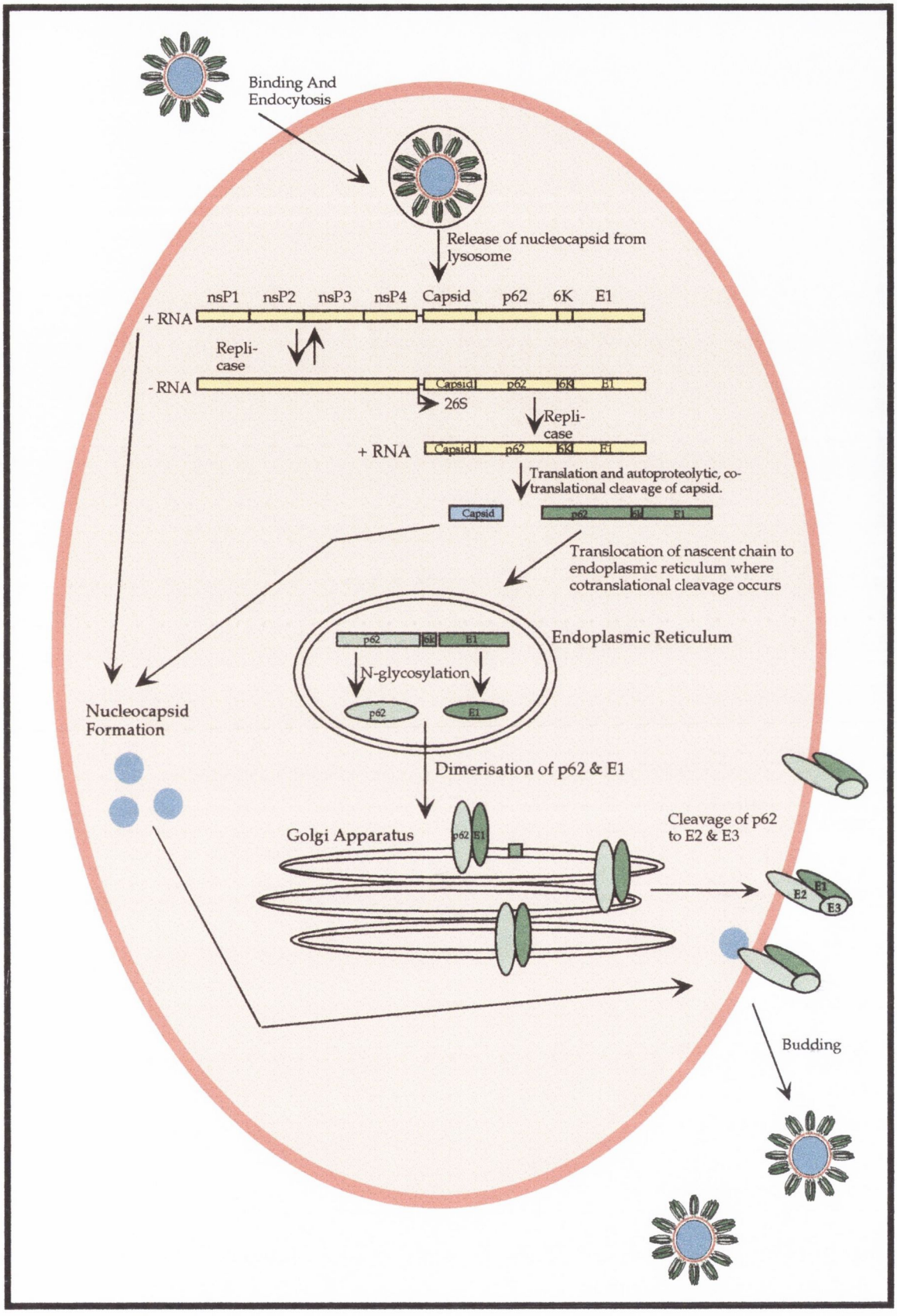

Figure 1.6 - Semliki Forest virus life cycle.

1.4.6 Expression vectors

1.4.6.1 Basic expression vector

The construction of full-length cDNA clones of SIN (Rice *et al,* 1987) and SFV (Liljestrom *et al,* 1991) viruses has led to their development as expression vectors. Several features of alphaviruses are advantageous for this. These include the wide host range of the virus and high levels of cytoplasmic

RNA and protein expression. Infectious RNA transcripts can be produced from the cDNA clones and transfected into cells at very high efficiencies. The basic SFV replicon is based on the SFV infectious clone pSP6-SFV4. The subgenomic coding region from the infectious clone pSP6-SFV4 has been deleted and a *Bam*H I - *Xma* I - *Sma* I polylinker sequence and a cassette of translational stop codons in all three reading frames inserted downstream from the 26S promoter site. The nonstructural coding regions are retained, as are the short 5′ and 3′ regions required for RNA replication (Levis *et al,* 1986). Expression levels of heterologous proteins from these vectors were significantly enhanced when the capsid protein translational enhancer (Forsell *et al,* 1995; Sjoberg and Garoff, 1996) was fused to the heterologous gene (Sjoberg *et al,* 1994).

1.4.6.2 Packaging of recombinant RNA

Cotransfecting cells with RNAs transcribed from helper plasmids and recombinant plasmids achieves efficient packaging of RNA replicons (Liljestrom and Garoff 1991b; Bredenbeek et al, 1993). The replicase function, expressed by the recombinant RNA, leads to its own amplification and acts *in trans* to allow the replication and transcription of helper RNA. The helper RNA retains the 5′ and 3′ regions required for replication and the complete structural coding region, but lacks the signal in NSP1 (Weiss *et al*, 1989; Levis *et al*, 1986) required to package RNA into nucleocapsids. The virus particles produced by co-transfection will only contain recombinant derived RNA. A potential biosafety problem is the production of wild type virus by recombination during *in vivo* packaging (Geigenmuller-Gnirke *et al*, 1991; Berglund *et al*, 1993). A new helper system was subsequently developed to overcome this recombination. This system was based upon a cleavage deficient variant of the SFV spike glycoprotein (Salminen *et al*, 1992). The infectivity of virus particles produced using this mutant helper can be restored by treatment with α-chymotrypsin (Berglund *et al*, 1993). Alternatively, the capsid and spike helper regions can be expressed from two separate RNA molecules (Smerdou and Liljestrom, 1999). Recombination between the three RNA molecules transfected into cells would be negligible and biosafety is further increased by the capsid S219A mutation which abolishes self-cleavage of the capsid protein (Hahn and Strauss, 1990; Melancon and Garoff, 1987)

1.4.6.3 Challenge models.

Several features of the alphavirus life cycle make it an ideal candidate for vaccine design. High levels of intracellular antigen expression result in strong immune responses. Also, expression of vector structural proteins is minimal and therefore immune responses to the vector itself will be negligible. The broad host range of the virus is also a clear advantage. Recombinant RNA encoding the heterologous antigen may be used directly as a vaccine (Johanning *et al*, 1995; Zhou *et al*, 1994) or recombinant particles produced by the *in vivo* packaging procedure described above may be injected (Zhou *et al*, 1995; Mossman *et al*, 1996; Fleeton *et al*, 1999). Both methods result in the generation of a strong humoral and cellular immune response with prolonged memory. Heterologous antigens that have been shown to elicit protection when expressed by recombinant SFV particles include influenza nucleoprotein (Zhou *et al*, 1994; Zhou *et al*, 1995; Berglund *et al*, 1999), simian immunodeficiency virus (SIV) gp160 (Mossman *et al*, 1996) and louping ill prME and NS1 proteins (Fleeton *et al*, 1999).

1.4.6.4 Layered DNA/RNA system.

The alphavirus genome has also been used to further enhance the effects of DNA vaccines. Conventional DNA vaccines are composed of bacterial plasmids that

encode a strong viral promoter, such as the cytomegalovirus (CMV) immediate early promoter, and the gene of interest with control elements including polyadenylation and transcription termination signals. A typical DNA expression plasmid is pcDNA3 (figure 1.7). DNA vaccines are administered in one of the following manners:

(a) Direct injection of plasmids dissolved in saline into muscle/skin (Montgomery *et al*, 1994; Fynan *et al*, 1993).

(b) DNA is complexed with lipids before inoculation into cells (Wheeler *et al*, 1996).

(c) Gold beads are coated with DNA and fired into cells using a gene-gun (Tang *et al*, 1992; Fynan *et al*, 1993; Klein and Fitzpatrick-McElligott, 1993; Montgomery *et al*, 1994).

The direct intracellular synthesis of desired antigens by DNA immunisations results in the generation of protective antibody and cell mediated immune responses (Fynan *et al*, 1993; Donnelly *et al*, 1997). The immune response from DNA vaccination appears to be long-lived, with plasmid DNA and encoded proteins detectable as late as 19 months after injection (Wolff *et al*, 1992). The efficacy of conventional DNA vaccines is greatly enhanced through the use of plasmids that place alphavirus replicons within RNA polymerase II cassettes (Herweijer *et al*, 1995; Dubensky *et al*, 1996; Berglund *et al*, 1998). Unlike conventional DNA vaccines, the CMV promoter does not drive the expression of the heterologous gene directly but directs synthesis of the recombinant alphaviral RNA transcript. This RNA is transported from the nucleus to the cytoplasm where the autocatalytic amplification of the alphavirus vector proceeds and leads to high levels of heterologous antigen expression. The layered DNA/RNA vectors have been used for *in vivo* challenge models (Hariharan *et al*, 1998; Berglund *et al*, 1998) and have been

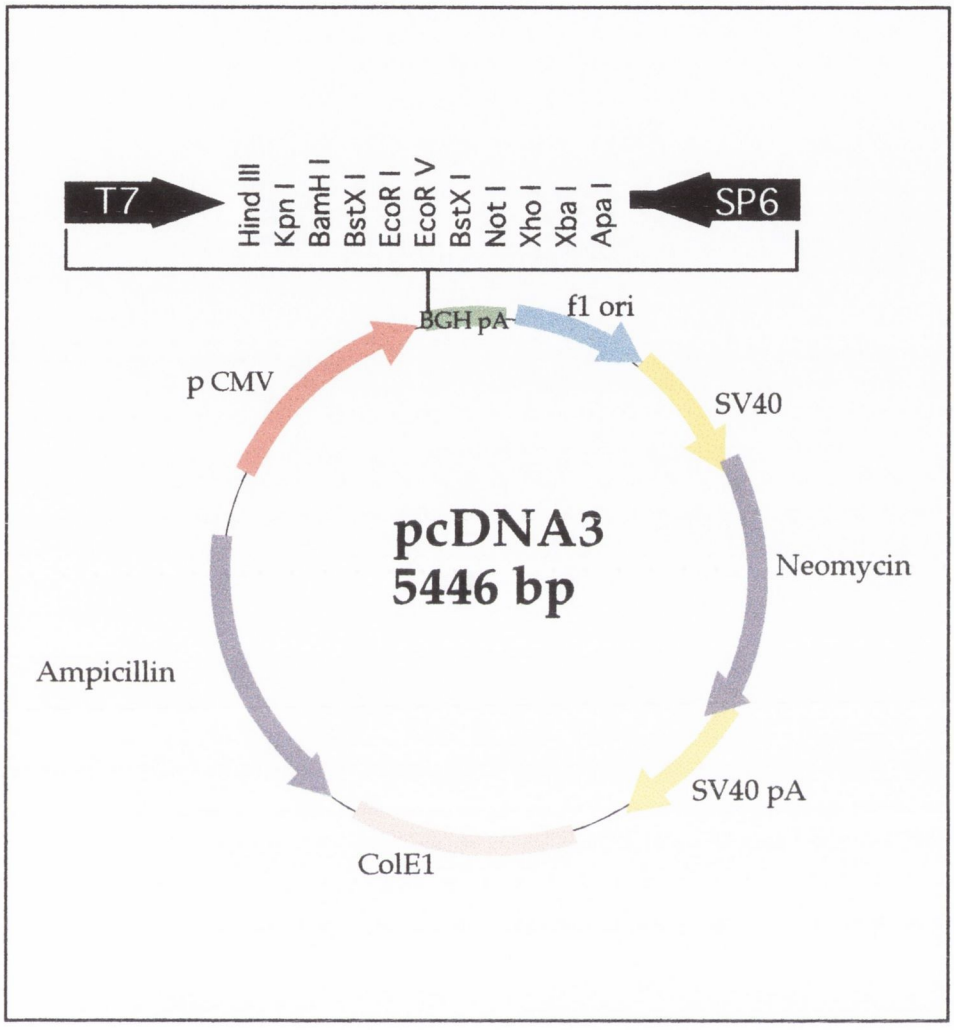

Figure 1.7 - pcDNA3 expression vector. The diagram shows the positions of restriction enzyme sites. The cytomegalovirus promoter drives expression of heterologous antigen that is inserted in the multiple cloning site flanked by T7 And SP6 promoters used for *in vitro* transcription. The plasmid contains an ampicillin resistance marker and the SV40 and ColE1 origins of replication. BGH pA: bovine growth hormone polyadenylation signal.

shown to elicit equal or superior protective immune responses against influenza and herpes simplex virus in mice. In general 10-1000 fold less DNA is required to stimulate the immune response.

. The SFV vector system is depicted in figure 1.8.

Figure 1.8 – SFV vector systems. A number of strategies are available to express heterologous antigens with alphavirus vectors. RNA transcribed *in vitro* from the basic SFV expression vector can be electroporated into cells. RNA produced in this way can also be packaged into recombinant particles using a helper vector. The recombinant particles are released from cells in the normal manner and can be purified and used to infect cells for heterologous expression of antigens. The SFV replicase can also be placed under the control of a eukaryotic promoter. DNA is directly transfected into cells where transcription occurs in the nucleus to produce mRNA, which is transported to the cytoplasm. In all three strategies, the RNA is released into the cytoplasm and produces the replicase, which is responsible for the production of full length minus strand RNAs and subgenomic RNA species. Translation of the subgenomic RNA is responsible for the expression of heterologous antigens.

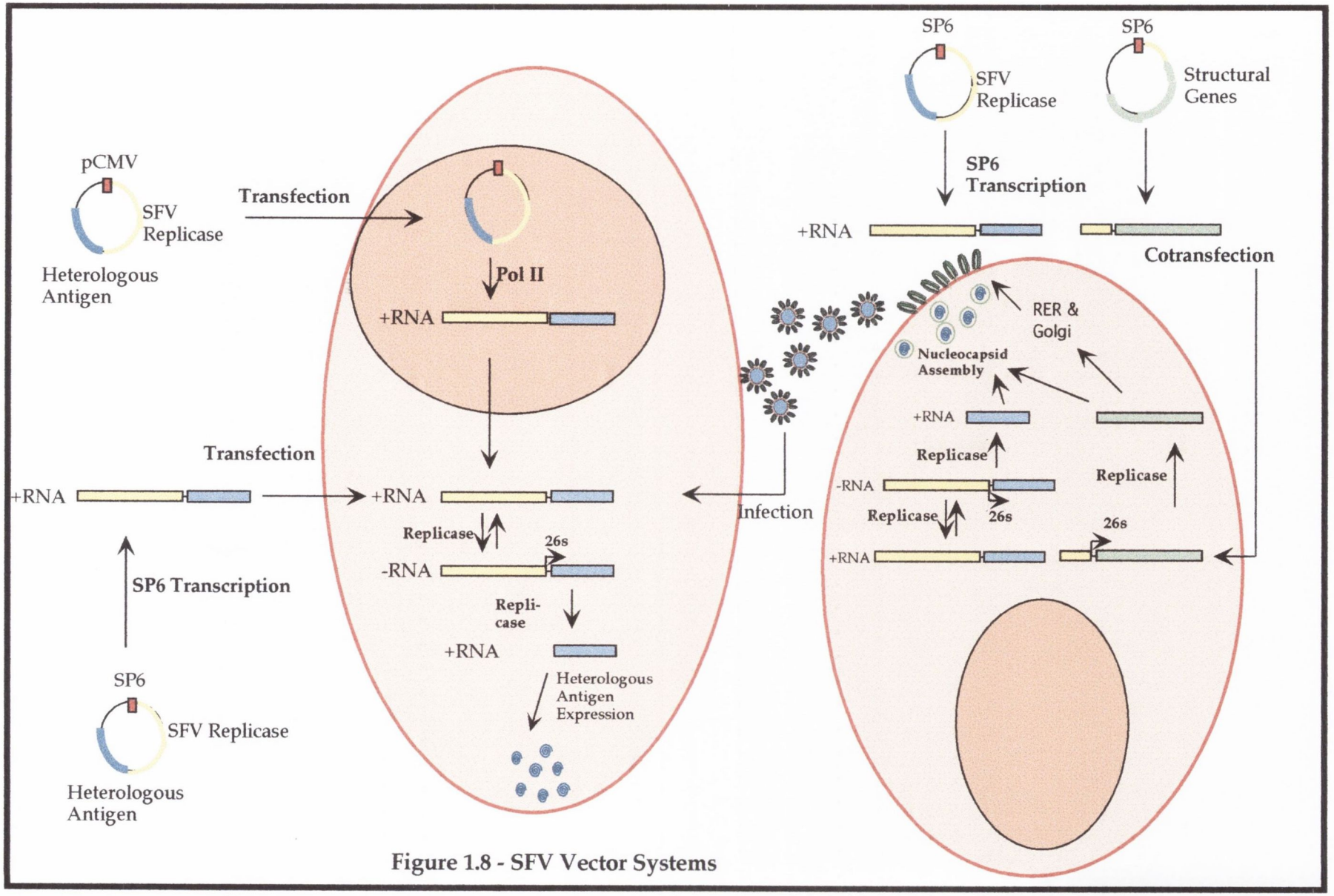

Figure 1.8 - SFV Vector Systems

1.5 Aims of project

The main objective of this project was to clone and over express IPNV genes in the SFV and layered SFV expression system. A Norwegian field isolate was used in the study. The A-segment of the RNA genome was amplified by RT-PCR and its sequence determined. Expression of the A-segment from the various systems was subsequently evaluated. This work was part of a consortium project set up and funded by the E.U. FAIR Programme in 1996. The consortium partners were :

Participant 1: National Pharmaceutical Biotechnology Centre, Dublin.

Participant 2: Karolinska Institute, Huddinge, Sweden.

Participant 3: Veterinary Sciences Division, Dept. Agriculture for N. Ireland, Belfast.

Participant 4: Central Veterinary Laboratory, Oslo, Norway.

Participant 5: VESO Vikan AkvaVet Research Station, 7815 Alhusstrand, Norway.

Consortium partner 2 was responsible for the development of the SFV expression system and provision of training in its use. The ability of the expression systems to deliver expressed antigens to fish cells in culture and to the immune system of salmonid fish was assessed by partner 3. Partners 4 and 5 were responsible for the development of standardised IPNV infection challenge models and laboratory assays to measure immune responses to IPNV. These were subsequently used to test the protective efficacy of candidate recombinant vaccines developed by partner 1 during the course of the project.

2.1 Introduction

The initial aim of the project was to clone and sequence the A-segment of a Norwegian field isolate of IPNV. The strain originated from a clinical outbreak of the disease in a hatchery on the western coast of Norway. Initial serotyping of the strain by project partners in Norway revealed that it was of the Sp serotype. A similar reaction to subtype N1 was observed when this strain was tested against a panel of MAbs. The cloning procedure was designed to initially amplify and clone the 1.5 kb VP2 region into a sequencing plasmid such as pCR-Script. This corresponds to the 5′ half of the A-segment. The 3′ end of the A-segment would subsequently be amplified and cloned in the same manner and the full-length sequence generated by "walking across" the genome in a stepwise fashion.

2.2 Materials and methods

2.2.1 Virus

The Sp strain of IPNV (IMP-986) was supplied by partner 4 (Central Veterinary Laboratory, Oslo, Norway).

2.2.2 Cells

The salmonid cell line, CHSE-214, was used for viral growth and RNA isolation. The cells were maintained in Eagle's MEM supplemented with 20mM glutamine, 1% non-essential amino acid, 10mM Hepes, 10% foetal calf serum (FCS) and 100 IU/ml penicillin, 100μg/ml streptomycin (GMEM). Maintenance medium (MMEM) consisting of GMEM with a reduced FCS concentration of 2% was used during virus growth.

2.2.3 Virus growth and RNA isolation

CHSE-214 cells were grown to confluency in 75 cm^2 flasks and inoculated with the IPNV field isolate. Following absorption of the virus for one hour at 15°C, 15 ml fresh MMEM were added and the cells incubated at 15°C until a cytopathic effect (cpe) was observed. At approximately 4 days post-infection total RNA was extracted from IPNV infected CHSE cells using the Genosys RNA isolator according to the protocol recommended by the supplier. RNA was placed at -70°C under 95% ethanol for long term storage.

2.2.4 Reverse transcription-PCR

Single stranded cDNA was synthesised from 400 ng total RNA using 150 ng HPLC purified primers and 15 units avian myeloblastis virus reverse transcriptase according to the protocol recommended by the supplier (Promega). Precipitated double-stranded RNA was resuspended in 10 µl of primer and denatured at 95°C for 10 minutes before the remainder of the reverse transcription mixture was added.

Standard PCR reaction mixtures contained 1.5 mM $MgCl_2$, 1x Thermo DNA poly buffer, 200 mM each dATP, dCTP, dGTP and dTTP and 100 ng each of forward and reverse primers along with 2 µl first strand cDNA mixture. The reaction was performed in an automated DNA thermal cycler (Hybaid Omnigene) programmed for 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and polymerisation at 72°C for 2 min. The final cycle included a polymerisation step at 72°C for 10 min.

"Hot-start" PCR was performed in all cases. In this procedure 2.5 units Taq polymerase (Promega) were added after denaturation and preheating of the sample to 94°C for 10 min. The primer pairs, with 5'-*Xma* I or *Hpa* I restriction ends (sequence not shown), are described in Table 1. Primer pairs 100F and 600R were used as positive controls for the RT-PCR reaction.

Primer name	Primer sequence: 5′-3′
IPNVF1	TACCCCGGGCCAACAATTCTTATCTRCATGAAT
IPNV R1	ATACCCGGGRGCTGCRTGTGACAGGTAGC
SPF1	ATACCCGGGGGTCTATATCAATRCAAG
SPR1	TATCCCGGGGTRGCGSCCTCCKGCKGC
3′F1	ACGGTTAACTACTGTCCACGCTGTTTCC
3′R1	GCGGTTAACGAAAGAGAGTAGTCGTTAC
100F	CAGATTCTGGCCGTCGTCC
600R	ATTTGTGTCAGATTCCAGGG

Table 2.1 - Oligonucleotide primers used in PCR amplification of IPNV (Norwegian field isolate). R= A or G; K= G or T; S= G or C.

2.2.5 DNA Cloning

2.2.5.1 Purification and ligation of PCR insert.

PCR products were checked by electrophoresis of a 20 µl amount on a 1.2% (w/v) agarose gel and subsequently purified using the Qiagen PCR purification kit (Figure 2.1). Ten µl of the purified PCR product were "polished" in order to remove A-overhangs using 0.5 units cloned *Pfu* polymerase (Stratagene), 1 µl 10 mM dNTP mix and 1.3 µl 10x polishing buffer (Stratagene) at 72°C for 30 min. The resulting blunt-ended PCR product was ligated to the pre-digested, blunt-ended pPCR-script vector according to the supplier's instructions (Stratagene). Two µl of the ligation mixture were used to transform Epicurian Coli XL1-Blue MRF Kan supercompetent cells (Stratagene) according to the manufacturer's protocol. Transformed cells were plated onto L-agar containing 100 µg/ml ampicillin, 40 µg/ml X-gal and 50 mM IPTG. Plates were incubated overnight at 37°C to allow bacterial growth.

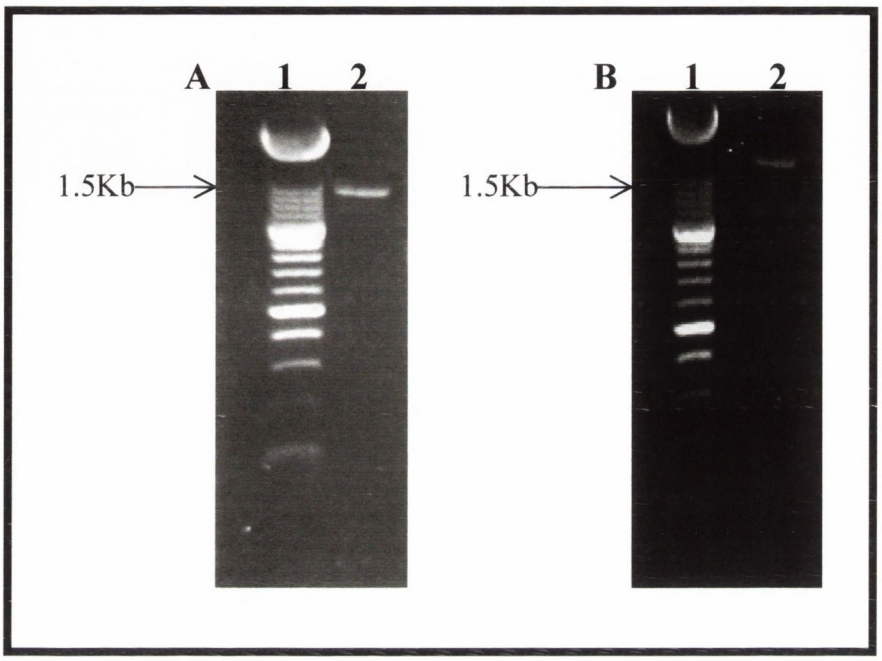

Figure 2.1 – RT-PCR products. Primers were designed to amplify the A-segment of IPNV in two stages. RT-PCR products were run on 1.2% agarose gels stained with ethidium bromide. Panel A: Lane1: 100 bp ladder. Lane 2: 1.5 kb RT-PCR product amplified using primers designed to amplify the VP2 region of the A-segment. Panel B: Lane 1: 100 bp ladder. Lane 2: 1.6 kb RT-PCR product amplified using primers designed to amplify the 3′ end of the A-segment.

2.2.5.2 Screening for recombinant plasmids.

Screening for recombinant clones was carried out by standard blue/white screening where positive clones containing IPNV inserts were identified as white colonies. Negative clones were identified as blue colonies. To confirm the presence of inserts, a putative positive white colony was inoculated into 10 ml L broth containing 100μg/ml ampicillin and incubated with shaking at 220 r.p.m at 37°C overnight. Plasmid DNA was isolated using the Qiagen Miniprep DNA purification system, digested with the appropriate enzyme to drop out the insert, and run on a 1% (w/v) agarose gel. (Figure 2.2)

2.2.6 DNA Sequencing.

A sequencing strategy to obtain the full-length A-segment sequence is depicted in figure 2.3.

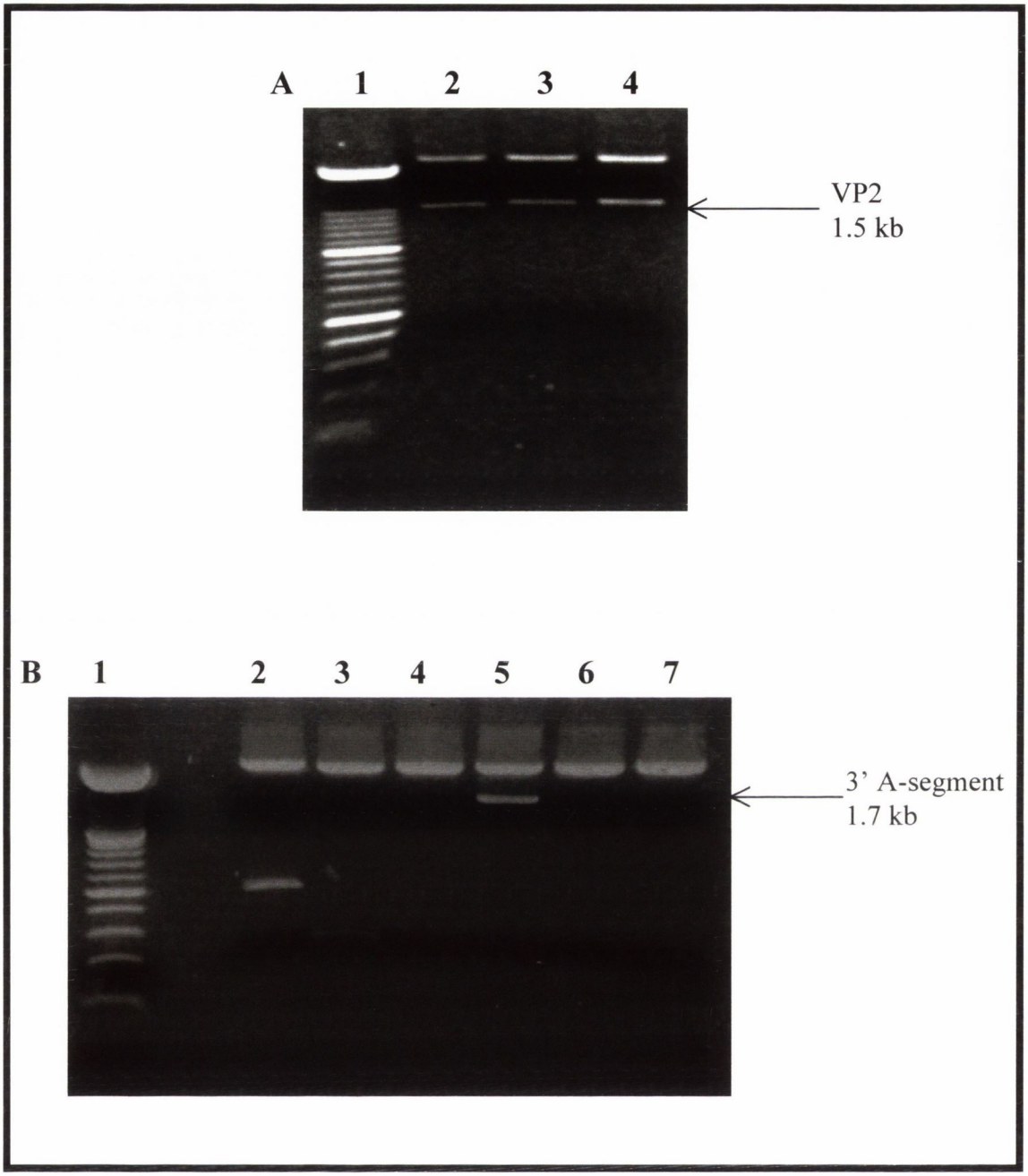

Figure 2.2 – Screening for recombinant clones. 1% agarose gels stained with ethidium bromide. Panel A: Lane 1: 100 bp ladder. Lanes 2-4: Putative pPCR-Script-VP2 clones digested with *Xma* I. A 1.5 kb fragment corresponding to the VP2 coding sequence is indicated by an arrow in lanes 2-4. Panel B: Lane 1: 100 bp ladder. Lanes 2-7 putative pPCR-Script-3'Asegment clones digested with *Hpa* I. A 1.7 kb fragment corresponding to the 3'end of the A-segment is indicated by an arrow in lane 5. Lanes 2-4 and 6-7 were false positive clones.

Figure 2.3 – Sequencing strategy. The VP2 and 3′ regions of the A-segment were cloned into the sequencing vector pPCR-Script. Panel A: *Xma* I, *Eco*R I and *Hin*d III deletions were made in pPCR-Script-VP2 clones of opposite orientations. These deletion mutants were sequenced using universal forward and reverse primers. Designing custom primers from the sequence already obtained completed the double stranded sequence of the VP2 region. Panel B: The 3′ end of the A-segment sequence was obtained using universal forward and reverse primers together with custom primers designed to complete the sequence. Red arrows denote M13 forward primer. Green arrows denote universal reverse primer. Custom designed primers are in bold. Sequencing reactions from pPCR-Script deletion mutants are in dotted lines.

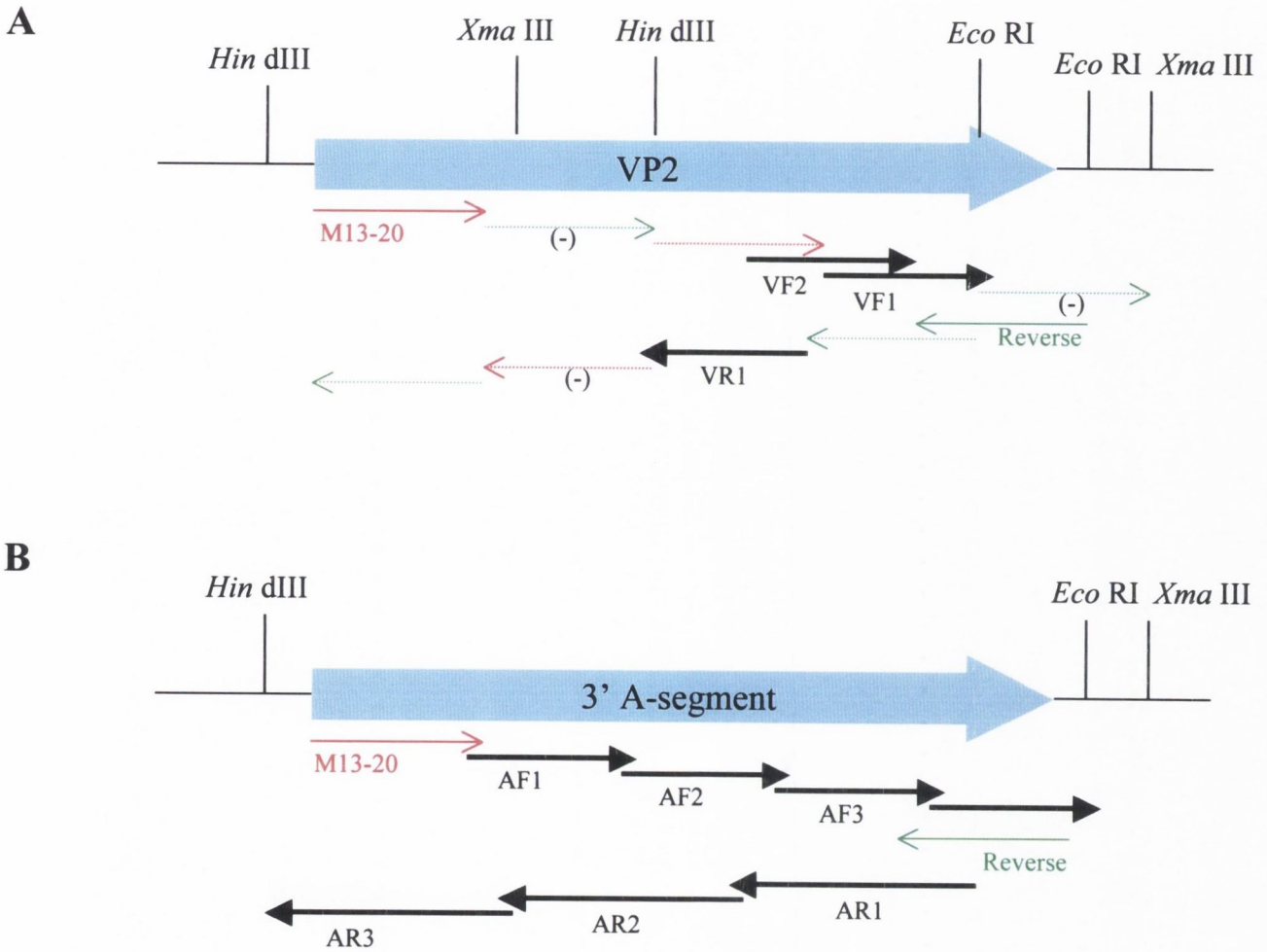

Figure 2.3 - Sequencing strategy.

2.2.6.1 Construction of pPCR-Script-VP2 deletions

A complete restriction analysis of Sp-VP2 (Leong, 1992) and Sp-IPNV sequences were performed using the GeneJockey II program. Using pPCR-Script-VP2 clones with the VP2 insert in both orientations *Hind* III, *Xma* I and *Eco*R I deletions were constructed using conventional methods of DNA manipulation (Sambrook *et al*, 1989).

2.2.6.2 Isolation and purification of recombinant DNA

For DNA sequencing, bacterial colonies containing the recombinant DNA plasmid were grown in 25 ml L broth supplemented with 100 μg/ml ampicillin, shaking at 222 r.p.m. for 16 h at 37°C. The bacteria were pelleted by centrifugation at 6,000 g for 10 min. Plasmid DNA was isolated from the bacterial pellet using the Qiagen Midiprep kit. The concentration of DNA obtained by the procedure was determined by agarose gel electrophoresis with a sample of plasmid DNA of known concentration.

2.2.6.3 Dye terminator sequencing reactions.

Sequencing reactions were performed using Thermosequenase sequencing kit (Amersham). Custom oligonucleotides were designed using the published sequences of Sp strains of IPNV. Sequencing was carried out using automated cycle sequencing and an ABI 373A automated DNA sequencer. The sequence was viewed by computer generated colour coded chromatograph. Both strands of the DNA were sequenced.

2.2.6.4 Sequence analysis

Multiple alignments of sequences were generated using the CLUSTAL W alignment program.

2.3 Results

2.3.1 RT-PCR

A CLUSTAL alignment, comparing the amino acid sequences of 24 IPNV strains, was performed and a consensus sequence chosen for primer design. Both primers IPNVF1 and IPNVR1 were used in an RT-PCR reaction, however, no cDNA products were obtained, suggesting that the oligonucleotide sequences

were not sufficiently similar to prime cDNA synthesis. Primer pair 100F and 600R, salmon genomic primers designed to amplify cDNA encoding a 500 bp fragment of the ribosomal protein, L18, confirmed that the reverse transcription and PCR steps were working. Subsequent serological analysis by project partners in Norway indicated that the Norwegian field isolate was an Sp-like serotype virus. A second set of primers (SPF1 and SPR1) based on the published Sp-VP2 sequence (Leong, 1992) was designed. These primers successfully amplified the 1.5 kb fragment encoding the IPNV VP2 gene. The 1.6 kb 3′ end of the A-segment was amplified using the HPLC purified primers 3′F1 and 3′R1. This primer pair was designed using published Sp-like sequences.

2.3.2 DNA cloning.

Three clones containing the 1.5 kb VP2 cDNA insert were obtained. These were subjected to restriction analysis with *Sma* I, *Bam*H I, *Eco*R I, *Hin*d III and *Sal* I and found to be identical to a published VP2 gene sequence. The cDNA insert in one of the clones was present in the opposite orientation to the others. To enable the full-length sequence of the VP2 clones to be obtained a number of deletions were constructed in two clones of opposite orientation.

Three clones containing the 1.6 kb 3′ A-segment cDNA insert were obtained.

2.3.3 Sequencing.

Each clone and deletion mutant was partially sequenced from both ends using universal forward and reverse primers. Designing custom primers from the sequence previously obtained completed gaps in the double stranded sequence of VP2. Custom primers were also designed to complete the sequence of the 3′ end of the A-segment.

The nucleotide and deduced amino acid sequence of the A-segment is shown in figure 2.4. The deduced amino acid sequence was compared with a number of previously published sequences including the N1 (Havarstein *et al,* 1990) and Sp (Leong, 1992) sequences (figure 2.5) using the CLUSTAL W alignment program.

2.4 Discussion

A virulent Norwegian field isolate was characterised by project partners in Norway and found to be an Sp-like serotype of IPNV. The virus was grown in tissue culture and total RNA extracted. cDNA from the virus was amplified and the A-segment open reading frame cloned and sequenced. Initially a CLUSTAL alignment of known IPNV sequences was performed and a consensus sequence chosen as primers to amplify the VP2 gene in a RT-PCR reaction. However no cDNA products were obtained with these primers. This suggested that the

Figure 2.4 – Nucleotide and deduced amino acid sequence of IPNV field isolate A-segment. The initiation methionine of the polyprotein is indicated in red type face. The site of the mutation resulting in truncated VP2 protein is indicated in blue.

```
GGT CTA TAT CAA TAC AAG ATG AAC ACA AAC AAG GCA ACC GCA ACT TAC CTG AAA TCC ATT
 G   L   Y   Q   Y   K   M   N   T   N   K   A   T   A   T   Y   L   K   S   I

ATG CTT CCA GAG ACT GGA CCA GCA AGC ATC CCG GAC GAC ATA ACG GAG AGA CAC ATC TTA
 M   L   P   E   T   G   P   A   S   I   P   D   D   I   T   E   R   H   I   L

AAA CAA GAG ACC TCG TCA TAC AAC TTA GAG GTC TCC GAA TCA GGA AGT GGC ATT CTT GTT
 K   Q   E   T   S   S   Y   N   L   E   V   S   E   S   G   S   G   I   L   V

TGT TTC CCT GGG GCA CCA GGC TCA CGG ATC GGT GCA CAC TAC AGA TGG AAT GCG AAC CAG
 C   F   P   G   A   P   G   S   R   I   G   A   H   Y   R   W   N   A   N   Q

ACG GGG CTG GAG TTC GAC CAG TGG CTG GAG ACG TCG CAG GAC CTG AAG AAA GCC TTC AAC
 T   G   L   E   F   D   Q   W   L   E   T   S   Q   D   L   K   K   A   F   N

TAC GGG AGG CTG ATC TCA AGG AAA TAC GAC ATC CAA AGC TCC ACA CTA CCG GCC GGT CTC
 Y   G   R   L   I   S   R   K   Y   D   I   Q   S   S   T   L   P   A   G   L

TAT GCT CTG AAC GGG ACG CTC AAC GCT GCC ACC TTC GAA GGC AGT CTG TCT GAG GTG GAG
 Y   A   L   N   G   T   L   N   A   A   T   F   E   G   S   L   S   E   V   E

AGC CTG ACC TAC AAC AGC CTG ATG TCC CTA ACA ACG AAC CCC CAG GAC AAA GTC AAC AAC
 S   L   T   Y   N   S   L   M   S   L   T   T   N   P   Q   D   K   V   N   N

CAG CTG GTG ACC AAA GGA GTC ACA GTC CTG AAT TTA CCA ACA GGG TTC GAC AAA CCA TAC
 Q   L   V   T   K   G   V   T   V   L   N   L   P   T   G   F   D   K   P   Y

GTC CGC CTA GAG GAC GAG ACA CCC CAG GGT CTC CAG TCA ATG AAC GGG GCC AAG ATG AGG
 V   R   L   E   D   E   T   P   Q   G   L   Q   S   M   N   G   A   K   M   R

TGC ACA GCT GCA ATT GCA CCG CGG AGG TAC GAG ATC GAC CTC CCA TCC CAA CGC CTA CCC
 C   T   A   A   I   A   P   R   R   Y   E   I   D   L   P   S   Q   R   L   P

CCC GTT ACT GCG ACA GGA GCC CTC ACC ACT CTC TAC GAA GGA AAC GCC GAC ATC GTC AAC
 P   V   T   A   T   G   A   L   T   T   L   Y   E   G   N   A   D   I   V   N

TCC ACG ACA GCG ACG GGA GAC ATA AAC TTC AGT CTG ACA GAA CAA CCC GCG GTC GAG ACC
 S   T   T   A   T   G   D   I   N   F   S   L   T   E   Q   P   A   V   E   T

AAG TTC CAC TTC CAG CTG GAC TTC ATG GGC CTT GAC AAC GAC GTC CCA GTT GTC ACA GTG
 K   F   H   F   Q   L   D   F   M   G   L   D   N   D   V   P   V   V   T   V

GTC AGC TCC GTG CTG GCC ACA AAT GAC AAC TAC AGA GGA GTC TCA GCC AAG ATG ACC CAG
 V   S   S   V   L   A   T   N   D   N   Y   R   G   V   S   A   K   M   T   Q

TCC ATC CCG ACC GAG AAC ATC ACC AAG CCG ATC ACC AGG GTC AAG CTG TCA TAC AAG ATC
 S   I   P   T   E   N   I   T   K   P   I   T   R   V   K   L   S   Y   K   I

AAC CAG CAG ACA GCA ATC GGC AAC GTC ACC ACC CTG GGC ACA ATG GGT CCA GCA TCC GTC
 N   Q   Q   T   A   I   G   N   V   T   T   L   G   T   M   G   P   A   S   V

TCC TTC TCA TCA GGG AAC GGA AAT GTC CCC GGC GTG CTC AGA CCA ATC ACA CTG GTG GCC
 S   F   S   S   G   N   G   N   V   P   G   V   L   R   P   I   T   L   V   A

TAT GAG AAG ATG ACA CCG CTG TCC ATC CTG ACC GTA GCT GGA GTG TCC AAT TAC GAG CTG
 Y   E   K   M   T   P   L   S   I   L   T   V   A   G   V   S   N   Y   E   L
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ATC CCA AAC CCA GAA CTC CTC AAG AAC ATG GTG ACA CGC TAT GGC AAG TAC GAC CCC GAA
 I   P   N   P   E   L   L   K   N   M   V   T   R   Y   G   K   Y   D   P   E

GGT CTC AAC TAT GCC AAG ATG ATC CTG TCC CAC AGG GAA GAG CTG GAC ATC AGG ACA GTG
 G   L   N   Y   A   K   M   I   L   S   H   R   E   E   L   D   I   R   T   V

TGG AGG ACA GAG GAG TAC AAG GAG AGG ACC AGA GTC TTC AAC GAA ATC ACG GAC TTC TCC
 W   R   T   E   E   Y   K   E   R   T   R   V   F   N   E   I   T   D   F   S

AGT GAC CTG CCC ACG TCA AAG GCA TGG GGC TGG AGA GAC ATA GTC AGA GGA ATT CGG AAA
 S   D   L   P   T   S   K   A   W   G   W   R   D   I   V   R   G   I   R   K

GTC GCA GCT CCT GTA CTG TCC ACG CTG TTT CCA ATG GCA GCA CCA CTC ATA GGA ATG GCA
 V   A   A   P   V   L   S   T   L   F   P   M   A   A   P   L   I   G   M   A

GAC CAA TTC ATT GGA GAT CTC ACC AAG ACC AAC GCA GCA GGC GGA AGG TAC CAC TCC ATG
 D   Q   F   I   G   D   L   T   K   T   N   A   A   G   G   R   Y   H   S   M

GCC GCA GGA GGG CGC CAC AAA GAC GTG CTC GAG TCC TGG GCA AGC GGA GGG CCC GAC GGA
 A   A   G   G   R   H   K   D   V   L   E   S   W   A   S   G   G   P   D   G

AAA TTC TCC CGA GCC CTC AAG AAC AGG CTG GAG TCC GCC AAC TAC GAG GAA GTC GAG CTT
 K   F   S   R   A   L   K   N   R   L   E   S   A   N   Y   E   E   V   E   L

CCA CCC CCC TCA AAA GGA GTC ATC GTC CCT GTG GTG CAC ACA GTC AAG AGT GCA CCA GGC
 P   P   P   S   K   G   V   I   V   P   V   V   H   T   V   K   S   A   P   G

GAG GCA TTC GGG TCC CTG GCA ATC ATA ATT CCA GGG GAG TAC CCC GAG CTT CTA GAT GCC
 E   A   F   G   S   L   A   I   I   I   P   G   E   Y   P   E   L   L   D   A

AAC CAG CAG GTC CTA TCC CAC TTC GCA AAC GAC ACC GGG AGC GTG TGG GGC ATA GGA GAG
 N   Q   Q   V   L   S   H   F   A   N   D   T   G   S   V   W   G   I   G   E

GAC ATA CCC TTC GAG GGA GAC AAC ATG TGC TAC ACT GCA CTC CCA CTC AAG GAG ATC AAA
 D   I   P   F   E   G   D   N   M   C   Y   T   A   L   P   L   K   E   I   K

AGA AAC GGG AAC ATA GTA GTC GAG AAG ATC TTT GCT GGA CCA ATC ATG GGT CCC TCT GCT
 R   N   G   N   I   V   V   E   K   I   F   A   G   P   I   M   G   P   S   A

CTA CTA GGA CTG TCC CTA CTT GTG AAC GAC ATC GAG GAC GGA GTT CCA AGG ATG GTA TTC
 L   L   G   L   S   L   L   V   N   D   I   E   D   G   V   P   R   M   V   F

ACC GGC GAA ATC GCC GAT GAC GAG GAG ACA ATC ATA CCA ATC TGC GGT GTA GAC ATC GAA
 T   G   E   I   A   D   D   E   E   T   I   I   P   I   C   G   V   D   I   E

GCC ATC GCA GCC CAT GAA CAA GGG CTG CCA CTC ATC GGC AAC CAA CCA GGA GTG GAC GAG
 A   I   A   A   H   E   Q   G   L   P   L   I   G   N   Q   P   G   V   D   E

GAG GTG CGA AAC ACA TCC CTG GCC GCA CAC CTG ATC CAG ACC GGA ACC CTG CCC GTA CAA
 E   V   R   N   T   S   L   A   A   H   L   I   Q   T   G   T   L   P   V   Q

CGC GCA AAG GGC TCC AAC AAG AGG ATC AAG TAC CTG GGA GAG CTG ATG GCA TCA AAT GCA
 R   A   K   G   S   N   K   R   I   K   Y   L   G   E   L   M   A   S   N   A

TCC GGG ATG GAC GAG GAA CTG CAA CGC CTC CTG AAC GCC ACA ATG GCA CGG GCC AAA GAA
 S   G   M   D   E   E   L   Q   R   L   L   N   A   T   M   A   R   A   K   E
```
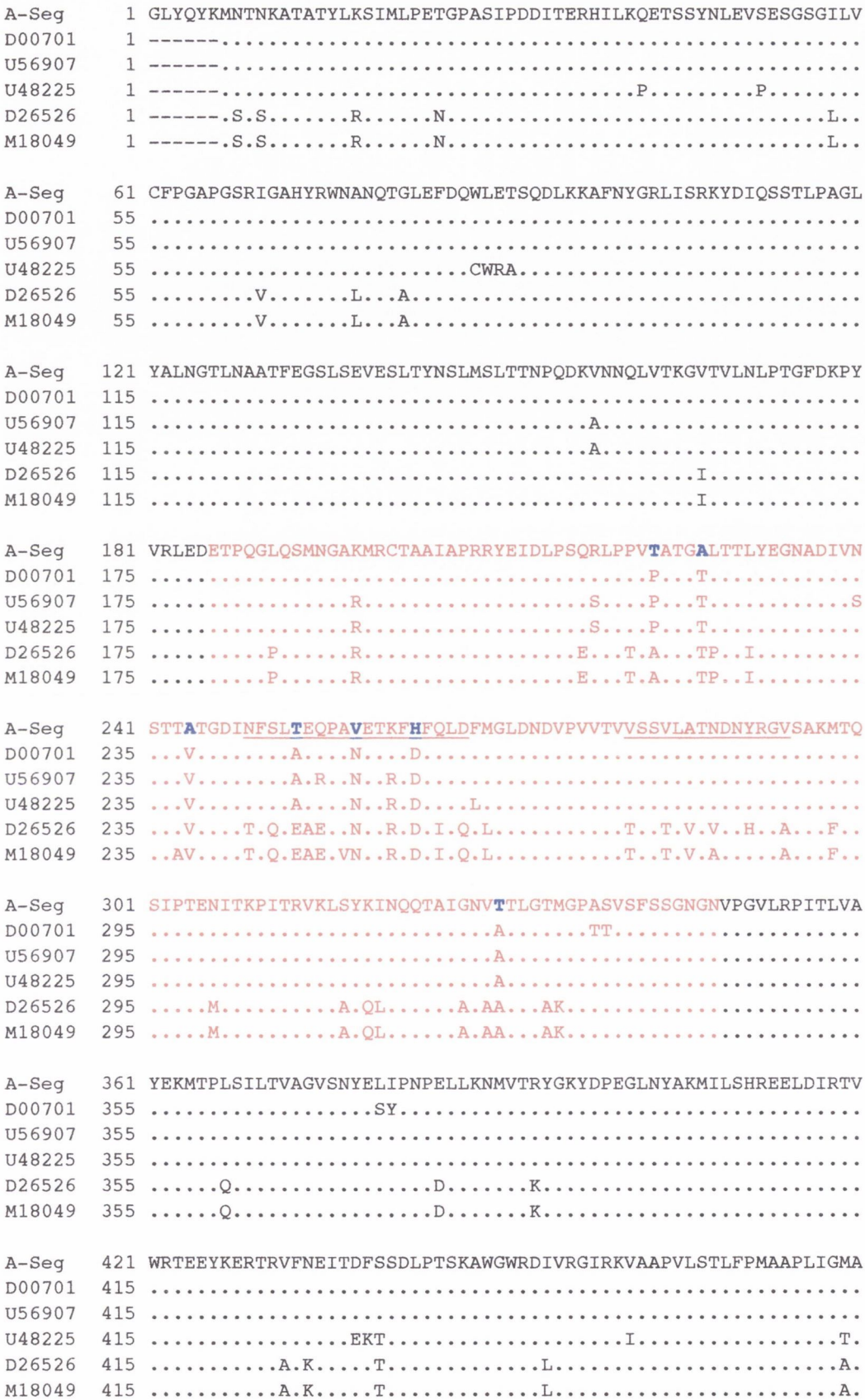

```
GTC CAG GAC GCC GAG ATC TAC AAA CTT CTT AAG CTC ATG GCA TGG ACC AGA AAG AAC GAC
 V   Q   D   A   E   I   Y   K   L   L   K   L   M   A   W   T   R   K   N   D

CTC ACC GAC CAC ATG TAC GAG TGG TCA AAA GAG GAC CCC GAT GCA CTA AAG TTC GGA AGG
 L   T   D   H   M   Y   E   W   S   K   E   D   P   D   A   L   K   F   G   R

CTC ATC AGC ACG CCA CCA AAG CAC CCC GAG AAG CCC AAA GGA CCA GAC CAA CAC CAC GCC
 L   I   S   T   P   P   K   H   P   E   K   P   K   G   P   D   Q   H   H   A

CAA GAG GCG AGA GCC ACC CGC ATA TCA CTG GAC GCC GTG AGA GCC GGG GCG GAC TTC GCC
 Q   E   A   R   A   T   R   I   S   L   D   A   V   R   A   G   A   D   F   A

ACA CCG GAA TGG GTC GCG CTG AAC AAC TAC CGC GGC CCA TCT CCC GGG CAG TTC AAG TAC
 T   P   E   W   V   A   L   N   N   Y   R   G   P   S   P   G   Q   F   K   Y

TAC CTG ATC ACT GGA CGA GAA CCA GAA CCA GGT GAT GAG TAC GAG GAC TAC ATA AAA CAA
 Y   L   I   T   G   R   E   P   E   P   G   D   E   Y   E   D   Y   I   K   Q

CCC ATT GTG AAA CCG ACC GAC ATG AAC AAA ATC AGA CGT CTA GCC AAC AGT GTG TAC GGC
 P   I   V   K   P   T   D   M   N   K   I   R   R   L   A   N   S   V   Y   G

CTC CCA CAC CAG GAA CCA GCA CCA GAG GAG TTC TAC GAT GCA GTT GCA GCT GTA TTC GCA
 L   P   H   Q   E   P   A   P   E   E   F   Y   D   A   V   A   A   V   F   A

CAG AAC GGA GGC AGA GGT CCC GAC CAG GAC CAA ATG CAA GAC CTC AGG GAG CTC GCA AGA
 Q   N   G   G   R   G   P   D   Q   D   Q   M   Q   D   L   R   E   L   A   R

CAG ATG AAA CGA CGA CCC CGG AAC GCC GAT GCA CCA CGG AGA ACC AGA GCG CCA GCG GAA
 Q   M   K   R   R   P   R   N   A   D   A   P   R   R   T   R   A   P   A   E

CCG GCA CCG CCC GGA CGC TCA AGG TTC ACC CCC AGC GGA GAC AAC GCT GAG GTG
 P   A   P   P   G   R   S   R   F   T   P   S   G   D   N   A   E   V
```

Figure 2.4 – Nucleotide and deduced amino acid sequence of IPNV field isolate A-segment.

Figure 2.5 – Amino acid sequence alignment of the A-segment polyprotein from Norwegian field isolate with others from the database. D00701: strain N1, U56907: strain Sp, U48225: strain Sp, D26526: strain DRT, M18049: strain Jasper. The VP2 variable region is marked by red type-face and hypervariable domains are underlined. Amino acid changes are indicated by blue type-face.


A-Seg 1 GLYQYKMNTNKATATYLKSIMLPETGPASIPDDITERHILKQETSSYNLEVSESGSGILV
D00701 1 ------
U56907 1 ------
U48225 1 ------P.....P.....
D26526 1 ------.S.S.....R.....N.....L..
M18049 1 ------.S.S.....R.....N.....L..
A-Seg 61 CFPGAPGSRIGAHYRWNANQTGLEFDQWLETSQDLKKAFNYGRLISRKYDIQSSTLPAGL
U48225 55 .....CWRA.....
D26526 55 .....V.....L...A.....
M18049 55 .....V.....L...A.....
A-Seg 121 YALNGTLNAATFEGSLSEVESLTYNSLMSLTTNPQDKVNNQLVTKGVTVLNLPTGFDKPY
U56907 115 .....A.....
U48225 115 .....A.....
D26526 115 .....I.....
M18049 115 .....I.....
A-Seg 181 VRLEDETPQGLQSMNGAKMRCTAAIAPRRYEIDLPSQRLPPVTATGALTTLYEGNADIVN
D00701 175 .....P...T.....
U56907 175 .....R.....S....P...T.....S
U48225 175 .....R.....S....P...T.....
D26526 175 .....P.....R.....E...T.A...TP..I.....
M18049 175 .....P.....R.....E...T.A...TP..I.....
A-Seg 241 STTATGDINFSLTEQPAVETKFHFQLDFMGLDNDVPVVTVVSSVLATNDNYRGVSAKMTQ
D00701 235 ...V.....A....N....D.....
U56907 235 ...V.....A.R..N..R.D.....
U48225 235 ...V.....A....N..R.D....L.....
D26526 235 ...V....T.Q.EAE..N..R.D.I.Q.L.....T..T.V.V..H..A...F..
M18049 235 ..AV....T.Q.EAE.VN..R.D.I.Q.L.....T..T.V.A.....A...F..
A-Seg 301 SIPTENITKPITRVKLSYKINQQTAIGNVTTLGTMGPASVSFSSGNGNVPGVLRPITLVA
D00701 295 .....A.....TT.....
U56907 295 .....A.....
U48225 295 .....A.....
D26526 295 .....M.....A.QL.....A.AA...AK.....
M18049 295 .....M.....A.QL.....A.AA...AK.....
A-Seg 361 YEKMTPLSILTVAGVSNYELIPNPELLKNMVTRYGKYDPEGLNYAKMILSHREELDIRTV
D00701 355 .....SY.....
U56907 355 .....
U48225 355 .....
D26526 355 .....Q.....D.....K.....
M18049 355 .....Q.....D.....K.....
A-Seg 421 WRTEEYKERTRVFNEITDFSSDLPTSKAWGWRDIVRGIRKVAAPVLSTLFPMAAPLIGMA
D00701 415 .....
U56907 415 .....
U48225 415 .....EKT.....I.....T.
D26526 415 .....A.K.....T.....L.....A.
M18049 415 .....A.K.....T.....L.....A.

```
A-Seg   481 DQFIGDLTKTNAAGGRYHSMAAGGRHKDVLESWASGGPDGKFSRALKNRLESANYEEVEL
D00701  475 ............................................................
U56907  475 ........................Y...................................
U48225  475 ........................Y...................................
D26526  475 ..........S.....L.H.....YH..MD.....SEA.SY.KH..T....N.......
M18049  475 ..........S.....L.H.....YH..MD.....SEA.SY.KH..T....N.......

A-Seg   541 PPPSKGVIVPVVHTVKSAPGEAFGSLAIIIPGEYPELLDANQQVLSHFANDTGSVWGIGE
D00701  535 ............................................................
U56907  535 ............................................................
U48225  535 ..........................R....SF..........................
D26526  535 .K.T....F......E..........VVV...AH.....P......Y.K....CI.....
M18049  535 .K.T....F......E..........VVV..EA......P......Y.K....C......

A-Seg   601 DIPFEGDNMCYTALPLKEIKRNGNIVVEKIFAGPIMGPSALLGLSLLVNDIEDGVPRMVF
D00701  595 ........................................Q...................
U56907  595 ........................................Q...................
U48225  595 ........................................Q...................
D26526  595 .......D............................A....SQ.A........DE.I.....
M18049  595 .......D............................A....SQ.A........DE.I.....

A-Seg   661 TGEIADDEETIIPICGVDIEAIAAHEQGLPLIGNQPGVDEEVRNTSLAAHLIQTGTLPVQ
D00701  655 ..................K......P..............................
U56907  655 ..................K.....................................
U48225  655 ..................K.....................................
D26526  655 ..........V........K.M....H......C......M.A.....S....S.A....
M18049  655 ..........V........K......H......C......M.A.....S....G.A....

A-Seg   721 RAKGSNKRIKYLGELMASNASGMDEELQRLLNATMARAKEVQDAEIYKLLKLMAWTRKND
D00701  715 ............................................................
U56907  715 ............................................................
U48225  715 ............................................................
D26526  715 K.Q.ACR......Q..RTT.....A...G..Q.........K...VF......S......
M18049  715 K.Q.ACR......Q..RTT.....A...G..Q.........K...VF......S......

A-Seg   781 LTDHMYEWSKEDPDALKFGRLISTPPKHPEKPKGPDQHHAQEARATRISLDAVRAGADFA
D00701  775 ..................K.....................................
U56907  775 ..................K.....................................
U48225  775 ..................K.....................................
D26526  775 ..............I............Q.........T....K.........K......
M18049  775 ..............I.....V......Q.........T....K.........K......

A-Seg   841 TPEWVALNNYRGPSPGQFKYYLITGREPEPGDEYEDYIKQPIVKPTDMNKIRRLANSVYG
D00701  835 ............................................................
U56907  835 ..........................................................T.....
U48225  835 ..............................Q.............................
D26526  835 S...I.E..............M....V.N..E.....VRK..TR....D...........
M18049  835 S...I.E..............M....V.N..E.....VRK..TR....D...........

A-Seg   901 LPHQEPAPEEFYDAVAAVFAQNGGRGPDQDQMQDLRELARQMKRRPRNADAPRRTRAPAE
D00701  895 ............................................................
U56907  895 ............................................................
U48225  895 ............................................................GK
D26526  895 ........DD..Q..VE...E..............D..........P.ETR.Q.KT.PR
M18049  895 ........DD..Q..VE...E..............D..........P.ETR.Q.KT.PR
```

```
A-Seg    961 PAPPGRSRFTPSGDNAEV
D00701   955 ..................
U56907   955 ..................
U48225   955 ....DV............
D26526   955 A.TSSG........DG..
M18049   955 A.TSSG........DG..
```

Figure 2.5 – Amino acid sequence alignment of the A-segment polyprotein from Norwegian field isolate with others from the database.

oligonucleotide sequences were not sufficiently similar to prime cDNA synthesis. RNA fingerprinting analysis of IPN viruses demonstrates that IPNV Sp strains may be regarded as an evolutionary outgroup (Hsu *et al*, 1995). This may account for the failure of the first oligonucleotide pair to prime cDNA synthesis. Primers subsequently designed using the Sp serotype sequence successfully amplified VP2.

European isolates mainly belong to the Sp, Ab, He or Te serotypes (Hill and Way, 1995). IPNV was first isolated in Norway in 1976 as an Ab isolate with N1 serotype first discovered in 1986 (Christie *et al*, 1988). Initially N1 was believed to form a separate serogroup, A10 (Christie *et al*, 1988). Subsequently the N1 and Sp type strains were found to differ in reaction with only one of a panel of six MAbs and the two strains were placed in the same serogroup, A2 (Melby and Christie, 1994). Serological tests based on the reaction with a panel of MAbs indicated that the majority of IPNV field strains in Norway are antigenically indistinguishable from the N1 strain and therefore belong to the Sp serotype (Christie *et al*, 1990; Melby and Christie, 1994; Melby *et al*, 1994). The Sp strain may be associated with outbreaks of IPN in Atlantic salmon post-smolts (Smail *et al*, 1992; Melby *et al*, 1994). The inclusion of N1 as a member of the Sp serotype is confirmed by genomic analysis. Restriction fragment profiles of a 359 bp region amplified from 37 IPNV strains resulted in the formation of three major groups of IPNV strains (Heppel *et al*, 1992). These groups are termed genogroups and each contains several distinct subgroups. The restriction fragment profiles of Sp and N1 place the two strains in the same subgroup. When the same region is sequenced the

resulting amino acid sequence of the Sp and N1 strains are almost identical (Heppel *et al*, 1993). A larger segment of the VP2 region of Sp and N1 has also been sequenced. The two strains were found to be 97.4% identical at the nucleotide level over a 578 bp region, with only two amino acid changes (Pryde *et al*, 1993).

The full-length sequence of the A-segment from the Norwegian field isolate shows extensive homology to Sp strains. The alignment in figure 2.6 indicates that the field isolate is most homologous to the N1 strain, D00701. A BLAST homology search using the deduced amino acid sequence indicated that our field isolate was 98.9% identical to the published nucleotide sequences for strains N1 and Sp, and 99.0 % identical to the deduced amino acid sequence of these strains. When amino acid sequences of different serotypes are compared it is evident that VP2 is the most conserved protein encoded by the genome. The VP2 proteins of birnaviruses have highly conserved N and C termini with less conserved internal regions (Havarstein *et al*, 1990; Kibenge *et al*, 1990; Bayliss *et al*, 1990). The major neutralisation epitopes of birnaviruses are also located within VP2. The serotype specific epitopes, responsible for the induction of neutralising protective antibodies, are highly conformation dependent (Azad *et al*, 1987; Caswell-Reno *et al*, 1986) and are localised to the variable region of the gene (Azad *et al*, 1987; Bayliss *et al*, 1990). Looking at the CLUSTAL alignment in figure 2.6, it is evident that the majority of sequence replacements occur in the variable region of the VP2 gene. The hydrophobic fragment linking the two hypervariable regions has been

shown to tolerate a certain variation in amino acid sequence within serotypes without explicit consequences for antigenic properties or replication of the virus. However even one single amino acid change within the hypervariable region was sufficient to alter the specificity of the epitope in IBDV (Schnitzler *et al*, 1993). Serogroup A strains of IPNV were shown to contain at least three partly overlapping neutralisation epitopes (Frost *et* al, 1995). Initial reports stated that MAbs H8 and B9, against variable neutralisation epitopes were unable to recognise the Sp strain (Christie *et al*, 1990). The high variability of epiotpes H8 and B9 was confirmed when later clones of the same passage of Sp type strain gave a positive reaction with MAb-H8 and reacted variably with MAb-B9 (Melby and Christie, 1994). Of European isolates only Sp and N1 strains contain the variable H8 epitope. The same epitope is also found on the Canadian Jasper strains and American West-Buxton strains despite the high degree of sequence variation between these and the European isolates over the H8 epitope region.

Initial sequencing over the VP2 region in clone pBLVP236 revealed a point mutation changing a Trp codon (TGG), positioned approximately 50 amino acids from the probable end of the VP2 protein, to an amber stop codon (TAG). This would result in the premature termination of the VP2 protein. The location of this mutation is marked in bold on figure 2.5. Following repeated cDNA synthesis and sequence analysis a clone was obtained which did not contain the amber stop codon (pBLVP28). Premature termination of the VP2 protein has also been detected when this region is cloned into pUC19 and expressed in *E. coli*. The

polypeptides produced react with IPNV and VP2 polyclonal antisera in Western blots (Leong, 1992; Manning and Leong, 1990) and would therefore be expected to confer immunity to IPNV infection if used in a recombinant vaccine.

The A-segment of birnaviruses is produced as a polyprotein that is co-translationally cleaved to produce VP2, VP3 and VP4 (Duncan and Dobos, 1986; Hudson *et al*, 1986). VP4 is believed to be the viral autoprotease, which cleaves the polyprotein. Comparisons of IPNV and IBDV A-segment sequences reveals no apparent homology in the VP4 region (Kibenge *et a*l, 1990) and neither protein shows significant homology to known proteases (Hudson *et al*, 1986). A number of putative cleavage sites have been suggested. For IBDV these include the dibasic residues, R-R or K-R, and the motif A-X-A-A-S which is repeated three times between residues 483-503 and also appears at 752-756 (Hudson *et al*, 1986). Postulated cleavage sites for IPNV include Y-L dipeptide (Duncan *et al*, 1987). This motif is replaced by a Y-H dipeptide at the VP2-VP4 junction in Sp or N1 sequences and also in our field isolate. Although the motif is present at the VP3-VP4 junction it also appears in the middle of the VP3 coding region. Isolates that contain the Y-L dipeptide at the VP2-VP4 junction also have an A-A-G-G-R-Y motif repeated on either side of the dipeptide (Heppel *et al*, 1993). The field isolate and Sp/N1 sequences are again slightly different with A-A-G-G-R-Y present on one side and A-A-G-G-R-H on the other. This motif is not found in the VP4-VP3 junction region.

Chapter Three

Expression of IPNV Antigens

3.1 Introduction

The full length A-segment and VP2 could be cloned into the SFV expression vector pSFV1 or into plasmid vectors under the control of strong eukaryotic promoters. The expression and antigenicity of IPNV proteins by the various constructs was then to be investigated in both animal and fish cells using various immunodetection procedures. If the constructs are to have any potential as vaccines, IPNV proteins should be expressed in fish cells in a conformation capable of reacting with and stimulating the production of IPNV-specific antibodies.

3.2 SFV in fish

Although SFV does not normally infect fish it does have a broad host range and is known to infect mammalian, insect and avian species. Project partner three, Department of Agriculture for Northern Ireland, performed preliminary experiments. This group infected a fish cell line, CHSE-214, with SFV and found that infection resulted in pronounced cpe with viral protein detectable using immunofluorescence. The ability of the SFV vector system to express IPNV antigens was evaluated both in BHK-21 and CHSE cells.

3.3 Materials and methods

3.3.1 SFV-VP2 cloning

3.3.1.1 Proof-reading PCR

A primer was designed to incorporate the native SFV ribosome-binding sequence in IPNV clones. The sequence of primers used in a proof reading PCR are as follows, with the SFV ribosome-binding sequence underlined and the VP2 start codon in bold in the forward primer:

SFVF1 5'-GCG GGA TCC TAT AGCACC **ATG** AAC ACA AAC AAG GC-3'

SFVRVP25'- (AG)GCG(GC)CCTCC(GT)GC(GT)GC-3'

A standard proof-reading PCR reaction contained 1x cloned *Pfu* polymerase buffer (Stratagene), 200 μM each dATP, dCTP, dGTP and dTTP and 250 ng each of the forward and reverse primers along with 100-200 ng linearised DNA template. The reaction was performed in an automated DNA thermal cycler programmed for 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and polymerisation at 72°C for 3.5 min. The final cycle included a polymerisation step at 72°C for 10 min. "Hot start" PCR was performed as described earlier using 2.5 units cloned *Pfu* polymerase. PCR products were analysed by electrophoresis of a 20μl aliquot on a 1.2 % (w/v) agarose gel and subsequently purified using the Qiagen PCR purification kit. (Figure 3.1)

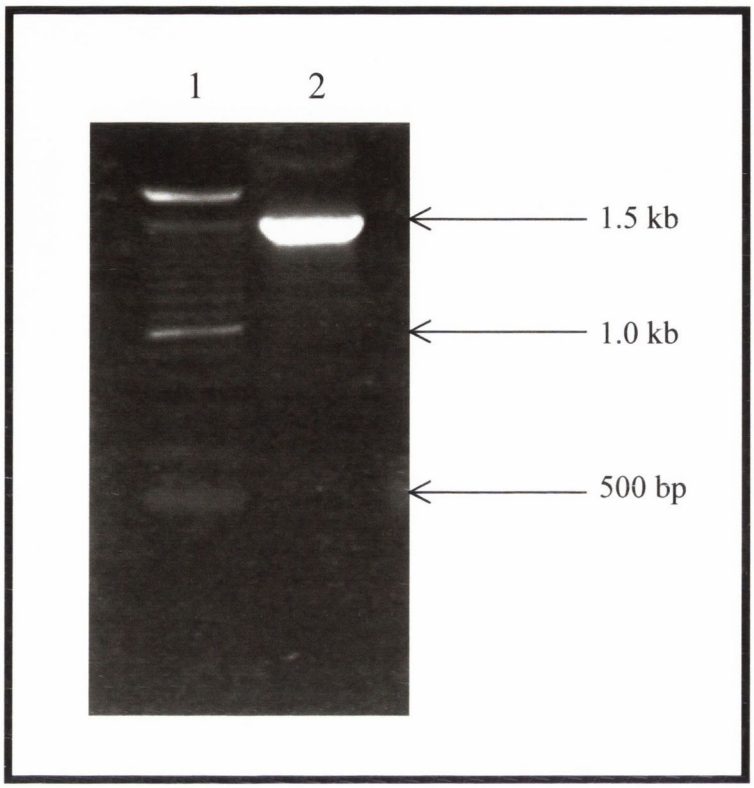


Figure 3.1 – Proofreading PCR. The VP2 coding sequence was reamplified from pPCR-Script-VP2 using *Pfu* polymerase and a primer designed to incorporate the native SFV ribosome binding sequence. The PCR product was run on a 1.2% agarose gel stained with ethidium bromide. Lane 1: 100 bp ladder. Lane 2: 1.5 kb reamplified VP2 coding sequence.

3.3.1.2 Preparation of vector

The 11.3 kb plasmid pSFV1 contains an ampicillin resistance site for bacterial screening and a MCS for use in the construction of recombinant plasmids. Prior to cloning the vector was digested with *Sma* I to linearise the circular plasmid and purified using the Qiagen nucleotide removal kit.

3.3.1.3 Preparation of competent cells

Cells chosen for transformation of recombinant constructs were *E. coli* strain DH5α. Five hundred ml of *E.coli* cells were grown, with shaking, in L broth at 37°C and were periodically checked until the optical density at 600 nm reached 0.45-0.55. The culture was then chilled on ice for 2 h after which the culture was centrifuged at 4,000 g for 20 min at 4°C. The bacterial pellet was resuspended in 500 ml titration buffer (100 mM $CaCl_2$, 70 mM $MgCl_2$, 40 mM NaOAc) and incubated on ice for 45 min. The bacteria were further pelleted by centrifugation at 3,500 g for 10 min and the pellet resuspended in 50 ml titration buffer. Eighty % glycerol (Sterilised by dry-heat) was added to the bacterial suspension in a drop wise fashion to a final concentration of 15% (v/v). The competent bacterial cell suspensions were then aliquoted and stored at -70°C.

3.3.1.4 Ligation and transformation

Ligation of the prepared linear vector to the purified blunt-ended PCR product was carried out at a ratio of 1:10 vector to insert, combined in a total volume of 20 µl, including 2 µl T4 DNA ligase reaction buffer and 1 unit T4 DNA ligase (Promega). The ligation mix was incubated at 18°C overnight before being used to transform *E.coli* strain DH5α using a 42°C heat shock period of 2 min. The transformed cells were plated onto L agar containing 100 µg/ml ampicillin and 40 µg/ml X-gal. Plates were incubated overnight at 37°C.

3.3.1.5 Screening for recombinant plasmids

Unlike the cloning vectors described in Chapter 2, pSFV1 does not contain a sequence which would permit the rapid visual detection of inserts. However, recombinant clones can be initially selected using a procedure which allows the rapid screening of uncut plasmid DNA directly from colonies on agar plates (Le Gouill and Dery, 1991). Putative positive clones are differentiated from negative clones by agarose gel electrophoresis of DNA extracted from cells transformed with the ligation mix alongside DNA extracted from vector-only colonies. Restriction digests of plasmid DNA can then be used to confirm the presence of the insert.

3.3.1.5.1 Rapid colony screening procedure

Recombinant clones were selected using a procedure that allows the rapid screening of uncut plasmid DNA directly from colonies on agar plates. Transformants were grown on selective plates as normal. Putative positive clones were picked off the agar plate using a sterile pipette tip and touched lightly onto a fresh plate before the remainder of the colony was resuspended in 8 µl of lysis buffer. The lysis solution contained 9 volumes [0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol; 25% (v/v) Ficoll type 400], 11 volumes water and 40 volumes [0.2 N NaOH, 1% (w/v) Sodium dodecyl sulphate (SDS)]. 1.5 µl buffer II (3 M potassium acetate, 1.8 M formic acid) was placed on the wall of the microcentrifuge tube before spinning at 14,000 g for four minutes. Supernatants were then loaded onto a 0.8% (w/v) agarose gel. Vector-only transformants were used as negative controls. (Figure 3.2)

3.3.1.5.2 Screening by restriction digests

To confirm the presence of inserts and their orientation in the vector a putative colony was inoculated into 10 ml L broth containing 100 µg/ml ampicillin and incubated with shaking at 220 r.p.m. at 37°C overnight. Plasmid DNA was isolated using the Qiagen Miniprep system,

Figure 3.2 – Rapid colony screening for recombinant clones. Putative positive clones were initially screened by running plasmid DNA isolated directly from transformant bacterial colonies on 0.8% agarose gels stained with ethidium bromide. Plasmid DNA isolated from transformants of known size was run alongside that from the ligation transformants. Lanes 1 & 15: Vector only transformants. Lanes 2 – 14: Plasmid DNA isolated from ligation tranformants. Lanes 9 & 14 contain plasmid DNA that is of a slightly higher molecular weight and therefore may contain a recombinant plasmid insert.

Figure 3.3 – Screening for recombinant clones by restriction digests. Putative positive colonies as determined by rapid colony screening were subjected to restriction analysis with *Sma* I to confirm the presence of recombinant plasmids. *Eco*R I digests will also determine the orientation of inserts if present. Digested plasmid DNA was run on 1.2% agarose gels stained with ethidium bromide. Panel A: Lane 1: λ (*Hin*d III) DNA marker. Lane 2: Putative positive clone digested with *Eco*R I. Lane 3: 100 bp ladder. Panel B: Lane 1: 100 bp ladder. Lane 2: Undigested plasmid DNA. Lanes 3 – 6: Putative positive clones digested with *Sma* I. Lanes 7 – 11: putative positive clones digested with *Eco*R I. Lane 12: λ (*Hin*d III) DNA marker. Recombinant clones with the insert in the correct orientation are shown in Panel A, Lane 2 and in Panel B, Lanes 7 and 8.

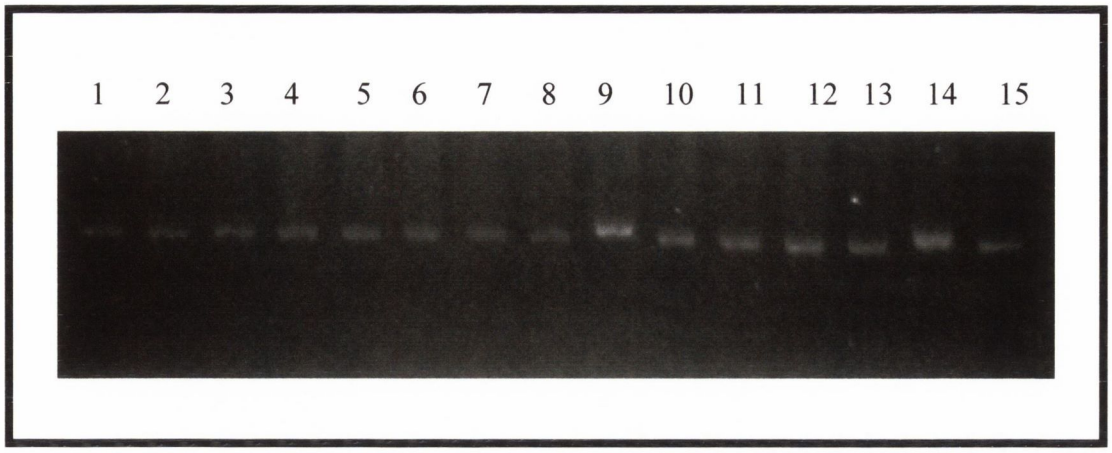


Figure 3.2 - Rapid colony screening for recombinant clones

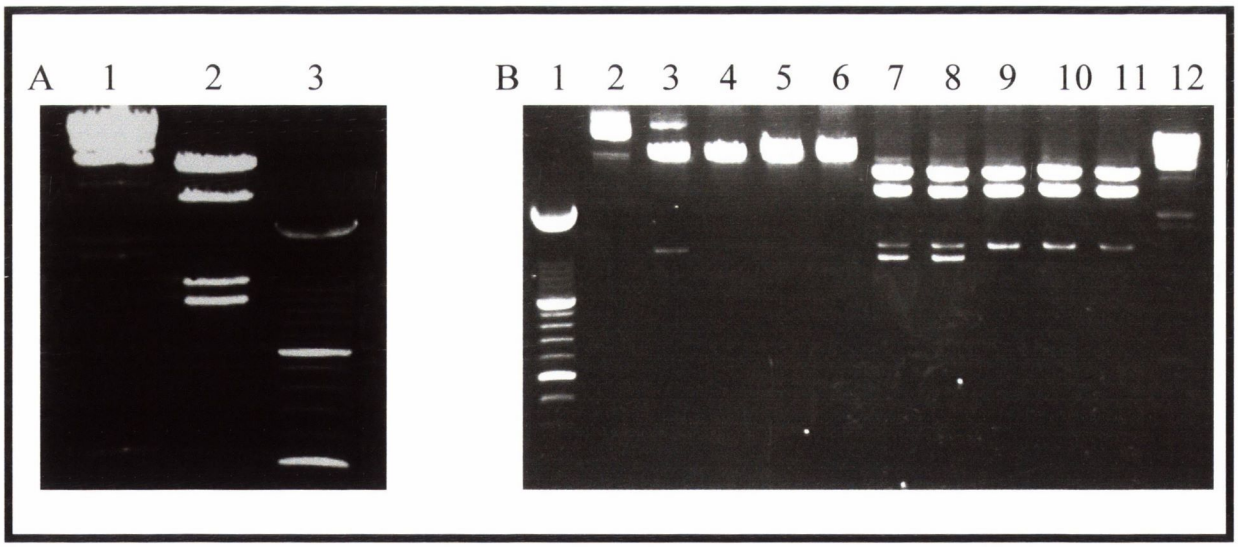


Figure 3.3 - Screening for recombinant clones by restriction digests.

digested with *Eco*R I and *Sma* I and run on a 1.2 % (w/v) agarose gel. (Figure 3.3)

3.3.2 A-segment clone construction

A strategy for cloning the 3′ end of the A-segment into pcDNA3-VP2 was designed, utilising a unique *Kpn* I restriction site. The strategy is illustrated in figure 3.4. From this full-length naked DNA clone the A-segment could then be transferred to the basic SFV vector and to the layered DNA/RNA vector system.

3.3.2.1 pcDNA3-A-segment construction

3.3.2.1.1 Isolation of A-segment 3′ end

The pCR-Script plasmid containing the 3′ end of the A-segment was digested with *Hpa* I and *Kpn* I. The digested plasmid was run on a 1% (w/v) agarose gel from which a 1.6 kb band, the 3′ end of the A-segment, was cut and purified using the Qiagen gel extraction kit.

Figure 3.4 – Construction of full length pcDNA3-Asegment clone. A strategy for cloning the 3'end of the A-segment into pcDNA3-VP2 was designed, utilising a unique *Kpn* I restriction site. The pcDNA clone expressing VP2 was cut with *Xba* I and then polished using *Pfu* polymerase to give blunt ends before digestion with *Kpn* I. The pPCR-Script clone containing the 3'end of the A-segment was digested with *Hpa* I and *Kpn* I and the 1.6 kb band cut from an agarose gel and purified using the Qiagen Gel extraction kit. The purified fragment was subsequently ligated to the *Kpn* I cut pcDNA3-VP2 clone.

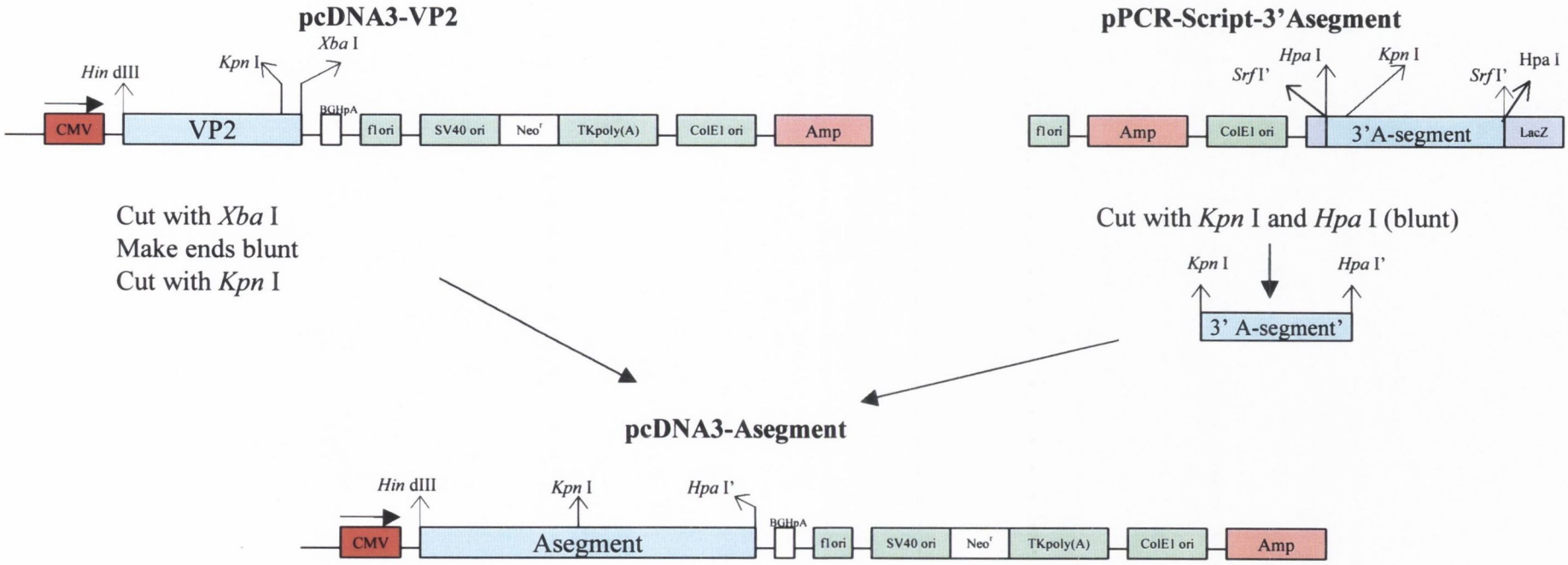


Figure 3.4 - Construction of full length pcDNA3-Asegment clone.

3.3.2.1.2 Preparation of pcDNA3-VP2 backbone

The pcDNA3 clone known to express VP2 was restricted with *Xba* I and overhangs removed, using *Pfu* polymerase (as described previously in section 2.2.5.1), to give blunt ends. The linearised vector backbone was then digested with *Kpn* I to facilitate the insertion of the prepared 3′ A-segment.

3.3.2.1.3 Ligation and transformation

The ligation and transformation procedures were performed as described in section 3.3.1.4.

3.3.2.1.4 Screening for recombinant plasmids

Screening for recombinant plasmids was carried out initially by the rapid colony screening procedure outlined in section 3.3.1.5.1. The presence of a full length A-segment was confirmed by *Hin*d III/*Kpn* I and *Hin*d III/*Sma* I restriction digests of plasmid DNA isolated as described in section 3.3.1.5.2 and run on 1% (w/v) agarose gels. (Figure 3.5)

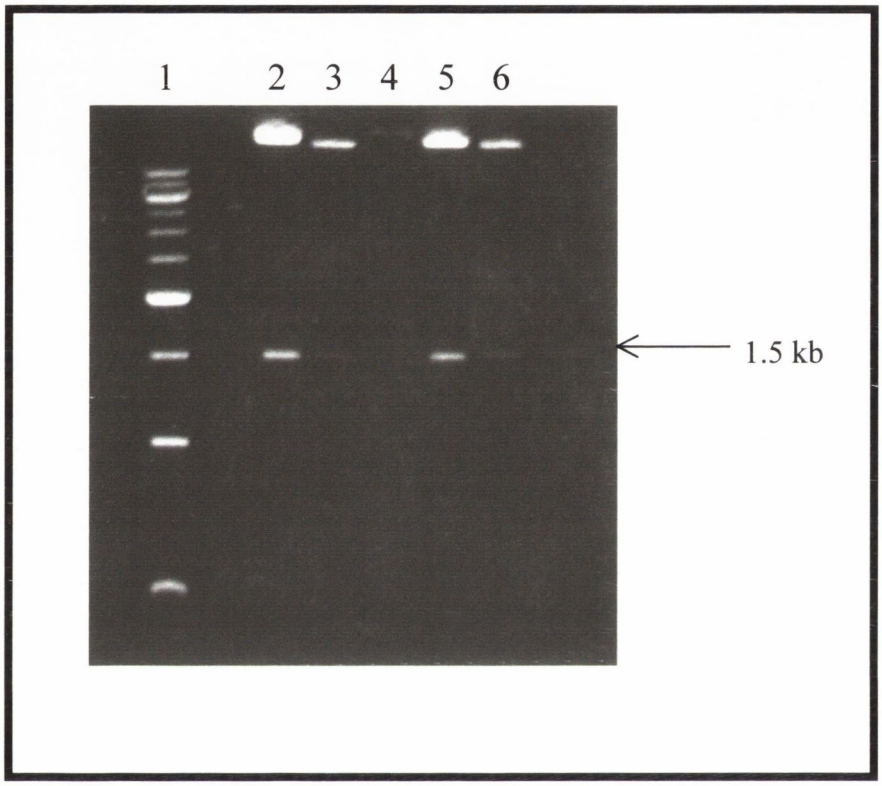


Figure 3.5 – Screening for pcDNA3 recombinant clones. Panel A: 1% agarose gel stained with ethidium bromide. Lane 1: 500 bp ladder. Lanes 2, 3, 5 and 6: Recombinant pcDNA3 clones containing A-segment insert digested with *Hpa* I. Lane 4: False positive pcDNA3 clone.

3.3.2.2 Proof-reading PCR

For construction of pSFV1-A-segment and pBKT-SFV.A-segment clones, a proof-reading PCR reaction was utilised to reamplify the full length A-segment from the pcDNA3-A-segment clone. The PCR product was purified using Qiagen PCR purification kit. PCR products were analysed by electrophoresis of a 20 μl aliquot on a 1% (w/v) agarose gel and subsequently purified using the Qiagen PCR purification kit. (Figure 3.6)

3.3.2.3 pSFV1-Asegment construction

The A-segment was inserted into the SFV expression vector pSFV1 as shown in figure 3.7.

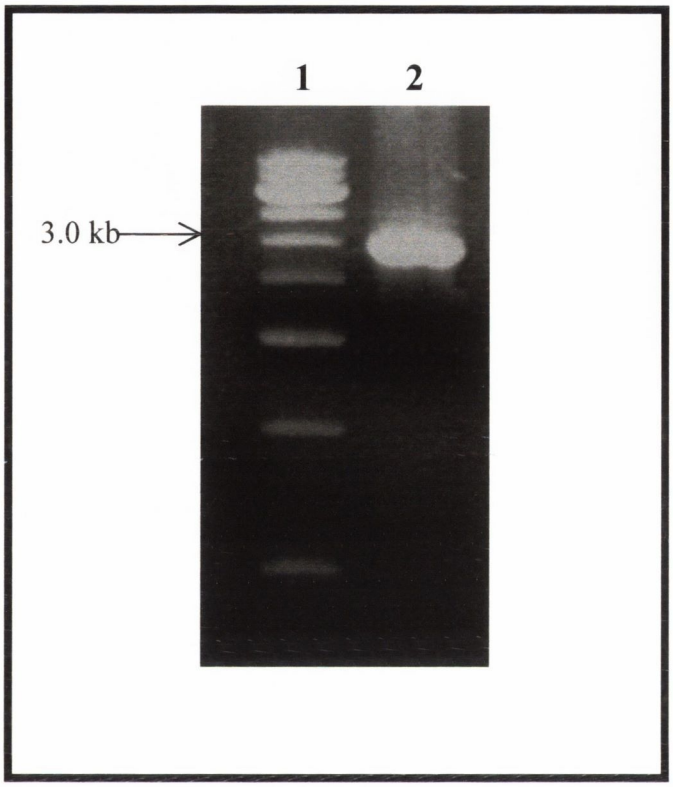


Figure 3.6 – Proofreading PCR of A-segment. The A-segment coding sequence was reamplified from pcDNA3-A-segment using *Pfu* polymerase and a primer designed to incorporate the native SFV ribosome binding sequence. The PCR product was run on a 1.2 % agarose gel stained with ethidium bromide. Lane 1: 500 bp ladder. Lane 2: 3.1 kb reamplified A-segment coding sequence.

Figure 3.7 – Construction of pSFV-I A-segment expression clones. The IPNV-A-segment coding sequence, reamplified from pcDNA3-A-segment, was cloned into the SFV expression vector pSFV1 at the *Sma* I site.

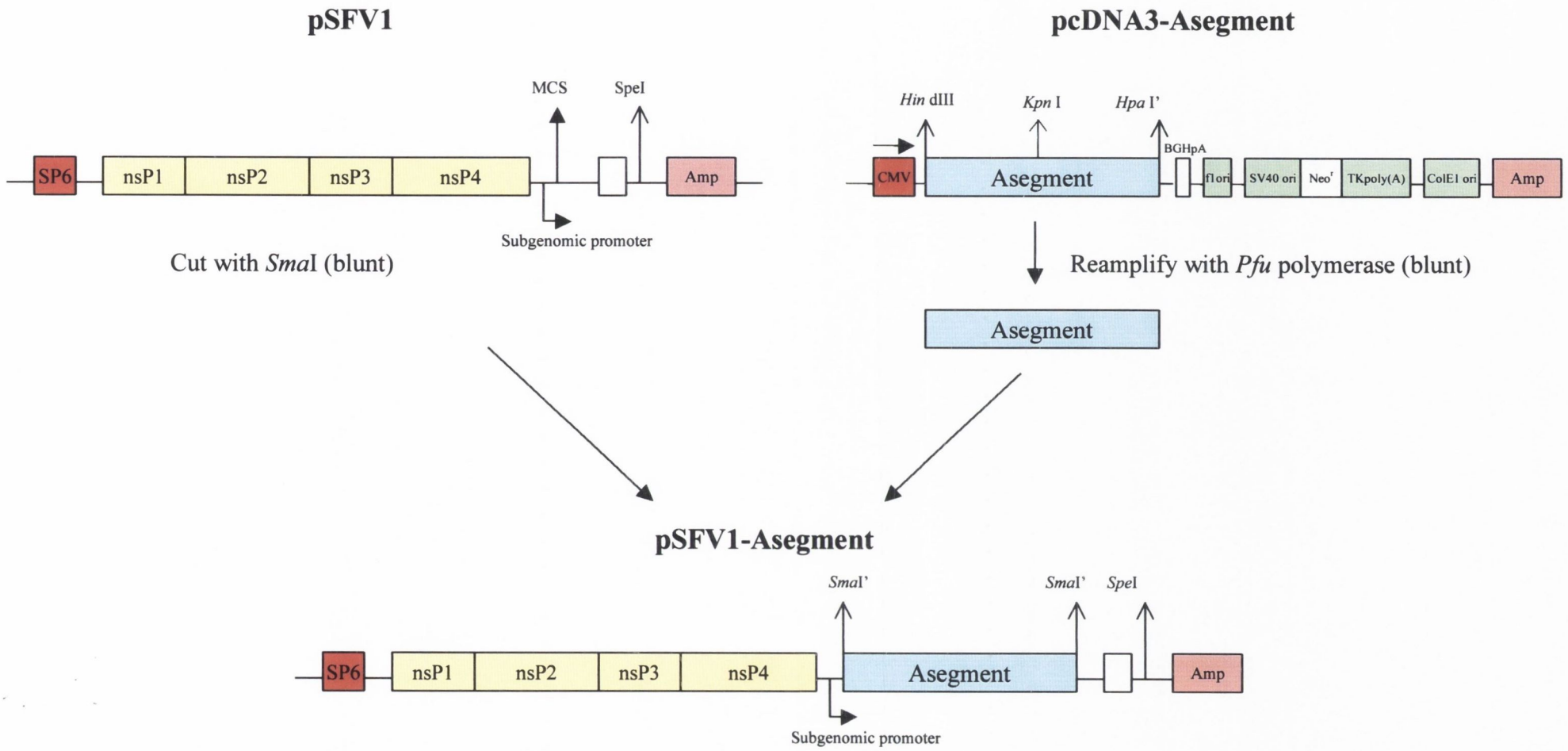


Figure 3.7 - Construction of pSFV1-Asegment expression clones.

3.3.2.3.1 Preparation of vector

As for the cloning of VP2 into pSFV1 the vector was digested with *Sma* I to linearise the circular plasmid and purified using the Qiagen nucleotide removal kit.

3.3.2.3.2 Ligation and transformation

The ligation and transformation procedures were performed as described in section 3.3.1.4.

3.3.2.3.3 Screening

Screening for recombinant plasmids was carried out initially by the rapid colony screening procedure outlined above. The presence of a full length A-segment and its orientation within the vector was confirmed by *Eco*R I and *Sma* I restriction digests of plasmid DNA isolated as described above and run on 1% (w/v) agarose gels (Figure 3.8).

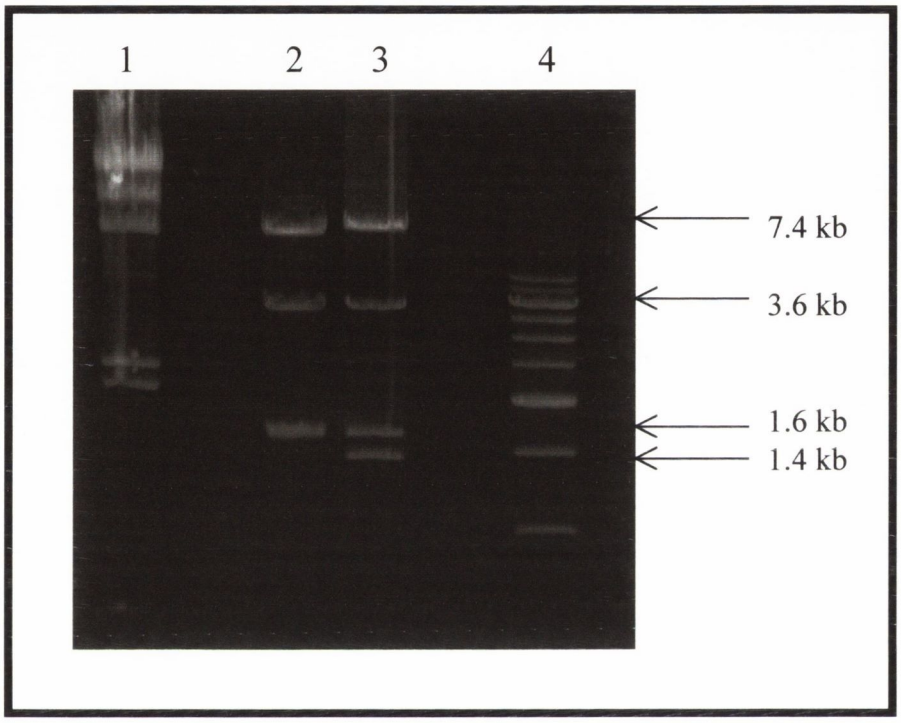


Figure 3.8 – Screening for pSFV1 recombinant clones. 1% agarose gel stained with ethidium bromide. Lane 1: λ (*Hin*d III) DNA marker. Lanes 2 and 3: putative positive clones digested with *Eco*R I. The clone in lane 3 contains the correct banding pattern for a pSFV1 recombinant with the A-segment insert present in the right orientation. Lane 4: 500 bp ladder.

3.3.2.4 pBKT-SFV.A-segment construction

The A-segment was inserted into the layered DNA-RNA expression vector pBKT-SFV as shown in figure 3.9.

3.3.2.4.1 Preparation of vector

Prior to cloning the vector was digested with *Bam*H I to linearise the circular plasmid and purified using the Qiagen nucleotide removal kit. Overhangs formed were subsequently removed using *Pfu* polymerase as described in section 2.5.5.1 and the blunt ended vector purified using Qiagen PCR purification kit.

3.3.2.4.2 Ligation and transformation

The ligation and transformation procedures were performed as described in section 3.3.1.4.

Figure 3.9 – Construction of pBKT-SFV A-segment expression clones. The IPNV-A-segment coding sequence reamplified from pcDNA3-A-segment was cloned into the layered expression vector pBKT-SFV at the *Bam*H I site which was polished with *Pfu* polymerase to give blunt ends.

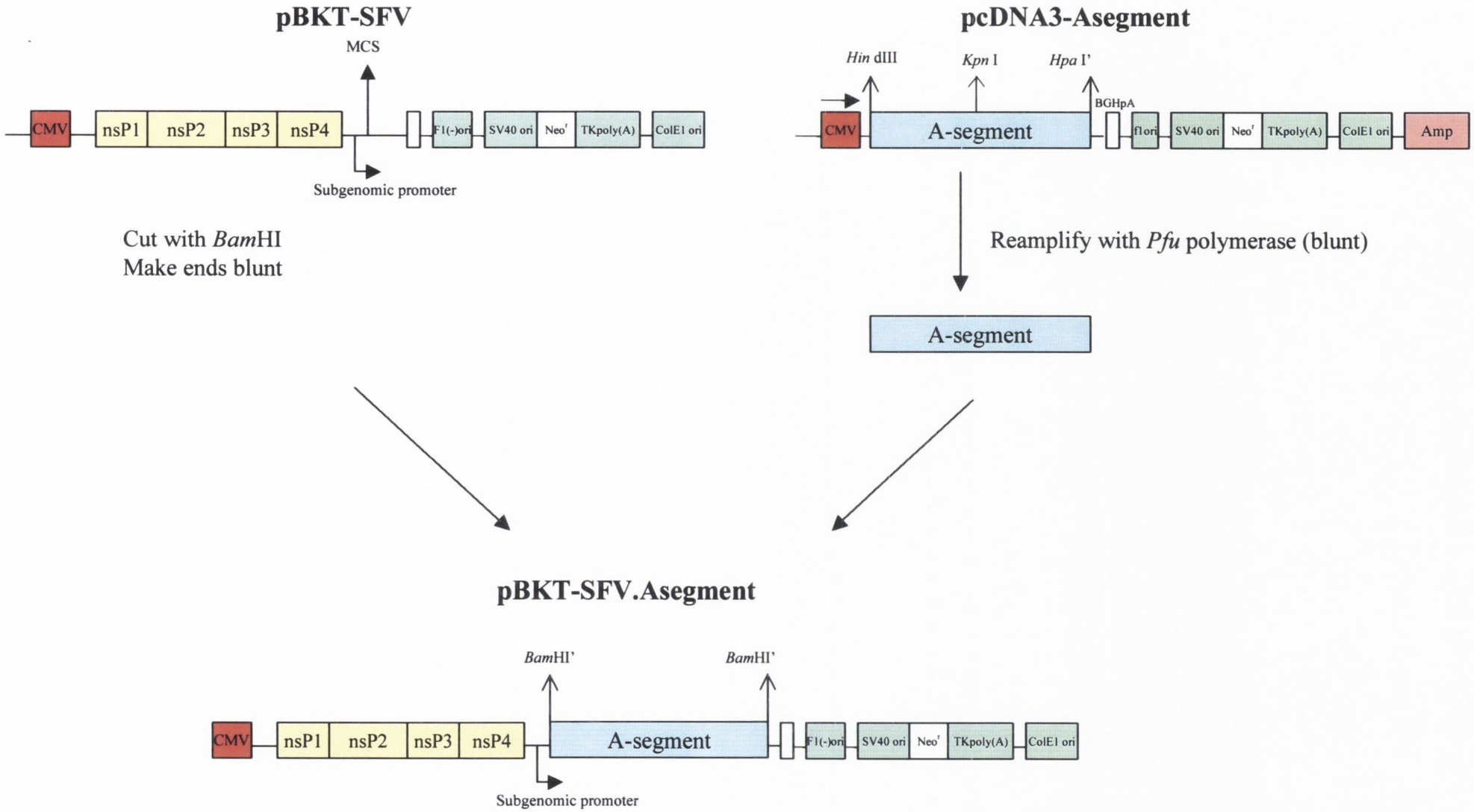


Figure 3.9 - Construction of pBKT-SFV.Asegment expression clones.

3.3.2.4.3. Screening

Screening for recombinant plasmids was carried out initially by the rapid colony screening procedure outlined above. The presence of a full length A-segment was confirmed by *Bam*H I restriction digests of plasmid DNA isolated as described above and run on 1% (w/v) agarose gels. (Figure 3.10) The orientation of inserts present was determined by *Eco*R I restriction digest of plasmid DNA.

3.3.3 Detection of expression

3.3.3.1 *In vitro* transcription

3.3.3.1.1 SP6 transcription

In vitro transcription of A-segment and VP2 RNA was performed as described by Liljestrom *et al*, 1991. Briefly, following *Spe* I linearisation of 1.5 μg plasmid DNA, SP6 RNA polymerase was used to initiate RNA transcription from each of the clones in a total reaction volume of 50 μl. Standard reaction mixtures contained 1.5 μg linearised DNA, 1x SP6 buffer [40 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid-KOH (Hepes-KOH), pH7.4, 6 mM MgOAc, 2 mM spermidine-HCl], 1 mM $m^7G(5')ppp(5')G$ (Pharmacia), 5 mM dithioreitol, 1 mM each

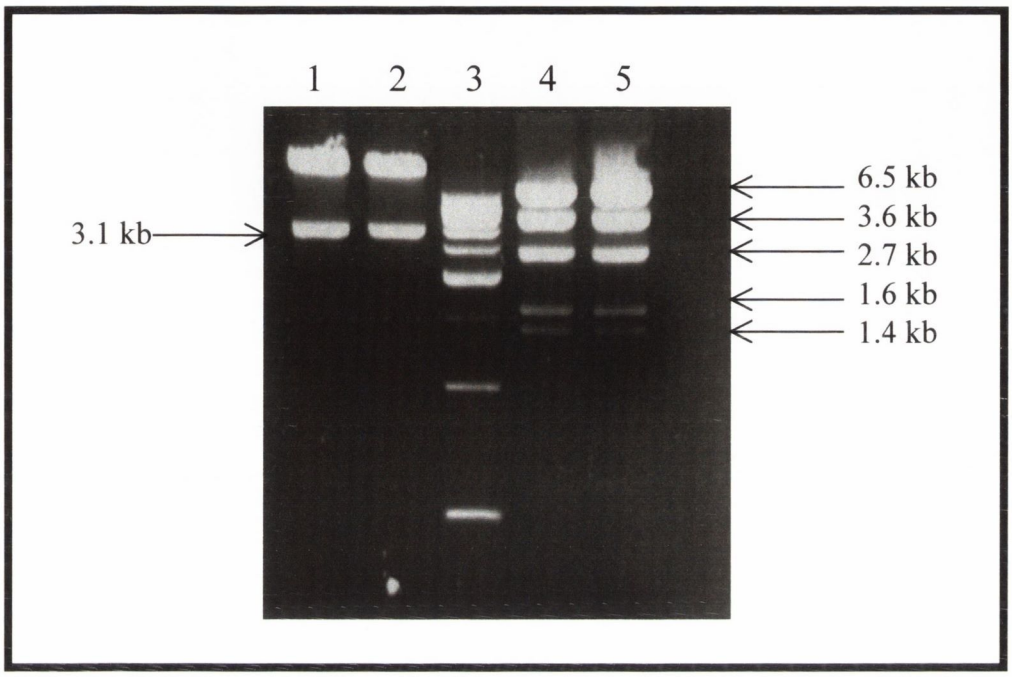


Figure 3.10 – Screening of pBKT-SFV recombinant clones. Putative positive clones were digested with *Bam*H I to confirm the presence of inserts and subsequently with *Eco*R I to determine the orientation of inserts present. Plasmid digests were run on 1% agarose gels stained with ethidium bromide. Lanes 1 and 2: Recombinant pBKT-SFV clones containing 3.1 kb A-segment insert; digested with *Bam*H I enzyme. Lane 3: 500 bp ladder. Lanes 4 and 5: Recombinant pBKT-SFV clones containing A-segment insert in the correct orientation; digested with *Eco*R I enzyme.

rATP, rCTP, rUTP and 500 μM rGTP, 50 units RNasin and 30 units SP6 RNA polymerase (Pharmacia). Transcripts were analysed by agarose gel electrophoresis. (Figure 3.11)

3.3.3.1.2 T7 transcription

For *in vitro* transcription of A-segment RNA from pcDNA3-A-segment clones T7 RNA polymerase was used as described by the manufacturer (Pharmacia). Briefly, following *Nde* I linearization of 1.5 μg plasmid DNA, T7 RNA polymerase was used to initiate RNA transcription from each of the clones in a total reaction volume of 50 μl. Standard reaction mixtures contained 1.5 μg linearized DNA, 1x T7 buffer (Promega), 1 mM $m^7G(5')ppp(5')G$, 5 mM dithoreitol, 1 mM each rATP, rCTP, rUTP and 500 μM rGTP, 50 units RNasin and 30 units T7 RNA polymerase (Promega). Transcripts were analysed by agarose gel electrophoresis. (Figure 3.12)

3.3.3.2 BHK cell culture

1 x 10^6 Baby Hamster kidney cells strain 21 (BHK-21) were grown in 75 cm^2 or 25 cm^2 tissue culture bottles or on 13 mm glass coverslips in 6 well plates. Cells were cultured at 37°C, 5% CO_2 in BHK-21 medium (GIBCO)

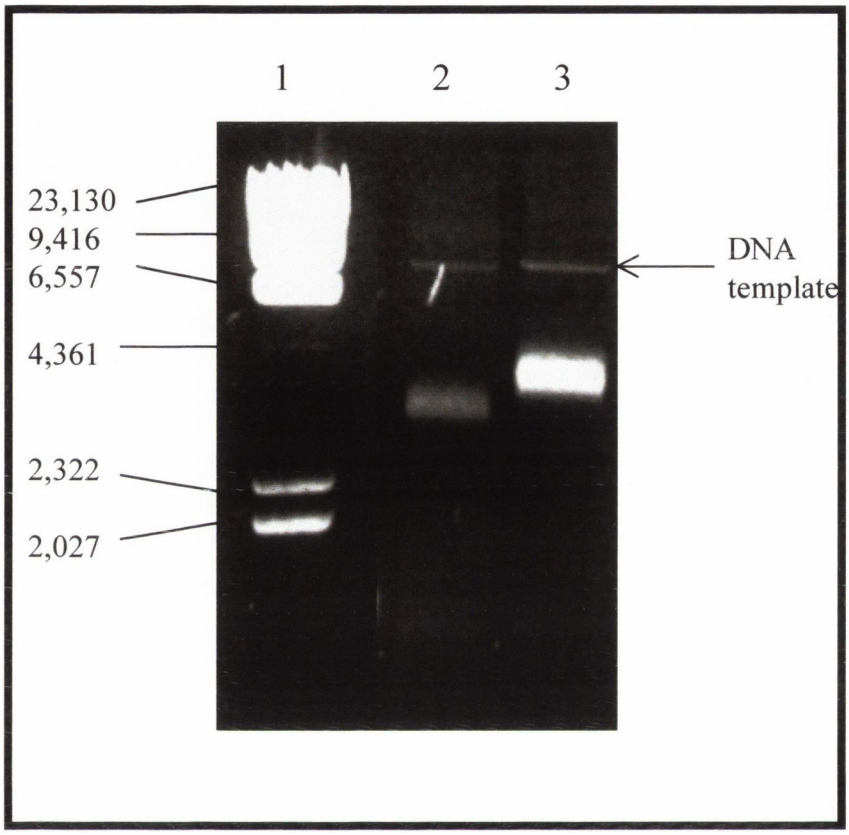


Figure 3.11 - *In vitro* RNA transcription by SP6 RNA polymerase. 1% agarose gels stained with ethidium bromide. Lane 1: λ (*Hin*d III) DNA Markers. Lane 2: *In vitro* transcribed RNA from Helper-2 plasmid. Lane 3: *In vitro* transcribed RNA from recombinant pSFV1-A-segment clone. An arrow indicates the linearised DNA template. Sizes of DNA markers are indicated in base pairs

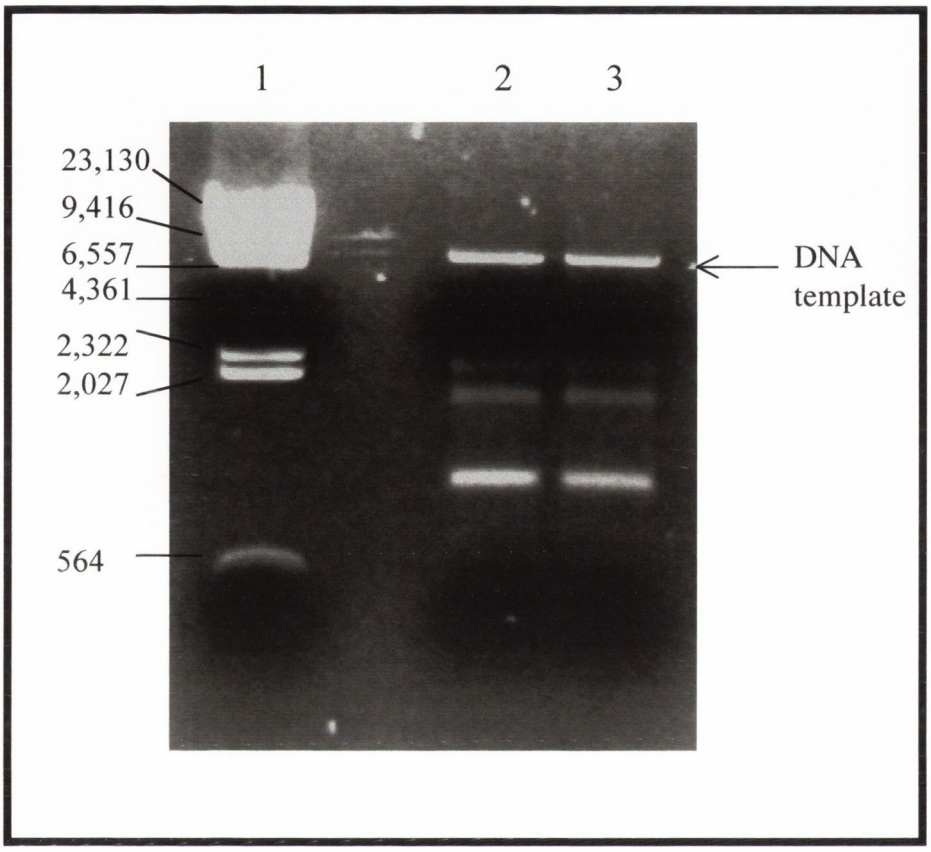


Figure 3.12 – *In vitro* RNA transcription by T7 RNA polymerase. 1% agarose gel stained with ethidium bromide. Lane 1: λ (*Hin*d III) DNA marker. Lanes 2 and 3: *In vitro* transcribed RNA from recombinant pcDNA3-A-segment clones. An arrow indicates the linearised DNA template. The size of DNA marker bands are indicated in base pairs

supplemented with 5% (v/v) newborn calf serum, 5% (v/v) tryptose phosphate broth, 0.1 % (v/v) penicillin/streptomycin solution and 2 mM glutamine.

3.3.3.3 Electroporation

To remove cells from the surface of 75 cm^2 tissue culture flasks confluent monoloayers were washed twice with phosphate buffered saline (PBS). One ml trypsin in PBS was added and the cells incubated at 37°C, 5% CO_2 for 5 min to disrupt the cells. Ten ml BHK-21 medium were added to neutralise the trypsin and cells centrifuged at 1,500 g for 5 min at room temperature. The cell pellet was washed once with 10 ml PBS before being resuspended in 0.8 ml PBS to give a cell suspension of approximately 10^7 cells/ml. Fifty µl *in vitro* transcribed RNA (approximately 4 µg) was added to the cell suspension and placed in a 4mm electroporation cuvette (EquiBio). The cuvette was pulsed twice at 850 V and 25 µF capacitance in a BioRad Gene Pulser with time constants reading approximately 0.4 after each pulse. Pulsed cells were diluted in 12 ml BHK-21 medium and plated in 3x 60 mm tissue culture plates before incubation at 37°C, 5% CO_2. Cells were harvested 24 h post electroporation using 500 ml lysis buffer containing 1% (v/v) Nonidet P-40 (NP-40), 50 mM Tris-HCl, pH7.6, 150 mM NaCl, 2 mM EDTA and 1 µg/ml Phenylmethylsulphonyl fluoride (PMSF).

3.3.3.4 Lipofection

When cells grown in $25cm^2$ tissue culture flasks were approximately 80% confluent they were transfected with 5 μg pcDNA3-Aseg or pBKT-SFV-Aseg using LIPOFECTAMINE reagent (GIBCO) and OPTI-MEM reduced serum medium (GIBCO) according to the instructions of the manufacturer. After 6 h incubation at 37°C normal culture growth medium was added and the cells incubated at 37°C. Cells were harvested 48 h post-transfection using 1 ml lysis buffer containing 1% (v/v) NP-40, 50 mM Tris-HCl, pH7.6, 150 mM NaCl, 2 mM EDTA and 1 μg/ml PMSF.

Alternatively, cells were grown on 13 mm glass coverslips in 35 mm tissue culture dishes and when approximately 80% confluent they were transfected with 1 μg pcDNA3-Aseg or pBKT-SFV-Aseg using LIPOFECTAMINE reagent (GIBCO) and OPTI-MEM reduced serum medium (GIBCO) according to the instructions of the manufacturer. After 6 h incubation at 37°C, with gentle agitation at 30 min intervals, 1 ml normal culture growth medium was added and the cells incubated at 37°C. Coverslips were harvested at 24 h post transfection, washed in PBS and fixed in acetone for 10 min.

3.3.3.5 Western Immunoblotting

SDS-PAGE was performed by the method of Laemmli (Laemmli,

1970) with pre-stained protein molecular weight standards (NEB). Gels were 10 x 12 cm. Thirty µl of cell lysates were stacked in, 4.5 % acrylamide and separated in 12% acrylamide at 50 mA. The cell lysates were from BHK-21 cells electroporated with pcDNA3-A-segment and pSFV1-VP2/A-segment constructs and from BHK-21 cells transfected with plasmid DNA from the pcDNA3 and pBKT-SFV A-segment constructs as described in sections 3.3.3 and 3.3.4. A standard protocol was followed for immunoblotting (Towbin et al, 1979). Proteins were transferred to PVDF membrane (Amersham) using the Bio-Rad semidry transfer apparatus in accordance with the manufacturer's recommendations. Following electroblotting, membranes were immersed in a 10% (w/v) blocking solution consisting of dried milk powder (Marvel™) in TS buffer (10 mM Tris-HCl pH 7.4, 0.9% (w/v) NaCl) and incubated at room temperature for two hours with gentle shaking. A 1:1000 dilution of IPNV rabbit polyclonal antisera or a 1:2000 dilution of a MAb to IPNV-VP3 in 10% (w/v) blocking solution were added to blots which were incubated at 4°C overnight. Blots were then washed twice for 10 min each in TS buffer, twice in TS buffer with 1% (v/v) Tween-20 and twice again with TS buffer. Blots were finally incubated in a 1:1000 dilution of horseradish peroxidase (HRP) -conjugated anti-rabbit IgG (DAKO) for the polyclonal primary antibody blot, or 1:1000 dilution HRP-conjugated anti-mouse IgG (DAKO) for the monoclonal antibody blot, in 10% (w/v) blocking solution for 2 h at room temperature with gentle shaking. Membranes were washed as above and developed using the ECL-Plus Western blotting kit (Amersham) according to manufacturers

instructions.

3.3.3.6 Immunofluorescence

Coverslips that had been transfected with plasmid DNA from pcDNA3-A-segment and pBKT-SFV-A-segment clones were incubated for 1 h at 37°C with a 1:1000 dilution of anti-IPNV polyclonal antisera, washed in PBS for 10 min and then incubated for a further 1 h at 37°C with a pig anti-rabbit FITC-conjugated antibody (DAKO). Cells were then washed for 10 min in PBS and mounted in Mowiol-DABCO and observed microscopically for the presence of specific fluorescence.

3.3.3.7 Metabolic labelling

VP2 and A-segment mRNAs transcribed from pSFV1 clones were electroporated into BHK cells as described in section 3.3.3.3 At 9 h post electroporation, growth medium was aspirated and the cells washed twice with PBS warmed to 37°C. Cells were overlayed with 2 ml starvation medium [methionine free MEM (GIBCO), 2 mM glutamine, 20 mM Hepes] and incubated at 37°C, 5% CO_2 for 30 min. The medium was aspirated and replaced with 500 µl of the same medium containing 100 µCi/ml ^{35}S-methionine (Amersham) and incubated for 30 min at 37°C,

5% CO_2. Pulse medium was aspirated and cells washed twice with 2 ml MEM-chase medium [E-MEM (GIBCO), 2mM glutamine, 20mM Hepes and 150 μg/ml unlabelled methionine], before being overlaid with 2 ml MEM-chase medium and incubated for 30 min. Following aspiration of medium, cells were washed twice with PBS before addition of 300-500 μl lysis buffer (1% (v/v) NP-40, 50 mM Tris-HCl, pH7.6, 150 mM NaCl, 2 mM EDTA, 1 μg/ml PMSF). Cells were placed on ice for 10 min before resuspension. They were then transferred into an Eppendorf tube and centrifuged at 14,000 g for 5 min to remove nuclei. Supernatants were transferred to fresh tubes for immunoprecipitation.

3.3.3.8 Immunoprecipitation

A solution of 0.3 g protein A-sepharose CL4B in 10 ml 100 mM Tris-HCl, pH7.5 was swollen overnight at 4°C, washed twice with the same buffer and resuspended in 10 mM Tris-HCl, pH 7.5. Forty μl of the protein A-sepharose slurry and 5 μl IPNV polyclonal antisera were added to 300 μl cell lysates, mixed and let stand on ice for 30 min with occasional mixing. Samples were spun at 14,000 g for 30 s and the pellets washed once with buffer A, once with buffer B and once with buffer C by adding 500 μl buffer, mixing and spinning for 30 seconds at 14,000 g. Buffer A contained 0.2% (v/v) NP40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 2 mM EDTA. Buffer B contained 0.2 % (v/v) NP-40, 10 MM Tris-HCl, pH 7.5, 0.5 mM NaCl and 2

mM EDTA and buffer C was 10 mM Tris-HCl, pH 7.5. The pellet was resuspended thoroughly in 50 µl SDS gel sample buffer (200 mM Tris-HCl, pH 8.8, 20% (v/v) glycerol, 5 mM EDTA, 0.02% (w/v) bromophenol blue and 3% (w/v) SDS) and heated to 95°C for 5 min. Samples were spun at 14,000 g for 2 min and 20 µl loaded on an SDS-polyacrylamide gel, which was run as described by Laemmli, 1970 with prestained protein molecular weight standards (NEB). The gel was dried using a vacuum gel drier (Savant) at 60 °C and exposed to X-ray film (Kodak) for 1-2 days.

3.4 Results

Table 3.1 summarises all expression constructs made for this study.

3.4.1 SFV-VP2 cloning and expression

The VP2 gene was re-amplified from pBLVP236 and pBLVP28 using cloned *Pfu* polymerase, a proofreading polymerase with 5'-3' exonuclease activity. The resulting blunt-ended PCR products were cloned into the SFV expression vector, pSFV1, at the *Sma* I site and recombinant clones screened using the rapid colony

Construct	Vector (size)	Insertion at site	Comments
pBLVP2-36	pPCR-Script (2.9 kb)	VP2 @ *Srf* I	VP2 with premature stop codon
pBLVP2-8	pPCR-Script (2.9 kb)	VP2 @ *Srf* I	Full length VP2
pSFV1VP2.I	pSFV1 (11 kb)	VP2 @ *Sma* I	From pBLVP2-8
pSFV1VP2.II	pSFV1 (11 kb)	VP2 @ *Sma* I	From pBLVP2-8
pSFV1VP2.III	pSFV1 (11 kb)	VP2 @ *Sma* I	From pBLVP2-36
pcDNA3VP2	pcDNA3 (5.4 kb)	VP2 @ *Hind* III	#From pBLVP2-8
pcDNA3A2	pcDNA3 (5.4 kb)	A-segment @ *Hind* III	
pcDNA3A14	pcDNA3 (5.4 kb)	A-segment @ *Hind* III	
pSFV1A7	pSFV1 (11 kb)	A-segment @ *Sma* I	From pcDNA3A2
pBKT-SFVA8	pBKT-SFV (12.7 kb)	A-segment @ *Bam* HI	From pcDNA3A2
pBKT-SFVA9	pBKT-SFV (12.7 kb)	A-segment @ *Bam* HI	From pcDNA3A2

Table 3.1 – Constructs from this study. The IPNV VP2 and A-segment expression constructs are detailed above. #: Constructed by Dr. Richard Fitzpatrick as part of this project.

screening procedure as described above. The presence of a 1.5 kb insert was confirmed by *Sma* I digestion of plasmid DNA and orientation of inserts was determined by *Eco*R I digestion. Three clones were obtained in the correct orientation. Two were obtained from the original clone pBLVP28, (pSFV1-VP2.I and pSFV-VP2.II) and the other from original clone pBLV236, (pSFV1-VP2.III). Expression of a 54 kDa protein, the presumptive VP2 protein, was detected in the clone pSFV1-VP2.I as shown in the autoradiograph in figure 3.13 panel A. A protein of the same size was also expressed from pSFV1-VP2.II (data not shown). Clone pSFV1-VP2.III did not express VP2 as shown in lanes 2 and 6. At 48 h post-electroporation, a higher degree of cell death was observed with many cells lifting off the monolayer.

3.4.2 Construction of A-segment

A full length A-segment was constructed as shown in figure 3.4 and recombinant clones screened using the rapid colony screening procedure as described above. *Hin*d III/*Kpn* I and *Hin*d III/*Sma* I digests confirmed the presence of the full length A-segment. Positive clones were designated pcDNA3-A2 and pcDNA3-A14. Initially, we hoped to detect expression of IPNV proteins expressed from pcDNA3 in the same manner as detection of expression of IPNV-VP2 from pSFV1 in section 3.6.1. However, no expression of IPNV proteins could be detected by immunoblotting of cells electroporated with RNA transcribed from the pcDNA3 clones (Figure 3.14, panel

Figure 3.13 – Western immunoblotting of SFV expression constructs. Cell lysates from cells electroporated with SFV expression constructs were run on SDS-polyacrylamide gels. Gels were subsequently blotted onto PVDF membrane and bound proteins detected using chemiluminesence with IPNV specific antibodies and a horseradish peroxidase conjugated secondary antibody. In all panels IPNV proteins are indicated with arrows.

Panel A: Western immunoblot with IPNV polyclonal antisera. Lane 1: pSFV1-VP2.I 24h post-electroporation. Lane 2: pSFV1-VP2.III (Non-expressing clone) 24h post-electroporation. Lane 3: pSFV1-lacZ 24h post-electroporation. Lane 4: Untransfected cells 24 h post-electroporation. Lane 5: pSFV1-VP2.I 48h post-electroporation. Lane 6: pSFV1-VP2.III 48 h post-electroporation. Lane 7: pSFV1-lacZ 48h post-electroporation. Lane 8: Untransfected cells 48 h post-electroporation. Lane 9: IPNV infected CHSE cells (positive control).

Panel B: Western immunoblot with IPNV polyclonal antisera Lane 1: IPNV-infected CHSE cells. Lane 2: pSFV1-VP2 24 h post-electroporation. Lane 3: pSFV1-A-segment 24 h post-electroporation. Lane 4: Untransfected cells (negative control).

Panel C: Western immunoblot with IPNV MAb to VP3. Lane 1: IPNV infected CHSE cells. Lane 2: Untransfected cells. Lane 3: pSFV1-Asegment 24 h post-electroporation

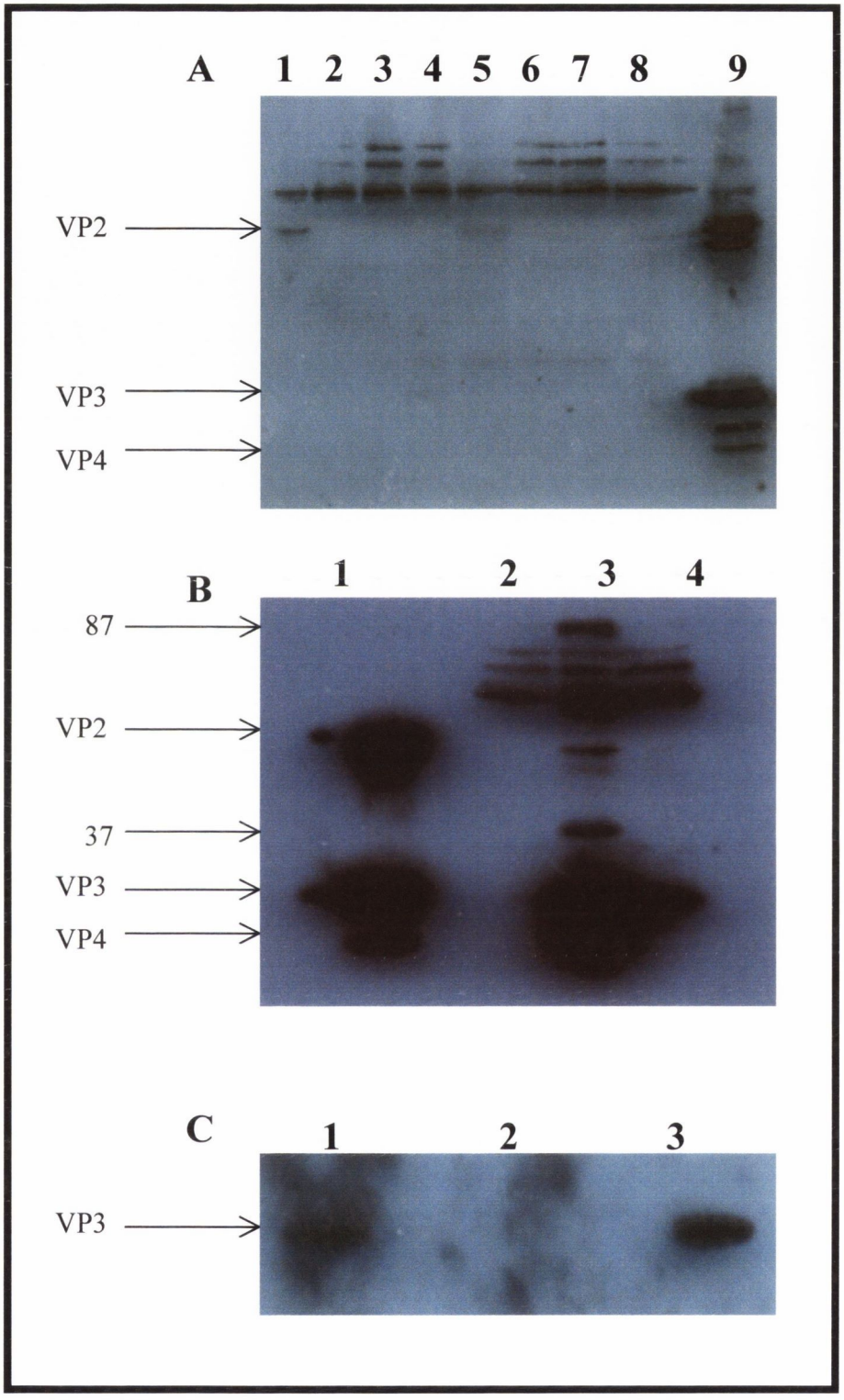


Figure 3.13 - Western immunoblots of SFV expression constructs.

Figure 3.14 – Western immunoblots of pcDNA3 expression constructs. Cell lysates from cells transfected with pcDNA3 expression constructs were run on SDS-polyacrylamide gels. Gels were subsequently blotted onto PVDF membrane and bound proteins detected using chemiluminesence with IPNV specific antibodies and a horseradish peroxidase conjugated secondary antibody. In all panels IPNV proteins are indicated with arrows.

Panel A: Western immunoblot of proteins from cells transfected by electroporation of T7 transcribed RNA probed with IPNV polyclonal antisera. Lane 1: IPNV infected CHSE cells (positive control). Lanes 2 - 6: pcDNA3-A-segment 24h post-electroporation. Lane 7: Untransfected cells (negative control).

Panel B: Western immunoblot of proteins from cells transfected by lipofection of plasmid DNA probed with IPNV polyclonal antisera. Lane 1: IPNV-infected CHSE cells. Lanes 2 and 3: pcDNA3-A-segment 48 h post-transfection. Lane 4: Untransfected cells. Lanes 5 and 6: pcDNA3-A-segment 72 h post-transfection.

Panel C: Western immunoblot of proteins from cells transfected by lipofection of plasmid DNA probed with IPNV MAb to VP3 Lane 1: IPNV infected CHSE cells. Lane 2: Untransfected cells. Lanes 3 and 4: pcDNA3-A-segment 48 h post-transfection.

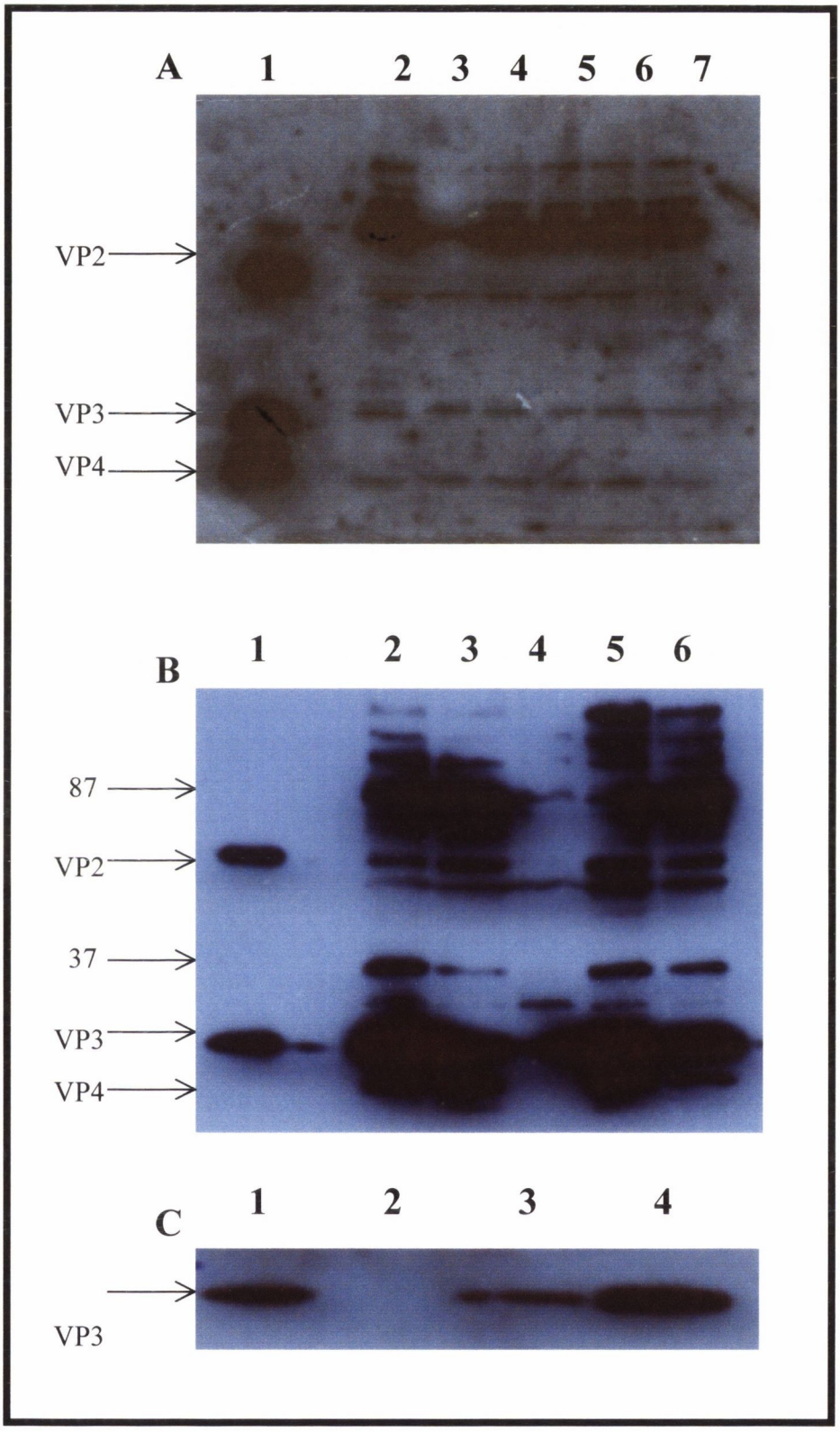


Figure 3.14 - Western immunoblots of pcDNA3 expression constructs.

A). T7 transcription resulted in the generation of a number of RNA bands possibly due to aberrant initiation or RNA instability (Figure 3.12). This may explain the failure of transfection by this method. Expression of IPNV-A-segment was detected by immunoblotting cell lysates from cells transfected by lipofection with plasmid DNA constructs. When labelled with polyclonal antisera, bands corresponding to VP2 at 54 kDa , VP3 at 32 kDa and VP4 at 28 kDa are present. In addition, bands at 38 kDa and 87 kDa react with the polyclonal antisera (Figure 3.14, panel B). Labelling with the MAb directed against VP3 results in the detection of a 32 kDa protein (Figure 3.14, panel C). Expression of the A-segment in BHK cells was also detected by immunofluorescence with polyclonal antiserum (Figure 3.15). Non-transfected cells were not immunofluorescent with IPNV antiserum (data not shown).

3.4.3 SFV-A-segment cloning and expression

The A-segment was re-amplified from pcDNA3-A2 using cloned *Pfu* polymerase, a proofreading polymerase with 5'-3' exonuclease activity. The resulting blunt-ended PCR product was cloned into the SFV expression vector, pSFV1, at the *Sma* I site and recombinant clones screened using the rapid colony screening procedure as described above. The presence and orientation of a 3.1 kb insert was confirmed by *Eco*R I and *Sma* I digestion of plasmid DNA. *In vitro* transcribed RNAs from clones containing the insert in the correct orientation were electroporated into BHK cells. 24 h post-electroporation cells were harvested and run on a standard SDS-

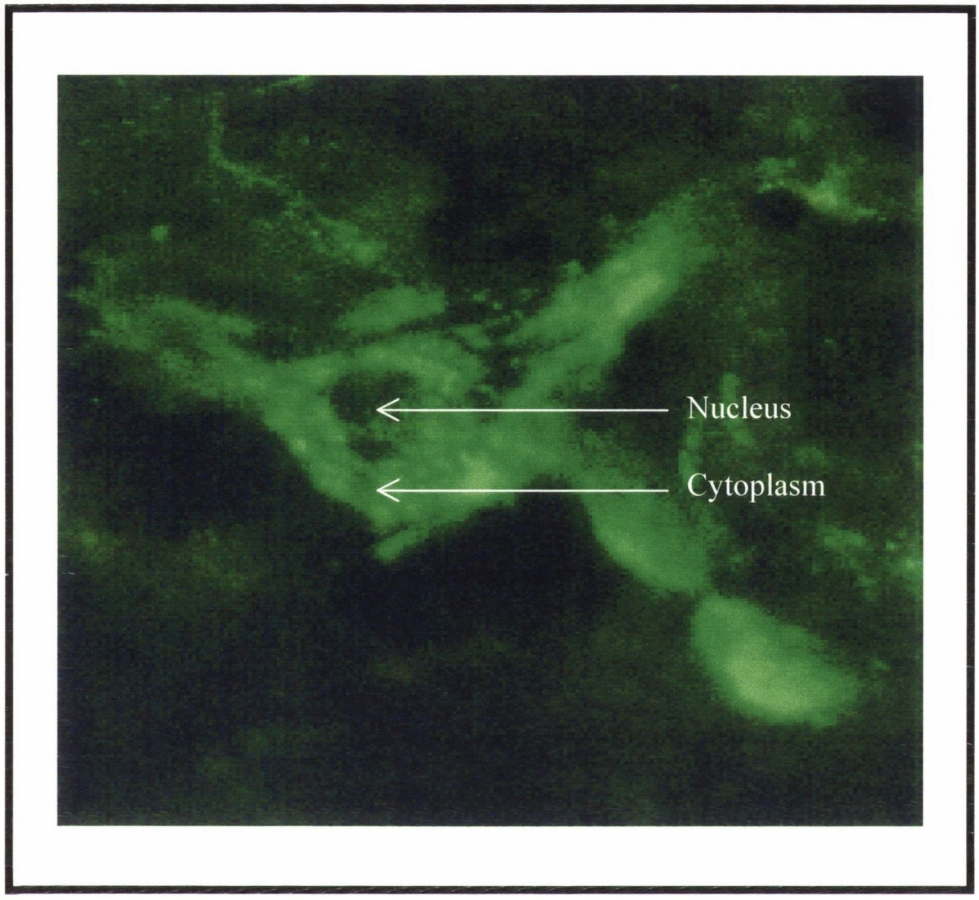


Figure 3.15 - Immunofluorescent staining of BHK cells transfected with pcDNA3.A-segment. The cells were fixed in acetone 48 h post-transfection and stained with IPNV polyclonal antiserum and a FITC-conjugated secondary antibody.

PAGE system. Immunoblots of the gels were obtained using IPNV polyclonal antiserum or a MAb to the VP3 protein and were developed using the ECL plus system (Amersham).

Expression of a number of IPNV proteins was detected in the pSFV clones as shown in the autoradiograph in figure 3.13, panel B. The proteins expressed are VP2 (50 kDa), VP3 (31.6 kDa) and VP4 (26.6 kDa). Cells electroporated with the pSFV1-A7 clone were also found to express proteins at 86.6 and 36.6 kDa. When a MAb to VP3 was used one protein at 31 kDa was detected in both purified virus and in cells electroporated with pSFV1-A7 (Figure 3.13, panel C) The autoradiograph in figure 3.16, shows that immunoprecipitation occurred with the pSFV1-VP2.I. and pSFV-A-segment VP2 protein expressed from pSFV1-VP2.II was also immuneprecipitated with IPNV polyclonal antisera (data not shown). The approximate sizes of proteins obtained were: VP2 - 54 kDa, VP4 - 27 kDa and VP3 - 32 kDa. Labelled but unprecipitated samples were run on the left side of the gel and untransfected cells were used as a negative control (lanes 1 and 4). pSFV1-VP2.III did not express the VP2 protein and no immunoprecipitate was detected (data not shown).

3.4.4 pBKT-SFV-A-segment cloning and expression

The full-length A-segment was reamplified from pcDNA3-A2 using cloned *Pfu* polymerase, a proof reading polymerase with 5'-3' exonuclease activity.

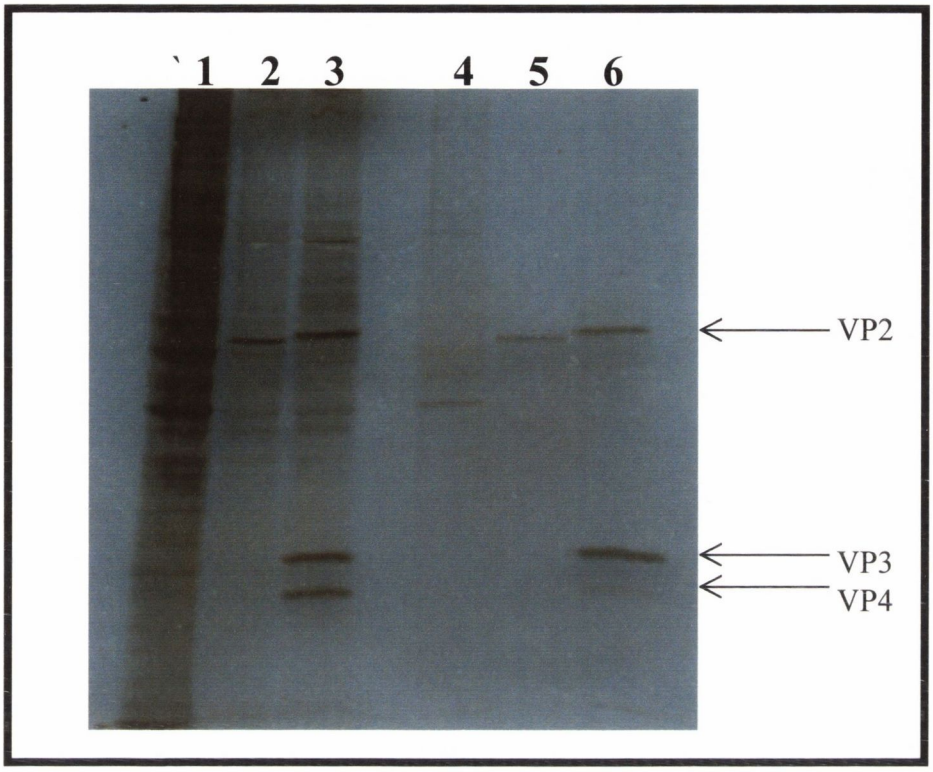


Figure 3.16 - Metabolic labelling and immunoprecipitation of proteins.
Cells transfected with pSFV1 expression constructs were radiolabelled with ^{35}S-methionine and proteins from cell lysates immunoprecipitated with IPNV polyclonal antisera. Lane 1: Untransfected cell lysate. Lane 2: Cell lysate from cells transfected with pSFV1-VP2. Lane 3: Cell lysate from cells transfected with pSFV1-A-segment. Lane 4: Untransfected cell lysate immunoprecipitated with IPNV polyclonal antisera. Lane 5: Cell lysate from cells transfected with pSFV1-VP2 and immunoprecipitated with IPNV polyclonal antisera. Lane 6: Cell lysate from cells transfected with pSFV1-A-segment and immunoprecipitated with polyclonal antisera.

The resulting blunt ended PCR product was cloned into the layered DNA/RNA expression vector pBKT-SFV at the *Sma* I site. The presence of a 3.1 kb insert was confirmed by *Bam*H I digestion and orientation of inserts determined by *Eco*R I restriction digests. BHK cells were transfected by lipofection with plasmid DNA from pBKT-SFV clones containing the A-segment insert in the correct orientation. Forty-eight hours post-transfection, cells were harvested and run on a standard SDS-PAGE system. Immunoblots of the gels were obtained using IPNV polyclonal antiserum or a MAb to the VP3 protein and were developed using the ECL plus system (Amersham).

Expression of a number of IPNV proteins was detected in the pBKT-SFV clones as shown in the autoradiograph in figure 3.17, panel A. The proteins expressed are VP2 (50 kDa), VP3 (31.6 kDa) and VP4 (26.6 kDa). When a MAb to VP3 was used one protein at 31 kDa was detected in both purified virus and in cells electroporated with pSFV1-A7 (Figure 3.17, panel B). Expression of the A-segment in BHK cells was also detected by immunofluorescence with polyclonal antiserum (Figure 3.18). Non-transfected cells were not immunofluorescent with IPNV antiserum (data not shown).

3.5 Discussion

When full-length cDNA encoding the VP2 gene is cloned into the SFV expression vector, pSFV1, a VP2 sized protein is detected in ^{35}S-labelled cells and in Western blots. The protein produced is approximately 54 kDa in size. However when

Figure 3.17 – Western immunoblots of pBKT-SFV expression constructs. Lysates from cells transfected with pcDNA3 expression constructs were run on SDS-polyacrylamide gels. Gels were subsequently blotted onto PVDF membrane and bound proteins detected using chemiluminesence with IPNV specific antibodies and a horseradish peroxidase conjugated secondary antibody. In all panels IPNV proteins are indicated with arrows.

Panel A: Western immunoblot of proteins from cells transfected by lipofection of plasmid DNA probedwith IPNV polyclonal antisera. Lane 1: IPNV-infected CHSE cells. Lanes 2 and 3: pBKT-SFV-A-segment 48 h post-transfection. Lane 4: Untransfected cells.

Panel B: Western immunoblot of proteins from cells transfected by lipofection of plasmid DNA probed with IPNV Mab to VP3. Lane 1: IPNV infected CHSE cells. Lane 2: Untransfected cells. Lanes 3 and 4: pcDNA3-A-segment 48 h post-transfection.

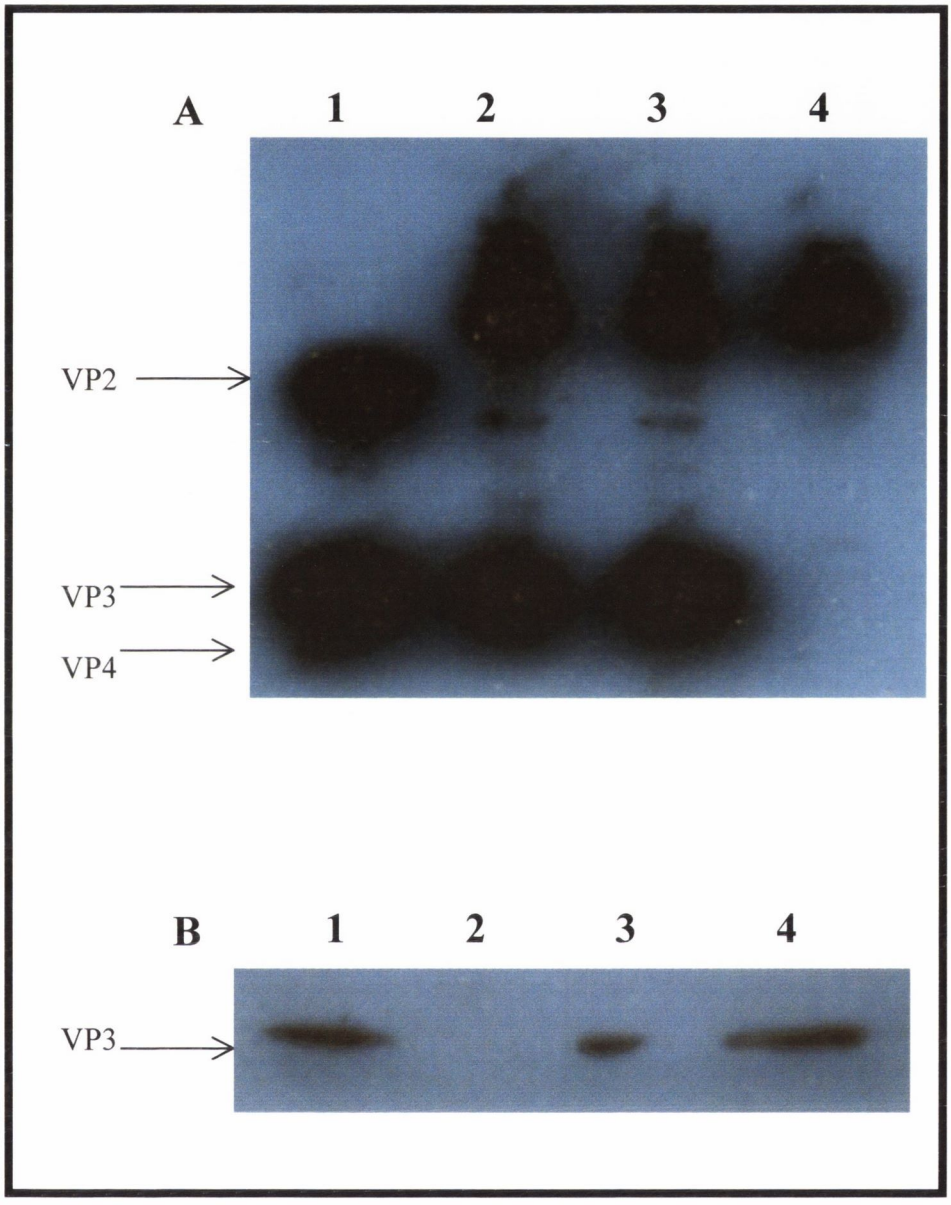


Figure 3.17 - Western immunoblots of pBKT-SFV expression clones.

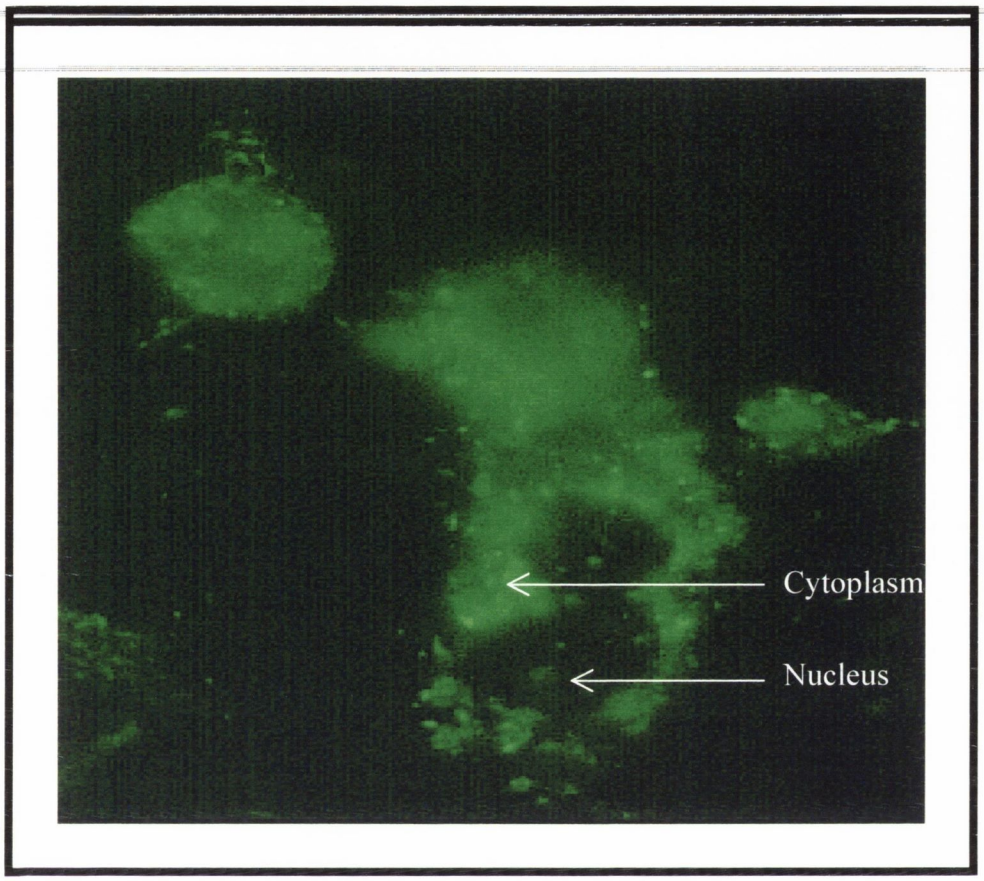


Figure 3.18 - Immunofluorescent staining of BHK cells transfected with pBKT-SFV-A-segment. The cells were fixed in acetone 48 h post-transfection and stained with IPNV polyclonal antiserum and a FITC-conjugated secondary antibody.

the smaller cDNA is cloned into pSFV1 no protein could be detected by either system. This could be due to rapid protein degradation due to incorrect folding of the recombinant product. Truncated cDNA clones have previously been shown to produce IPNV-VP2 when expressed in *E.coli* (Manning and Leong, 1990; Leong, 1992). As these proteins were found to react with IPNV specific antisera they were still considered to be immunogenic and distinctions were not drawn between the truncated and full-length cDNA clones in subsequent vaccination studies. A variety of deletions in VP2 have also been shown to react with conformation-dependent MAbs (Frost *et al*, 1995).

The cDNA encoding the entire A-segment was cloned into the expression vectors pSFV1, pcDNA3 and pBKT-SFV. Expression in BHK-21 cells from all three systems resulted in the processing of the polyprotein encoded by the A-segment. In addition to IPNV specific proteins which co-migrated with VP2, VP3 and VP4 proteins were produced with molecular weights of 89 and 37 kDa when expressed from pSFV1 and pcDNA3. *In vitro* translation of A-segment mRNA by Duncan and co-workers also resulted in the production of an 89 kDa protein (Duncan *et al*, 1987). The protein is believed to be an amino truncated polyprotein produced by internal initiation, probably at Met5. VP4 mediated cleavage of this protein results in the production of a 37 kDa protein, an amino truncated VP2 polypeptide. Recombinant baculovirus expressing IPNV VP2 or A-segment was also found to produce a protein of this size (Magyar and Dobos, 1994a). Internal initiation of translation of IPNV Sp RNA, *in vitro* and in infected cells, is also responsible for the

production of a 38 kDa protein. This protein is an amino truncated VP4-VP3 polypeptide.

In infected cells, the polyprotein is rapidly cleaved into pVP2, VP4 and VP3. Further processing of pVP2 to VP2 and VP3 to VP3a is slow and is most likely due to host cell proteases rather than the further proteolytic action of the VP4 protease. Although cloning of IPNV A-segment into baculovirus expression vectors resulted in the correct processing of the polyprotein to pVP2, VP4 and VP3 in insect cells, neither VP2 nor VP3a were detected (Magyar and Dobos, 1994a). Expression of IPNV A-segment from the vectors in this study appears to result in the correct processing of VP2 to pVP2. Processing of VP3 to VP3a also appears to occur efficiently.

Recombinant IPNV proteins expressed from *E. coli* have successfully been used in vaccination experiments. Rainbow trout fry were immersed in crude bacterial lysates that expressed the IPNV Sp-A-segment. Subsequent challenge with a heterologous strain of IPNV 3 weeks later resulted in a RPS of 91% (Manning and Leong, 1990). The cloning of N1-VP2 into *E.coli* also results in the production of a recombinant protein, which appears to be correctly processed as it reacts with several neutralising MAb's recognising conformationally dependent epitopes (Frost *et al*, 1995). VP2 produced in this manner has been included in a commercial oil/glucan adjuvanted multivalent vaccine, against furunculosis, vibriosis and cold-water vibriosis, developed by Intervet Norbio. In field trials RPS in the vaccinated Atlantic salmon group was evaluated as 60% (Christie, 1997).

One of the newest approaches in fish vaccine design is genetic immunisation. Strong expression of reporter genes can be detected following intramuscular injection of plasmid DNA (Hansen *et al*, 1991; Heppell *et al*, 1998). DNA vaccines against IPNV have not yet been evaluated although prototype vaccines against other viruses have been developed. Plasmid expressed proteins appear to retain their native structure as they are recognised by conformation dependent MAbs in cell-culture expression studies (Heppell *et al*, 1998). The viral glycoprotein of IHNV and the glycoprotein and nucleocapsid protein of VHSV, when placed under the control of CMV promoter, induced protective immunity against viral challenge (Anderson *et al*, 1996a; Anderson *et al*, 1996b; Lorenzen *et al*, 1998). Combined DNA immunisation against the two pathogens resulted in an antibody reaction of equivalent quality and intensity to single immunisations (Boudinot *et al*, 1998).

The expression constructs developed in this study were sent to consortium partner 5 to evaluate their protective efficacy in a large scale challenge model with Atlantic salmon *(Salmo salar L.)*.

Chapter Four

Production of Recombinant SFV Particles

4.1 Introduction

In addition to *in vitro*-produced SFV RNA used directly for transfection of cells, a helper system has been developed to allow the packaging of recombinant RNA into infectious "suicide" SFV particles. Particles are produced by the co-transfection of BHK cells with *in vitro* transcribed recombinant SFV RNA and helper RNA. The replication deficient particles can then be used to infect cells in a manner identical to infectious virus. Preliminary experiments indicate that SFV particles are stable in water and therefore may be a feasible approach for immersion vaccination.

4.2 Materials and methods

4.2.1 SFV particle production

In vitro transcription of VP2 and A-segment RNA from pSFV1 clones was performed as described in section 3.5.3.1.1. In addition, mRNA was transcribed from the split helper plasmids pSFV-HelperS2 and pSFV-HelperCS219A (Smerdou and Liljestrom, 1999). Equimolar amounts of helper RNAs and A-segment RNA were electroporated into BHK cells as described in section 3.3.3.3. Twenty-four h post-electroporation, tissue-culture supernatants were harvested and cell debris removed by centrifuging at 1000 g

for 15 min. Particles in the supernatant were then aliquoted on ice and rapidly frozen in liquid nitrogen before storing at -70°C.

4.2.2 BHK cell infection

4.2.2.1 Particle titration

Particle titres were obtained by infecting 80% confluent BHK cell monolayers grown on 13 mm glass coverslips in six-well tissue culture plates with serial dilutions of virus in infection medium (MEM, 0.2% BSA, 20 mM Hepes, 2 mM glutamine). Virus particles were allowed to adhere for 1 h with shaking every 15 min. The inoculum was then aspirated and cells overlayed with normal BHK-21 medium and incubated at 37°C, in 5% CO_2 overnight. Coverslips were washed twice with PBS before fixing in acetone for 10 min. The coverslips were then incubated for 1 h at 37°C with a 1:1000 dilution of the anti-IPNV polyclonal antiserum, washed in PBS for 10 min and then incubated for a further 1 h at 37°C with a pig anti-rabbit FITC-conjugated antibody (DAKO). Cells were then washed for 10 min in PBS and mounted in Mowiol-DABCO. Finally cells were observed microscopically for the presence of specific fluorescence. (Figure 4.1)

4.2.2.2 Monoclonal antibodies

BHK cells were infected with particles and fixed in acetone as described in section 4.2.2.1. Coverslips were incubated for 1 h at 37°C with either a 1:50 dilution of the anti-VP2 MAbs B9 and F2, a 1:100 dilution of the anti-VP2 MAb H8 or a 1:100 dilution of the anti-VP3 MAb C12. The coverslips were then washed in PBS for 10 min and incubated for a further 1 h at 37°C with a pig anti-mouse FITC-conjugated secondary antibody (DAKO). Cells were then washed for 10 min in PBS, mounted in Mowiol-DABCO and observed microscopically for the presence of specific fluorescence. (Figure 4.2)

4.2.3 CHSE cells

4.2.3.1 Infection of CHSE cells with recombinant particles.

CHSE-214 cells were maintained in growth medium as described in section 2.5.2. Cells were propagated on 13 mm glass coverslips in 35 mm tissue culture dishes and were incubated at 20°C in closed containers in a 3% CO_2 atmosphere. Serial dilutions of virus in infection media (MEM, 0.2% BSA, 20 mM Hepes, 2 mM glutamine) were used to infect 80% confluent monolayers. Virus particles were allowed to adhere for 1 h at 20°C with shaking every 15 min. The inoculum was then aspirated and cells overlayed with growth medium that had a reduced FCS concentration of 2% and

incubated at 25°C, 3% CO_2 for 48 h. Coverslips were washed twice with PBS before fixing in acetone for 10 min.

4.2.3.2 Immunofluorescence

The coverslips were subjected to indirect immunofluorescence as described in section 4.2 with both polyclonal and MAbs. (Figure 4.3)

4.2.4 Electron microscopy

4.2.4.1 Infection of CHSE cells with recombinant particles

CHSE-214 cells were maintained in growth medium as described in section 2.5.2. Cells were propagated on 2 cm^2 Melinex coverslips (Agar Scientific) in 35 mm tissue culture dishes and were incubated at 20°C in closed containers in a 3% CO_2 atmosphere. Cells were infected with recombinant particles as described in section 4.2.3.1, with the following exception. In order to increase the number of cells infected with particles, 30 µl of particles in 525 µl infection medium were used per well. This corresponds to $1.5x10^7$ particles/well. After 1 h the inoculum was aspirated and cells overlayed with growth medium with a reduced FCS concentration of 2% and incubated

at 25°C, 3% CO_2 for 4 days. Cells were fixed in warm glutaraldehyde fixative consisting of 2.5% glutaraldehyde in 0.09 M cacodylate buffer, pH7.2, containing 3 mM $CaCl_2$. Cells were fixed at the incubation temperature for 30 min, after which the fixative was aspirated and replaced with 0.1 M cacodylate buffer, pH7.2 containing 3 mM $CaCl_2$. Uninfected CHSE cells were processed in the same manner. Fixed cultures were stored at 4°C in buffer until embedding.

4.2.4.2 Embedding of cultured cells

Cultured cells on melinex coverslips were washed 6 times in 0.1 M cacodylate buffer before staining with 2% osmium tetroxide in 0.1 M cacodylate buffer for 30 min. Cells were dehydrated by successive incubations in 10, 30, 50, 75 and 95 % ethanol solutions, each for 10 min. Coverslips were then incubated overnight in 100% ethanol. The ethanol was then replaced with propylene oxide and incubated for 15 min followed by a second incubation step for 30 min. A solution of 50% Agar 100 Epoxy resin, containing DDSA hardener, MNA hardener and BDMA accelerator, in propylene oxide was prepared and used to cover the cells for 2 h. This was subsequently replaced with 100% resin for 2 h. Resin was placed in an embedding mould onto which the Melinex coverslip was placed face down. The resin was allowed to polymerise at 60°C for 24 h after which the Melinex film was peeled from the polymerised resin. Another layer of

resin was added to the monolayer of cells in the embedding mould and allowed to polymerise for 24 h, thereby forming a sandwich of the monolayer in resin.

2.2.4.3 Transmission electron microscopy

Ultra-thin sections cut from the embedded monolayers were placed on copper grids and stained with 0.5% aqueous uranyl acetate and Reynolds lead citrate. Stained grids were studied using the Hitachi H7000 electron microscope. (Figures 4.4)

4.3 Results

4.3.1 Particle production and titration in BHK cells

Particle titres were obtained by infecting 80% confluent BHK cell monolayers grown on glass coverslips in six-well tissue culture plates with serial dilutions of virus in infection media. Particles were allowed to adhere for 1 hour at 37°C before replacing inoculum with normal growth media and incubation at 37°C overnight. After fixing in acetone coverslips were incubated with a 1:1000 dilution of the IPNV polyclonal antiserum and subsequently with a pig anti-rabbit FITC conjugated antibody. Titres of approximately $1x10^7$ were routinely obtained. Conformational dependent MAbs to VP2

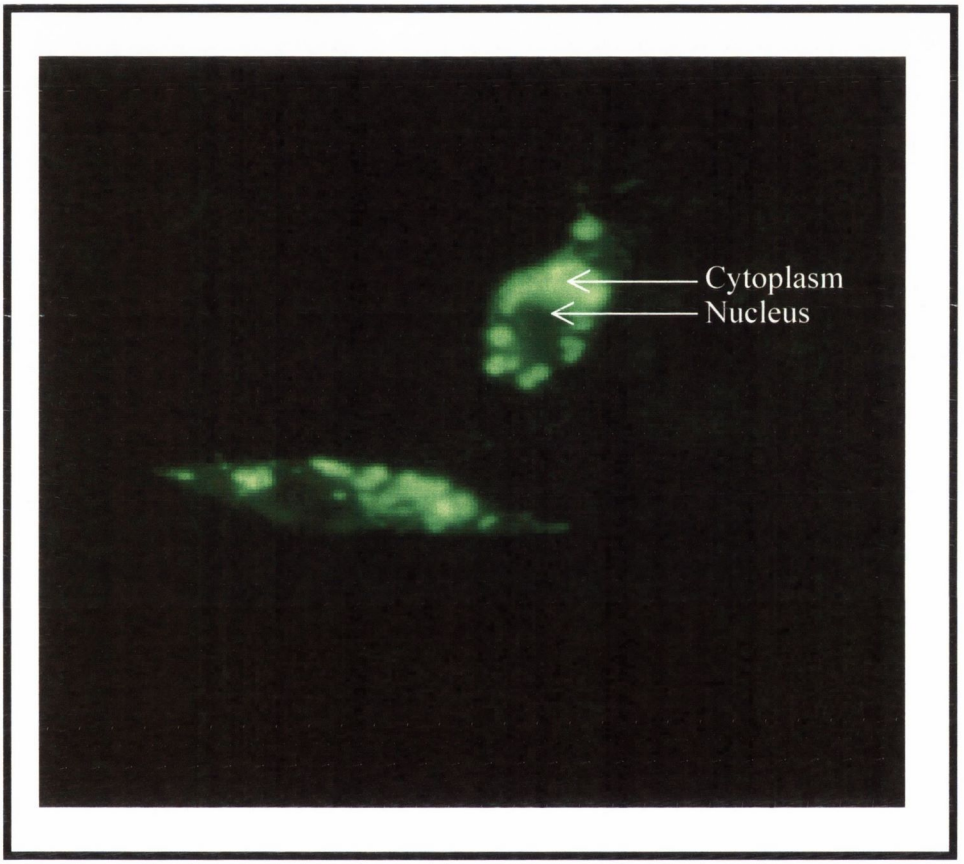


Figure 4.1 - Immunofluorescent staining of BHK cells infected with recombinant SFV particles expressing IPNV A-segment. BHK cells were grown to 80% confluency on 13 mm coverslips in 35 mm dishes and infected with recombinant SFV particles expressing IPNV A-segment. 24 h post-infection cells were fixed and incubated with IPNV polyclonal antiserum. Samples were subsequently incubated with FITC-conjugated pig anti-rabbit Ig and viewed by epifluorescence.

Figure 4.2 – Immunofluorescent staining of BHK cells infected with recombinant SFV particles expressing IPNV A-segment. BHK cells were grown to 80% confluency on 13 mm coverslips in 35 mm dishes and infected with recombinant SFV particles expressing IPNV A-segment. 24 h post-infection cells were fixed and incubated with IPNV MAbs. Samples were subsequently incubated with FITC-conjugated pig anti-mouse Ig and viewed by epifluorescence. Cytoplasmic staining was observed with the three IPNV MAbs used. Panel A: MAb C12 – anti VP3. Panel B: MAb B9 – anti VP2. Panel C: MAb H8G2 – anti VP2.

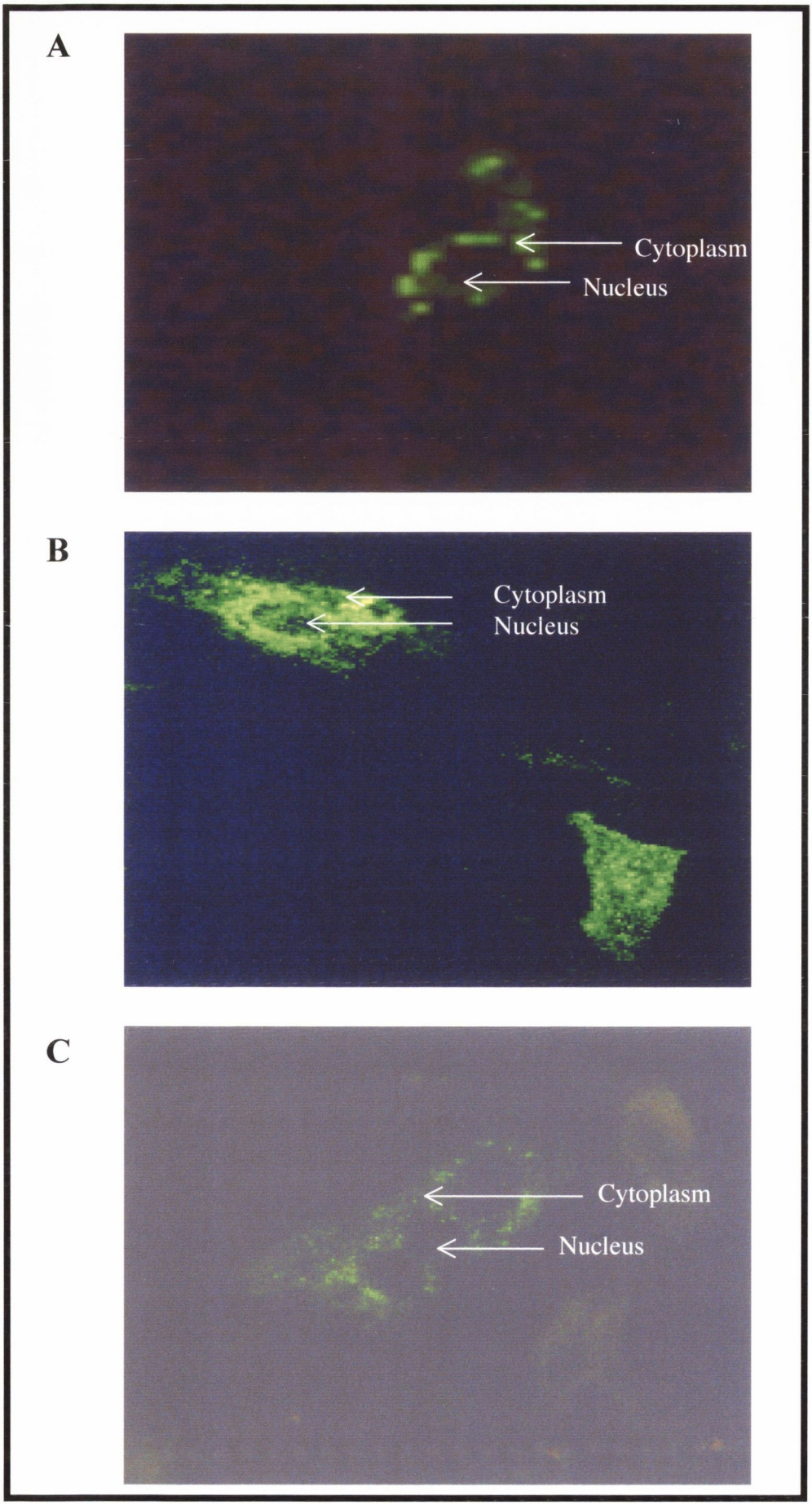


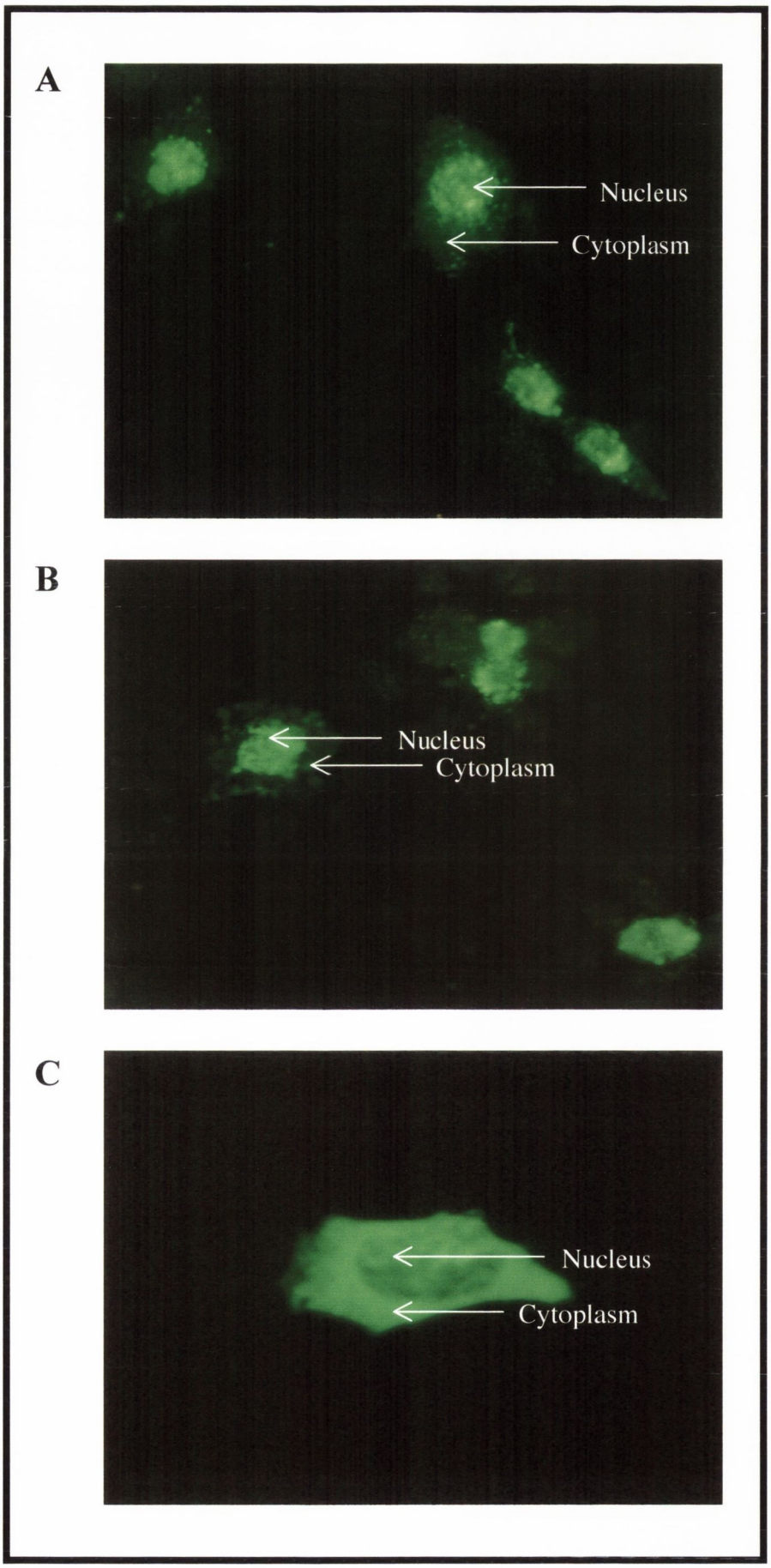
Figure 4.2 - Immunofluorescent staining of BHK cells infected with recombinant SFV particles expressing IPNV A-segment.

(B9, F2, H8G2) and a monoclonal to VP3 (C12) were also tested with infected BHK cells. Figure 4.1 shows typical staining patterns obtained with the polyclonal antiserum. The reaction with IPNV MAbs is shown in figure 4.2.

4.3.2 Infection of CHSE cells

The antigenicities of the pSFV1 constructs were also tested in CHSE cells. Cells were grown on glass coverslips as above and infected with recombinant particles when 80% confluent. Cells were infected at 20°C for 1 hour and then incubated at 25°C for 2 days. Following acetone fixation cells were incubated with the IPNV polyclonal antiserum or IPNV MAbs to VP2 and VP3 and incubated further with the appropriate FITC-conjugated secondary antibody. As figure 4.3 shows, the staining pattern with the poyclonal antiserum and C12 MAb was quite different to that observed in BHK cells with the majority of the staining occurring in the nucleus. Mainly cytoplasmic staining was observed with the VP2 monoclonal antibodies.

The antigenicities of the constructs are summarised in table 4.1.


A
Nucleus
Cytoplasm
B
Nucleus
Cytoplasm
C
Nucleus
Cytoplasm

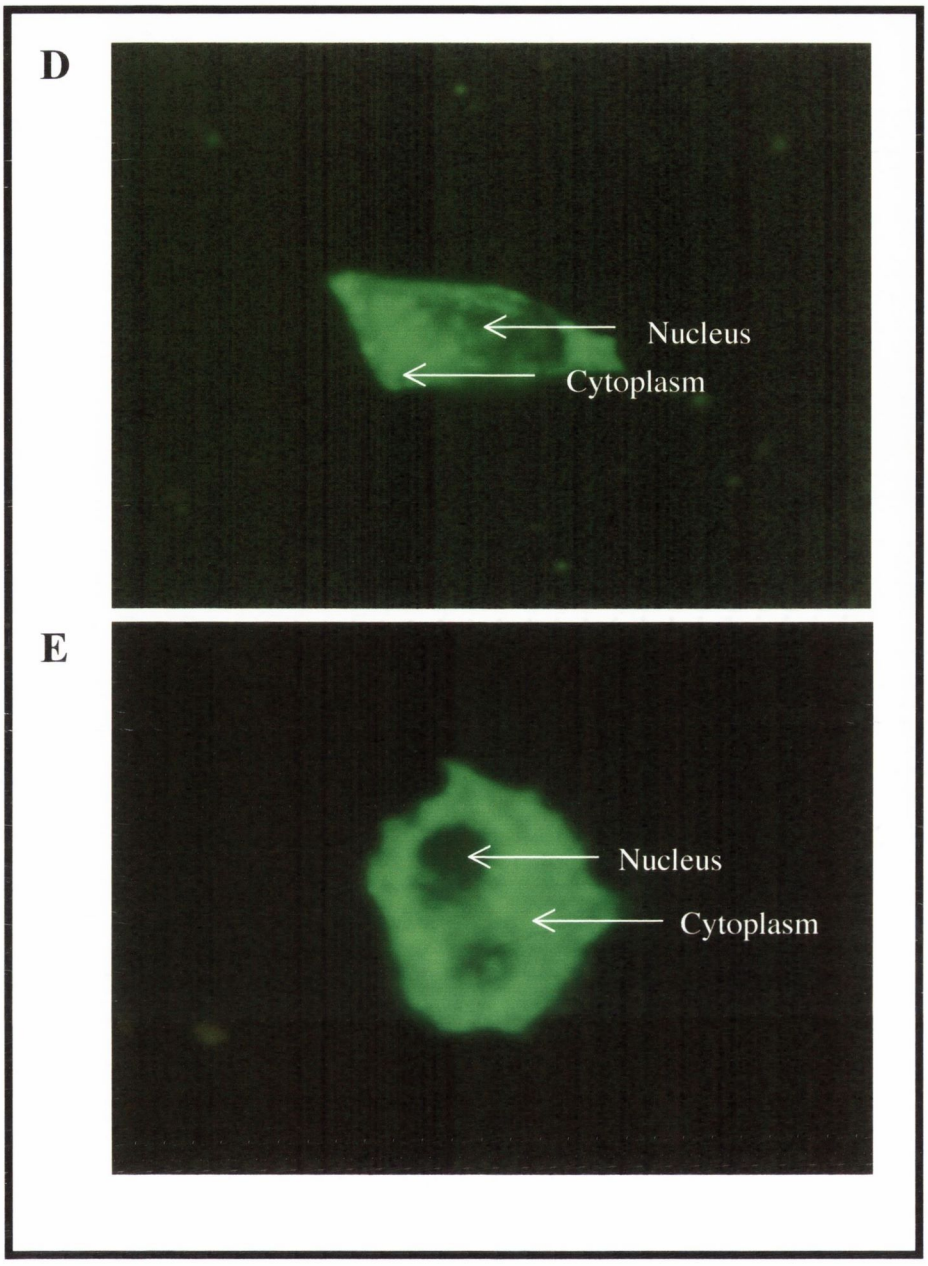


Figure 4.3 - Immunofluorescent staining of CHSE cells infected with recombinant SFV particles expressing IPNV A-segment. CHSE cells were grown to 80% confluency on 13 mm coverslips in 35 mm dishes and infected with recombinant SFV particles expressing IPNV A-segment. 72 h post-infection cells were fixed and incubated with IPNV polyclonal antiserum or IPNV MAbs. Samples were subsequently incubated with FITC-conjugated pig anti-rabbit Ig or FITC-conjugated pig anti-mouse Ig and viwed by epifluorescence. Panel A: IPNV polyclonal antiserum. Panel B: MAb C12 - anti VP3. Panel C: MAb B9 - anti VP2. Panel D: MAb F2 - anti VP2. Panel E: MAb H8G2 - anti VP2.

	BHK					CHSE				
antibody	***Poly***	***C12***	***B9***	***F2***	***H8***	***Poly***	***C12***	***B9***	***F2***	***H8***
pSFV-VP2	++++	-	+	+	+	++++	-	++	+	+
pSFV-A seg	++++	+++	+	+/-	+	++++	+++	+	+/-	+

Poly = polyclonal antiserum

++++ : 30% cells stained.
+++ : 5-10% cells stained.
++ : 1% cells stained.
+ : <1% cells stained.
+/- : faint staining.

Table 4.1 - Antigenic characterisation of SFV constructs expressing IPNV VP2 and A-segment in BHK and CHSE cell lines.

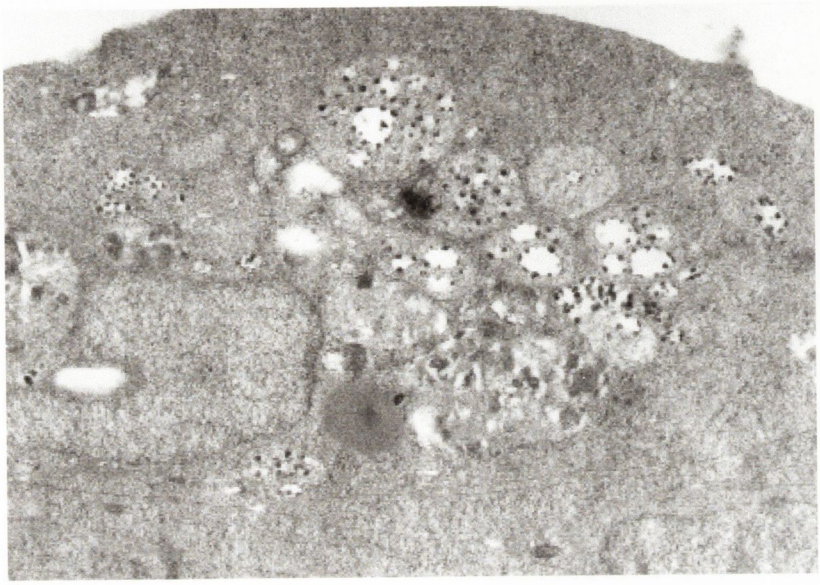
4.3.3 Electron microscopy.

Having established that expression of IPNV A-segment in mammalian and fish cells leads to the accumulation of the structural proteins VP2 and VP3, it was important to analyse whether they would assemble to form virus-like particles (VLP). To test this hypothesis CHSE-214 cells were infected with recombinant SFV particles expressing IPNV A-segment and maintained for 5 days before fixing and processing for thin-section transmission electron microscopy. As shown in figure 4.4 the cytoplasm of the infected cells contained membrane bound structures with accumulations of small isometric particles. The particles have a morphology and size (diameter of 60 nm) which strongly resemble the appearance of particles found within the cytoplasm of IPNV-infected cells.

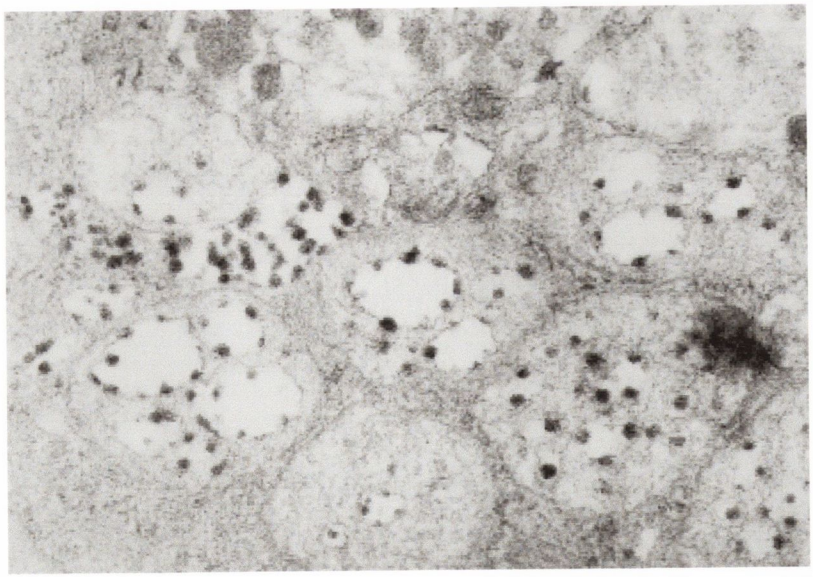
4.4 Discussion

Results from this study indicated that the SFV expression system functioned in BHK cells at 37°C and in the fish cell line, CHSE-214 at 25°C. This was demonstrated by the ability of recombinant SFV particles to express IPNV A-segment following infection of the cell lines. The expression levels in CHSE cells were lower than that in BHK cells. In addition to reacting with IPNV polyclonal antiserum, the expression products were

A



B



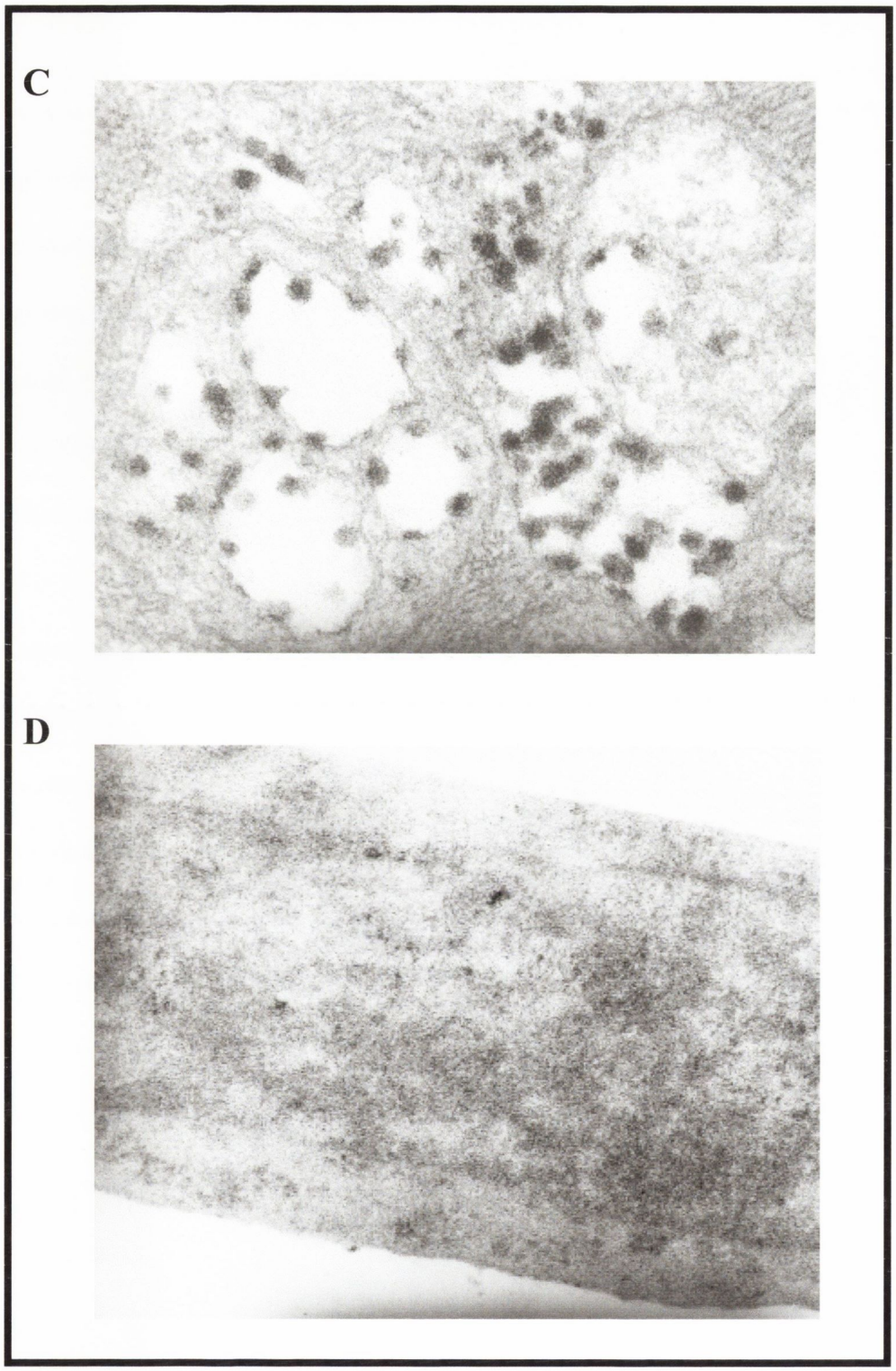


Figure 4.4 - TEM of CHSE cells infected with recombinant SFV particles expressing IPNV A-segment. Ultra-thin sections of CHSE cells were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy. The cytoplasm of infected cells show vacuoles containing virus like particles. Panel A: 20,000x magnification. Panel B: 40,000x magnification. Panel C: 100,000x magnification. Panel D: Uninfected cells, 30,000x magnification

recognised by the different IPNV MAbs. These MAbs are directed against conformation-dependent epitopes,

suggesting that the protein is processed correctly and that its antigenicity is conserved. In terms of vaccine development, the ability of an expression system to produce an antigen that is correctly processed and folded is important if the protein is to function as an effective immunogen. Such problems have been encountered by a baculovirus-produced G protein of VHSV (Lecocq-Xhonneux *et al*, 1994). Although only a fraction of the protein appeared to be correctly processed in this case, when tested in an experimental challenge, recombinant protein induced a level of protection comparable to that induced by conventional vaccines. The A-segment of IPNV has also been expressed using the baculovirus system, although the recombinant proteins produced have not been used in a vaccination trial (Magyar and Dobos, 1994a). Recombinant proteins produced by DNA expression vectors also appear to be correctly folded. VHSV-G-protein expressed from a pCMV expression vector in EPC cells is recognised by G-protein specific MAbs which recognise linear or conformational epitopes (Heppell *et al*, 1998). The minute quantitities of antigen expressed by DNA vaccines appear to be sufficient to induce strong and long-lasting immune responses in mammals (Donnelly *et al*, 1997) and fish (Heppell *et al*, 1998; Lorenzen *et al*, 1998; Anderson *et al*, 1996b). It is therefore possible that adequate levels of SFV expression may take place *in vivo* to stimulate an immune response following inoculation of fish with recombinant particles.

Expression of the A-segment of a related birnavirus, IBDV, from an inducible expression vector was shown to result in the formation of VLP in infected cells

(Fernandez-Arias *et al*, 1998). As immunofluorescence studies have shown, expression of IPNV A-segment from recombinant SFV particles in CHSE cells, leads to the accumulation of the structural proteins VP2 and VP3. The proteins formed are also found to react with conformation dependent MAbs, which suggests that they retain their native structure. Cells infected with recombinant particles were examined by transmission electron microscopy to determine if VLP were formed. VLP were detected in membrane bound structures within the cytoplasm of infected cells. The particles had a morphology and size similar to that reported for IPNV. In pancreatic tissue from diseased rainbow trout, IPNV particles are distributed randomly in the cell cytoplasm. In necrotic cells, particles were arranged in a crystalline array in membrane bound aggregates (Ball *et al*, 1971). Cultured cells infected with IPNV were also found to accumulate virus particles in crystalline arrays (Moss and Gravell, 1969; Granzow *et al*, 1997). No such aggregates were detected in this study. Co-transfection of cells with inducible vaccinia virus vectors expressing the A-segment and B-segment of IBDV results in the efficient encapsidation of VP1 into virus-like particles (Lombardo *et al*, 1999). Immunofluorescence, confocal laser scanning microscopy and immunoprecipitation analyses indicate that VP3 interacts with VP1, resulting in its efficient packaging into VLP. This interaction may be a key step in the morphogenesis of birnaviruses and may be critical for the formation of infectious virus.

A reverse genetics system for the recovery of infectious IPNV has been developed recently (Yao and Vakharia, 1998). Full length cDNA clones of IPNV virus A- and B-segments are used to produce infectious virus. T7 polymerase is used to generate plus

sense transcripts of the two genomic segments, which are co-transfected into CHSE-214 cells. As early as 4 days post-transfection infectious virus with a titre of $1.2x10^1$ PFU/ml could be recovered. This gradually increased to a maximum titre of $6.7x10^7$ PFU/ml 10 days post-transfection, causing severe cpe to infected cells. In addition, VP1 was discovered to be species specific as no infectious particles were generated if cells were co-transfected with transcripts derived from IPNV A-segment and IBDV B-segment or *vice versa*.

Chapter Five

Consortium Partner Results

5.1 Introduction

As described in section 1.5 this project was part of an E.U FAIR programme funded consortium.

5.2 Partner 2

Project participants in the Karolinska Institute provided the SFV expression vector system. Over the course of the project the original vector system was developed with further vectors emerging with enhanced biosafety characteristics (Smerdou and Liljestrom, 1999). The layered DNA/RNA vector was also developed and optimised (Berglund et al, 1998).

5.3 Partner 3

The main aim of the participant from the Dept. Agriculture for N. Ireland was to assess the ability of the SFV expression system to deliver reporter and IPNV antigens to fish cells in culture and to the immune system of fish. Their results indicated that the SFV expression system was able to function in

salmonid cell lines at 25°C but not below this temperature. Expression levels of *lacZ* and luciferase reporter genes following transfection of CHSE-214 cells were lower than those observed with BHK-21 cells. When IPNV-VP2 was expressed from the SFV expression system in BHK and CHSE cells the protein was found to react with IPNV MAbs which are directed against conformation-dependent epitopes, suggesting that the protein is correctly processed and its antigenicity conserved. The same protein expressed from a conventional DNA expression plasmid, pcDNA3, was found to react only weakly with one of the MAbs in CHSE cells. No reaction was observed in BHK cells. In contrast to the tissue culture results, expression of IPNV VP2 could not be detected following inoculation of fish using recombinant SFV particles, RNA or the layered DNA/RNA system. VP2 expressed from the DNA plasmid pcDNA3 could be detected and appeared to be correctly folded as it reacted with MAbs H8G2 and B9.

5.4 Partner 4

VESO Vikan AkvaVet was responsible for the development of a standardised IPNV infection challenge model, which could be used to determine the protective efficacy of candidate recombinant vaccines developed during the

course of the project. The research station investigated a series of challenge routes over the course of the project and although a standardised IPNV challenge model has not yet been established, they succeeded in inducing an acute IPN outbreak twice using a bath challenge model. The evaluation of candidate vaccines was possible in the last challenge. One year old Atlantic salmon (*Salmo salar* L.) smolts were kept in 1x1 m fibre glass tanks with a total volume of 450 l. One hundred fish were kept in each tank. Fish were vaccinated in June with a commercial IPN vaccine or one of the recombinant vaccines developed. Those included in this study were SFV-A-segment particles, pcDNA-VP2 and layered DNA/RNA VP2. DNA vaccines were administered intra-muscularly and other vaccines i.p. Fish were kept in the 1x1 m tanks for 8 weeks, tagged for identification and moved together to a 3 m diameter fibre glass tank for challenge. Water in this tank was kept at 13°C with 25% salinity. The water level in the tank was reduced to a water column of 10 cm (700 l) and 600 ml IPNV culture added. The water inlet was closed and water aerated for 5 h. The normal water column of 100 cm (7000 l) was then re-established and 24 h later salinity increased to 33%. Preliminary findings with the challenged fish showed an outbreak of IPN started 8 days post exposure and lasted for 10 days. The cumulative mortality in the non-vaccinated test group was 50 %. Among the vaccinated groups the commercial IPN vaccine had a RPS of 70 %. The best of the recombinant vaccines was SFV A-segment particles, which had a RPS of

22.2%. The RPS of the layered DNA/RNA and pcDNA3-VP2 were 14.3 % and 0% respectively. However it remains to be seen if these findings are significant.

5.5 Partner 5

The main contribution of Central Veterinary Laboratory, Oslo, was to work towards the development of laboratory assays for the measurement of humoral and cellular immune responses to IPNV in Atlantic salmon. These assays would then be used in order to measure and characterise the immune responses and protective immunity in fish after vaccination with recombinant vaccines against IPNV developed during the course of the project. An ELISA was established using the polyclonal rabbit antiserum as a catch for IPNV as antigen. The assay was used to evaluate the immune response in groups of fish vaccinated and/or challenged in trials at VESO Vikan AkvaVet. The immune response induced after virulent challenge with IPNV was detected and an increased response has been demonstrated in vaccinated fish compared to non-vaccinated fish after challenge. A lymphocyte stimulation assay was developed to monitor cell-mediated immune responses. The establishment of this assay was successful in that proliferation of lymphocytes from Atlantic salmon may be induced with mitogens. In the first year a preparation of a supernatant from IPNV infected cells was able to induce a lymphocyte response of at least the

same level as or higher than the known mitogens LPS from *E. coli* and PHA from *A. salmonicida*. Subsequent assays using similar infectious antigen preparations seemed to cause a marked and dose-dependent suppression of both B- and T-cell responses. The assay is not very stable and it has not been possible to demonstrate any increased lymphocyte proliferation in vaccinated fish.

Chapter Six

Discussion

6.1 Discussion

The aquaculture industry has expanded rapidly in recent years. Disease outbreaks are increasingly recognised as causing a significant constraint to aquaculture production. Over the last few years bacterial disease problems have been brought under control, largely due to the use of a new generation of oil-adjuvanted vaccines. Vaccine development for viral diseases such as IPNV, VHSV and IHV has proven to be more difficult. IPNV is the most important infectious disease in the Norwegian aquaculture industry. To date vaccine research has focused on the use of inactivated virus (Dorson, 1988; Bootland *et al*, 1990), live-attenuated strains (Dorson *et al*, 1978) or recombinant sub-unit vaccines (Manning and Leong, 1990). The traditional vaccines based on live IPNV are not deemed acceptable to the aquaculture industry because of their safety, environmental and economic disadvantages. Current methods of vaccine development have turned to recombinant DNA technology. It is hoped that this technology may provide efficient and inexpensive vaccines. Attempts to over-produce viral antigens in bacterial systems have not always been successful due to incorrect glycosylation or folding of the recombinant protein in hosts such as *E. coli*. Incorrect processing of recombinant proteins may lead to the loss or poor expression of important conformational epitopes, which may be protective. Despite these problems, the VP2 gene of IPNV over-expressed in *E. coli* and added to an oil-adjuvanted,

multivalent vaccine was shown to decrease viral multiplication and protects against viral challenge in field trials (Frost and Ness, 1997). Viral antigens have also been expressed in bacterial hosts and used directly as vaccines. Attenuated strains of *A. salmonicida* have been used to express IHNV and VHSV glycoproteins. Eukaryotic expression studies have also been examined. VHSV antigens, expressed from a recombinant baculovirus in insect cells, induce good protection in rainbow trout fry when administered by i.p. injection (Lecocq-Xhonneux *et al,* 1994). Recombinant baculovirus has also been used to express IHNV and IPNV antigens (Koener and Leong, 1990; Magyar and Dobos, 1994a). The proteins produced were found to be antigenic but have not been tested as vaccines as yet. Again, problems with incorrect folding may be responsible for a lack of antigenicity with some recombinant proteins produced by this system.

A consortium project was initiated in 1996 to investigate three novel approaches to the production of IPNV antigens and to evaluate the ability of these expression systems to deliver IPNV antigens to fish cells in culture and to the immune system of salmonid fish. The protective efficacies of candidate recombinant vaccines were determined using standardised challenge models and laboratory assays measuring immune responses to IPNV. The challenge model and laboratory assays were also developed during the course of the project.

One of the newest approaches in fish vaccinology is the use of naked DNA. The technique is based on the discovery that nucleic acid, with the correct eukaryotic expression signals can synthesise recombinant proteins encoded by the

DNA (Wolff *et al*, 1992; Tang *et al*, 1992). Immunisation with plasmid DNA encoding antigens elicits specific cellular and humoral immune responses (Donnelly *et al*, 1997). Although originally developed in mammals, the technique was found to be applicable to fish (Hansen *et al*, 1991). Intramuscular inoculation of rainbow trout with DNA plasmids expressing the G-glycoprotein of IHNV and the G and N proteins of VHSV provides protection in severe disease challenge models (Anderson *et al*, 1996b; Lorenzen *et al*, 1998). As yet there are no reports of a DNA vaccine expressing IPNV antigens.

In this study the potential of the SFV expression vector as a delivery system in fish was also investigated. SFV is known to have a very broad host range. Although not a natural host for SFV, the virus can infect cultured fish cells, producing viral protein. With this system, recombinant SFV RNA encoding IPNV proteins can be used directly for transfection of cells or for the production of replication defective particles when co-transfected with a helper plasmid.

The SFV vector design has extended to a DNA-based vector, in which the SFV replicon is placed under the control of the eukaryotic CMV immediate early promoter. Transfection of cells with DNA results in transcription of SFV RNA and high levels of expression of cloned heterologous genes.

The IPNV isolate chosen for this study was collected during a clinical outbreak of the disease in Norway. The isolate was shown to be representative of Norwegian isolates by serotyping. cDNA copies of the VP2 gene and the complete A-segment were constructed using RT-PCR. When sequenced, the field isolate

was found to be 95 % identical at the amino acid level to other Sp sequences, with the majority of substitutions occurring in the VP2 gene. This gene contains the major neutralising epitopes of the virus. The epitopes have been localised to a variable region of the genome that contains two hypervariable segments (Frost *et al*, 1995; Heppell *et al*, 1995; Tarrab *et al*, 1995). Amino acid substitutions outside the hypervariable regions are not believed to significantly affect the conformation-dependent neutralising epitopes. However, it has been noted that within serotypes the virus shows variable reactions with some conformation-dependent MAbs. This may be due to mutations, having arisen during cell passage, affecting the conformation of neutralising epitopes. Although some of the amino acid differences present in the field isolate may have arisen by genetic variation in the environment, mutations may also have occurred due to the RT-PCR reaction. The cDNA copy of the A-segment was then cloned into the various expression systems outlined above.

Results indicate that the SFV expression system is able to function in mammalian cells at 37°C. This was demonstrated by the ability of recombinant SFV RNA to express IPNV specific proteins following electroporation of BHK-21 cells. Replication defective particles were also produced and used to infect BHK cells. Immunofluorescence studies with conformation-dependent MAbs to IPNV confirmed that the recombinant proteins produced retained their antigenicity. When CHSE cells were infected with the replication defective particles the system was only able to function at 25°C. Again, conformation-dependent MAbs

confirmed that the recombinant protein produced was correctly processed and folded. The absence of expression in CHSE cells at lower temperatures indicates that the translation of SFV RNA or its replication is inhibited.

Recombinant protein expressed from pCMV-VP2 was found to react only with polyclonal antiserum in BHK cells. Infection of CHSE cells at 25°C resulted in the expression of recombinant protein that could be recognised by polyclonal antiserum and one of the conformation-dependent MAbs. Although recombinant protein expressed from the layered DNA-RNA vector could be detected with polyclonal antiserum in both BHK and CHSE cells, conformation dependent MAbs only recognised protein expressed in BHK cells. This could be an indication that the expression product is processed differently by the two expression systems.

In vivo studies were also performed. The various VP2 constructs were inoculated into fish muscle. Recombinant VP2 could only be detected in muscle sections from fish inoculated with pcDNA-VP2.

As previous studies have shown that only minute quantities of DNA are required to stimulate an immune response, it was decided to include all constructs in the vaccination trial regardless of the cell-culture findings. Evaluation of vaccines was only possible in the final challenge performed by VESO Vikan AkvaVet. The candidate vaccines included were pSFV-A-segment recombinant particles, pcDNA3-VP2 and pBKT-SFV-VP2. The commercial vaccine, Norvax Compact 5 was also included in the trial. Although the A-

segment particles gave better protection than the DNA based vaccines, there were only minor differences between the three candidate vaccines and negative control groups. Only the commercially available vaccine induced good protection. These preliminary results indicate that the candidate recombinant vaccines need to be further developed. The results from the challenge test were consistent with the findings of immune response assays performed by CVL. None of the candidate recombinant vaccines resulted in a significantly higher antibody response to IPNV than the control group when tested using an ELISA assay. Differences in lymphocyte proliferation between IPNV-vaccinated and non-vaccinated fish in challenge trials could not be detected when IPNV or other mitogens were used. IPNV is known to depress both T and B-cell immune responses (Knott and Munro, 1986; Novoa *et al*, 1996).

Expression of the IPNV polyprotein by recombinant SFV particles leads to the formation of IPNV VLP. The VLP accumulate in the cytoplasm of infected CHSE cells, with a morphology and size similar to that reported for IPNV. As cells infected with recombinant SFV particles expressing IPNV A-segment react with conformation-dependent MAb it is likely that the VLP produced may elicit an immune response similar to that induced by purified virus. Purified VLP may therefore be used as a candidate recombinant vaccine against IPNV. Purified IBDV VLP, from cells infected with a recombinant vaccinia virus vector expressing the A-segment, react strongly with anti-IBDV antiserum and are currently being assessed for vaccine potential (Fernández-Arias *et al*, 1998).

The *in vitro* and *in vivo* studies from this collaborative project have indicated that it is unlikely that SFV-based vectors will function effectively in salmonid fish maintained at temperatures below 15°C. The system may however be suited for evaluation as a vaccine in warm-blooded fish, such as carp or catfish. Diseases such as grass carp haemorrhage disease (GCHD) or channel catfish virus disease (CCVD) normally occur at temperatures between 25-34°C. Grass carp are cultured extensively in China and are responsible for 20% of the country's freshwater fish production. Outbreaks of GCHD severely affect production and can result in production losses of up to 30%. Although commercial inactivated and attenuated vaccines for this virus are available in China the disease is still a problem for this country's aquaculture industry. Channel catfish is the main aquaculture species in the United States. CCVD is caused by a herpesvirus and occurs mainly in fry and fingerlings. Attenuated virus vaccines for this disease have only had laboratory trials. There have been no reports of recombinant vaccine development for either of these economically important diseases (Dixon, 1997). Further studies into the ability of SFV expression systems to deliver recombinant antigens to fish under warm-water conditions may provide a solution to these problems.

An alphavirus has recently been isolated from salmonid fish. The classification is based upon sequence analysis of a 5.2 kb region of the genome of salmon pancreas disease virus. Sequence comparisons with other alphaviruses indicates that overall homology is uniform, ranging from 32-33% (Weston *et al*,

1999). Expression systems were readily developed for alphaviruses such as SFV, SIN and VEE (Frolov *et al*, 1996; Pushko *et al*, 1997; Tubulekas *et al*, 1997). It should therefore, be relatively easy to construct a similar system using this virus. If developed this system may be more applicable for aquaculture vaccines than the alphavirus vector systems currently available.

Chapter Seven

References

7.1 References

Amend, D. F. 1981. Potency testing of fish vaccines. Developments in Biological Standardisation. **49:**447-454.

Amend, D. F., and D. C. Fender. 1976. Uptake of bovine serum albumin by rainbow trout from hyperosmotic infiltration: a model for vaccinating fish. Science. **192:**793-794.

Anderson, E. D., D. V. Mourich, S. C. Fahrenkrug, S. LaPatra, J. Shepherd, and J. A. Leong. 1996b. Genetic immunisation of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology. **5:**114-122.

Anderson, E. D., D. V. Mourich, and J. A. Leong. 1996a. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology. **5:**105-113.

Anon. 1994. Distribution and economical impact of the disease IPN in Norway. Norwegian Centre for Veterinary Contract Research and Commercial Services (VESO/AS).

Anon. 1994-1995. Aquaculture - a motive force in the Norwegian coastal industry. Report to the Storting 48. ODIN - Norwegian Ministry of Fisheries.

Anon. 1997. Review of the state of world aquaculture. FAO Fisheries circular No.886 F1R1/C886. FAO Fisheries Department.

Anon. 1998. Annual report of aquaculture. Institute of Marine research.

Azad, A. A., S. A. Barrett, and K. J. Fahey. 1985. The characterisation and molecular cloning of the double stranded RNA genome of an Australian strain of infectious bursal disease virus. Virology. **143:**35-44.

Azad, A. A., M. N. Jagadish, M. A. Brown, and P. J. Hudson. 1987. Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. Virology. **161:**145-152.

Ball, H. J., A. L. S. Munro, E. A, K. G. R. Elson, W. Hodgkiss, and I. S. McFarlane. 1971. Infectious pancreatic necrosis in rainbow trout in Scotland. Nature. **234:**417-418.

Barth, B. U., and H. Garoff. 1997. The nucleocapsid-binding spike subunit E2 of Semliki Forest virus requires complex formation with the E1 subunit for activity. Journal of Virology. **71:**7857-7865.

Bayliss, C. D., U. Spies, K. Shaw, R. W. Peters, A. Papageorgiou, H. Muller, and M. E. G. Boursnell. 1990. A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. Journal of General Virology. **71:**1303-1312.

Berglund, P., M. N. Fleeton, C. Smerdou, and P. Liljestrom. 1999. Immunisations with recombinant Semliki Forest virus induces protection against influenza challenge in mice. Vaccine. **17:**497-507.

Berglund, P., M. Sjoberg, H. Garoff, G. J. Atkins, B. J. Sheahan, and P. Liljestrom. 1993. Semliki Forest virus expression system: production of conditionally infectious recombinant particles. Bio/Technology. **11:**916-920.

Berglund, P., C. Smerdou, M. N. Fleeton, I. Tubulekas, and P. liljestrom. 1998. Enhancing immune reponses using suicidal DNA vaccines. Nature Biotechnology. **16:**562-565.

Bootland, L. M., N. M. Allen, K. Edwards, H. V. Lorz, and L. Kirk. 1993. Efficacy of immunisation of rainbow trout fry with recombinant vaccines against IPNV. Bulletin of the Aquaculture Association of Canada. **93-94:**139-142.

Bootland, L. M., P. Dobos, and R. M. W. Stevenson. 1986. Experimental induction of the carrier state in yearling brook trout: A model challenge protocol for IPNV immunisation. Veterinary Immunology and Immunopathology. **12:**365-372.

Bootland, L. M., P. Dobos, and R. M. W. Stevenson. 1990. Fry age and size effects on immersion immunisation of brook trout, *Salvelinus fontinalis* Mitchell, against infectious pancreatic necrosis virus. Journal of Fish Diseases. **13:**113-125.

Bootland, L. M., P. Dobos, and R. M. W. Stevenson. 1995. Immunisation of adult brook trout, *Salvelinus fontinalis* (Mitchell), fails to prevent the infectious pancreatic necrosis virus (IPNV) carrier state. Journal of Fish Diseases. **18:**449-458.

Bootland, L. M., P. Dobos, and R. M. W. Stevenson. 1991. The IPNV carrier state and demonstration of vertical transmission in experimentally infected brook trout. Diseases of aquatic organisms. **10:**13-21.

Boudinot, P., M. Blanco, P. de Kinkelin, and A. Benmansour. 1998. Combined DNA immunisation with the glycoprotein gene of viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus induces double specific protective immunity and nonspecific response in rainbow trout. Virology. **249:**297-306.

Bredenbeek, P. J., I. Frolov, C. M. Rice, and S. Schlesinger. 1993. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. Journal of Virology. **67:**6439-6446.

Bron, R., J. M. Wahlberg, H. Garoff, and J. Wilschut. 1993. Membrane fusion of Semliki Forest virus in a model system: correlation between fusion kinetics and structural changes in the envelope glycoprotein. The European Journal of Molecular Biology. **12:**693-701.

Callis, J., M. Fromm, and V. Walbot. 1987. Expression of mRNA electroporated into plant and animal cells. Nucleic Acids Research. **15:**5823-5831.

Calvert, J. G., E. Nagy, M. Soler, and P. Dobos. 1991. Characterisation of the VPg-dsRNA linkage of infectious pancreatic necrosis virus. Journal of General Virology. **72:**2563-2567.

Caswell-Reno, P., V. Lipipun, P. W. Reno, and B. L. Nicholson. 1989. Use of group-reactive and other monoclonal antibodies in an enzyme immunodot assay for identification and presumptive serotyping of Aquatic birnaviruses. Journal of Clinical Microbiology. **27:**1924-1929.

Caswell-Reno, P., P. W. Reno, and B. L. Nicholson. 1986. Monoclonal antibodies to infectious pancreatic necrosis virus: Analysis of viral epitopes and comparison of different isolates. Journal of General Virology. **67:**2193-2205.

Cheng, R. H., R. J. Kuhn, N. H. Olson, M. G. Rossmann, H.-K. Choi, T. J. Smith, and T. S. Baker. 1995. Nucleocapsid and glycoprotein organisation in an enveloped virus. Cell. **80:**621-630.

Choi, H.-K., L. Tong, W. Minor, W. Dumas, U. Boege, M. G. Rossmann, and G. Wengler. 1991. Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. Nature. **354:**37-43.

Christie, K. E. 1997. Immunisation with viral antigens: infectious pancreatic necrosis virus., p. 191-199. *In* R. Gudding and A. Lillehaug and P. Midtlyng and F. Brown (ed.), Fish Vaccinology. Dev. Biol. Stand, vol. 90. International Association of Biological Standardisation, Basel.

Christie, K. E., L. S. Havarstein, H. O. Djupvik, S. Ness, and C. Endresen. 1988. Characterisation of a new serotpye of infectious pancreatic necrosis virus isolated from Atlantic salmon. Archives of Virology. **103:**167-177.

Christie, K. E., S. Ness, and H. O. Djupvik. 1990. Infectious pancreatic necrosis virus in Norway: partial serotyping by monoclonal antibodies. Journal of Fish Diseases. **13:**323-327.

Clegg, J. C. S., and S. I. T. Kennedy. 1974. Polyadenylic acid sequences in the virus RNA species of cells infected with Semliki Forest virus. Journal of General Virology. **22:**331-345.

Cohen, J. 1975. Ribonucleic acid polymerase activity in purified infectious pancreatic necrosis virus of trout. Biochemical and Biophysical Research Communications. **62:**689-695.

Cohen, J., A. Poinsard, and R. Scherrer. 1973. Physico-chemical and morphological features of infectious pancreatic necrosis virus. Journal of General Virology. **21:**485-498.

Dalgleish, A. G., P. C. L. Beverley, P. A Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London). **312:** 763-768.

de Curtis, I., and K. Simons. 1988. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. Proceedings from the National Academy of Sciences of the United States of America. **85:**8052-8056.

Dixon, P. 1997. Immunisation with viral antigens: viral diseases of carp and catfish. *In* R. Gudding and A. Lillehaug and P. Midtlyng and F. Brown (ed.), Fish Vaccinology. Dev. Biol. Stand. International Association of Biological Standardisation, Basel.

Dobos, P. 1993. *In vitro* guanylation of IPNV polypeptide VP1. Virology. **193:**403-413.

Dobos, P. 1976. Size and structure of the genome of infectious pancreatic necrosis virus. Nucleic Acids Research. **3:**1903-1924.

Dobos, P. 1977. Virus-specific protein synthesis in cells infected by infectious pancratic necrosis virus. Journal of Virology. **21:**242-258.

Dobos, P., R. Hallett, D. T. C. Kells, O. Sorensen, and D. Rowe. 1977. Biophysical studies of infectious pancreatic necrosis virus. Journal of Virology. **22:**150-159.

Dobos, P., B. J. Hill, R. Hallett, D. T. C. Kells, H. Becht, and D. Teninges. 1979. Biophysical and biochemical characterisation of five animal viruses with bisegmented double-stranded RNA genomes. Journal of Virology. **32:**593-605.

Dobos, P., and D. Rowe. 1977. Peptide map comparison of infectious pancreatic necrosis virus specific polypeptides. Journal of Virology. **24:**805-820.

Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. Annual Review of Immunology. **15:**617-648.

Dorson, M. 1988. Vaccination against infectious pancreatic necrosis, p. 162-171. *In* A. E. Ellis (ed.), Fish vaccination. Academic Press, London.

Dorson, M., J. Castric, and C. Torchy. 1978. Infectious pancreatic necrosis virus of salmonids: biological and antigenic features of a pathogenic strain and of a non-pathogenic variant selected in RTG-2 cells. Journal of Fish Diseases. **1:**309-320.

Dubensky, T. W., D. A. Driver, J. M. Polo, B. A. Belli, E. M. Latham, C. E. Ibanez, S. Chadda, D. Brumm, T. A. Banks, S. J. Mento, D. J. Jolly, and S. M. Chang. 1996. Sindbis virus DNA-based expression vectors: utility for *in vitro* and *in vivo* gene transfer. Journal of Virology. **70:**508-519.

Duff, D. C. B. 1942. The oral immunisation of trout against *Bacterium salmonicida.* Journal of Immunology. **44:**87-94.

Duncan, R., and P. Dobos. 1986. The nucleotide sequence of infectious pancreatic necrosis virus (IPNV) dsRNA segment A reveals one large ORF encoding a precursor protein. Nucleic Acids Research. **14:**5934.

Duncan, R., C. L. Mason, E. Nagy, J. Leong, and P. Dobos. 1991. Sequence analysis of infectious pancreatic necrosis virus genome segment B and its encoded VP1 protein: a putative RNA-dependent RNA polymerase lacking the Gly-Asp-Asp motif. Virology. **181:**541-552.

Duncan, R., E. Nagy, P. J. Krell, and P. Dobos. 1987. Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and fine structure mapping of genome segment A coding regions. Journal of Virology. **61:**3655-3664.

Eppstein, D. A., Y. V. Marsh, A. B. Schreiber, S. R. Newman, G. J. Todaro, and J. J. Nestor Jr. 1985. Epidermal growth factor receptor occupancy inhibits vaccinia virus infection. Nature (London). **318:** 663-665.

Fernandez-Arias, A., C. Risco, S. Martinez, J. P. Albar, and J. F. Rodriguez. 1998. Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. Journal of General Virology. **79:**1047-1054.

Fleeton, M. N., B. J. Sheahan, E. A. Gould, G. J. Atkins, and P. Liljestrom. 1999. Recombinant Semliki Forest virus particles encoding the prME or NS1 proteins of louping ill protect mice from lethal challenge. Journal of General Virology. **80:**1189-1198.

Forsell, K., M. Suomalainen, and H. Garoff. 1995. Structure-function relation of the NH2-terminal domain of the Semliki Forest virus capsid protein. Journal of Virology. **69:**1556-1563.

Frolov, I., T. A. Hoffman, B. M. Pragai, S. A. Dryga, H. V. Huang, S. Schlesinger, and C. M. Rice. 1996. Alphavirus-based expression vectors: strategies and applications. Proceedings of the National Academy of Sciences of the United States of America. **93:**11371-11377.

Frolova, E., I. Frolov, and S. Schlesinger. 1997. Packaging signals in alphaviruses. Journal of Virology. **71:**248-258.

Froshauer, S., J. Kartenbeck, and A. Helenius. 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. The Journal of Cell Biology. **107:**2075-2086.

Frost, P., L. S. Havarstein, B. Lygren, S. Stahl, C. Endresen, and C. E. Christie. 1995. Mapping of neutralization epitopes on infectious pancreatic necrosis viruses. Journal of General Virology. **76:**1165-1172.

Frost, P., and Ness. 1997. Vaccination of Atlantic salmon with recombinant VP2 of infectious pancreatic necrosis virus (IPNV), added to a multivalent vaccine, suppresses viral replication following IPNV challenge. Fish and Shellfish Immunology. **7:**609-619.

Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: protective immunisations by parenteral, mucosal

and gene-gun inoculations. Proceedings of the National Academy of Sciences of the United States of America. **90:**11478-11482.

Garoff, H., A. M. Frischauf, K. Simons, H. Lehrach, and H. Delius. 1980a. The capsid protein of Semliki Forest virus has clusters of basic amino acids and prolines in its amino-terminal region. Proceedings of the National Academy of Sciences of the United States of America. **77:**6376-6380.

Garoff, H., A. M. Frischauf, K. Simons, H. Lehrach, and H. Delius. 1980b. Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. Nature. **288:**236-241.

Garoff, H., and K. Simons. 1974. Location of the spike glycoproteins in the Semliki Forest virus membrane. Proceedings from the National Academy of Sciences of the United States of America. **71:**3988-3992.

Garoff, H., K. Simons, and B. Dobberstein. 1978. Assembly of the Semliki Forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum *in vitro*. Journal of Molecular Biology. **124:**587-600.

Garoff, H., K. Simons, and O. Renkonen. 1974. Isolation and characterisation of the membrane proteins of Semliki Forest virus. Virology. **61:**493-504.

Geigenmuller-Gnirke, U., B. Weiss, R. Wright, and S. Schlesinger. 1991. Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome. Proceedings of the National Academy of Sciences of the United States of America. **88:**3253-3257.

Glanville, N., M. Ranki, J. Morser, L. Kaariainen, and A. E. Smith. 1976. Initiation of translation directed by 42S and 26S RNAs from Semliki Forest virus *in vitro*. Proceedings of the National Academy of Sciences of the United States of America. **73:**3059-3063.

Gould, R. W., P. J. O'Leary, R. L. Garrison, J. S. Rohovec, and J. L. Fryer. 1978. Spray vaccination: a method for the immunisation of fish. Fish Pathology. **13:**63-68.

Granzow, H., F. Weiland, D. Fichtner, and P. J. Enzmann. 1997. Studies of the ultrastructure and morphogenesis of fish pathogenic viruses grown in cell culture. Journal of Fish Diseases. **20:**1-10.

Hahn, C. S., and J. H. Strauss. 1990. Site-directed mutagenesis of the proposed catalytic amino acids of the Sindbis virus capsid protein autoprotease. Journal of Virology. **64:**3069-3073.

Hansen, E., K. Fernandes, G. Goldspink, P. Butterworth, P. K. Umeda, and K. C. Chang. 1991. Strong expression of foreign genes following direct injection into fish muscle. FEBS Letters. **290:**73-76.

Hardy, W. R., and J. H. Strauss. 1989. Processing the nonstructural polyproteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in *cis* and in *trans*. Journal of Virology. **63:**4653-4664.

Hardy, W. R., and J. H. Strauss. 1988. Processing the nonstructural polyproteins of Sindbis virus: study of the kinetics *in vivo* by using monoclonal antibodies. Journal of Virology. **62:**998-1007.

Hariharan, M. J., D. A. Driver, K. Townsend, D. Brumm, J. M. Polo, B. A. Belli, D. J. Catton, D. Hsu, D. Mittelstaedt, J. E. McCormack, L. Karavodin, T. W. Dubensky, S. M. W. Chang, and T. A. Banks. 1998. DNA immunisation against herpes simplex virus: enhanced efficacy using a Sindbis virus-based vector. Journal of Virology. **72:**950-958.

Havarstein, L. S., K. H. Kalland, K. E. Christie, and C. Endresen. 1990. Sequence of the large double-stranded RNA segment of the N1 strain of infectious pancreatic necrosis virus: a comparison with other *Birnaviridae*. Journal of General Virology. **71:**299-308.

Helenius, A., B. Morein, E. Fries, K. Simons, P. Robinson, V. Schirrmacher, C. Terhorst, and J. L. Strominger. 1978. Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. Proceedings from the National Academy of Sciences of the United States of America. **75:**3846-3850.

Helenius, A., and K. H. von Bonsdorff. 1976. Semliki Forest virus membrane proteins. Preparation and characterisation of spike complexes soluble in detergent-free medium. Biochimica et Biophysica Acta. **436:**895-899.

Heppell, J., L. Berthiaume, F. Corbin, E. Tarrab, J. Lecomte, and M. Arella. 1993. Comparison of amino acid sequences deduced from a cDNA fragment obtained from Infectious pancreatic necrosis virus strains of different serotypes. Virology. **195:**840-844.

Heppell, J., L. Berthiaume, E. Tarrab, J. Lecomte, and M. Arella. 1992. Evidence of genomic variations between infectious pancreatic necrosis strains determined by restriction fragment profiles. Journal of General Virology. **73:**2863-2870.

Heppell, J., N. Lorenzen, N. K. Armstrong, T. Wu, E. Lorenzen, K. Einer-Jensen, J. Schorr, and H. L. Davis. 1998. Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. Fish and Shellfish Immunology. **8:**271-286.

Heppell, J., E. Tarrab, L. Berthiaume, J. Lecomte, and M. Arella. 1995a. Characterisation of the small open reading frame on genome segment A of infectious pancreatic necrosis virus. Journal of General Virology. **76:**2091-2096.

Heppell, J., E. Tarrab, J. Lecomte, L. Berthiaume, and M. Arella. 1995b. Strain variability and localisation of important epitopes on the major structural protein (VP2) of infectious pancreatic necrosis virus. Virology. **214:**40-49.

Herweijer, H., J. S. Latendresse, P. Williams, G. Zhang, I. Danko, S. Schlesinger, and J. A. Wolff. 1995. A plasmid-based self-amplifying Sindbis virus vector. Human Gene Therapy. **6:**1161-1167.

Hill, B. J., and K. Way. 1995. Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses. Annual Review of Fish Diseases. **5:**55-77.

Hosono, N., S. Suzuki, and R. Kusuda. 1996. Genogrouping of birnaviruses isolated from marine fish: a comparison of VP2/NS junction regions of genome segment A. Journal of Fish Diseases. **19:**295-302.

Hsu, Y., C. Chen, and J. Wu. 1995. Molecular relationships in infectious pancreatic necrosis virus. Virus research. **37:**239-252.

Huang, M. T. F., D. S. Manning, M. Warner, E. B. Stephens, and J. C. Leong. 1986. A physical map of the viral genome for infectious pancreatic necrosis virus Sp: Analysis of cell-free translation products derived from viral cDNA clones. Journal of Virology. **60:**1002-1011.

Hudson, P. J., N. M. McKern, B. E. Power, and A. A. Azad. 1986. Genomic structure of the large RNA segment of infectious bursal disease. Nucleic Acids Research. **14:**5001-5012.

Ivanova, L., and M. J. Schlesinger. 1993. Site-directed mutations in the Sindbis virus E2 glycoprotein identify palmitoylation sites and affect virus budding. Journal of Virology. **67:**2546-2551.

Jagadish, M. N., V. J. Staton, P. J. Hudson, and A. A. Azad. 1988. Birnavirus precursor polyprotein is procesed in *Escherichia coli* by its own virus-encoded polypeptide. Journal of Virology. **62:**1084-1087.

Johanning, F. W., R. M. Conry, A. F. LoBuglio, M. Wright, L. A. Sumerel, M. J. Pike, and D. T. Curiel. 1995. A Sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression *in vivo*. Nucleic Acids Research. **23:**1495-1501.

Johnson, K. A., J. K. Flynn, and D. F. Amend. 1982. Onset of immunity in salmonid fry vaccinated by direct immersion in Vibrio anguillarum and Yersinia ruckeri bacterins. Journal of Fish Diseases. **5:**197-205.

Kaariainen, L., K. Takkinen, S. Keranen, and H. Soderlund. 1987. Replication of the genome of Alphaviruses. Journal of Cell Science. **Supplement 7:**231-250.

Kalkinnen, N., H. Jornvall, and L. Kaariainen. 1981. Polyprotein processing of Alphaviruses: N-terminal structural analysis of Semliki Forest virus proteins p62, E3 and ns70. FEBS Letters. **126:**33-37.

Kalkinnen, N., M. Laaksonen, H. Soderlund, and H. Jornvall. 1981. Radio-sequence analysis of in vivo multilabelled nonstructural protein ns86 of Semliki Forest virus. Virology. **113:**188-195.

Kelly, R. K., and P. Loh. 1972. Electron microscopical and biochemical characterisation of infectious pancreatic necrosis virus. Journal of Virology. **10:**824-834.

Keranen, S., and L. Kaariainen. 1975. Proteins synthesized by Semliki Forest virus and its 16 temperature-sensitive mutants. Journal of Virology. **16:**388-396.

Kibenge, F. S. B., D. J. Jackwood, and C. C. Mercado. 1990. Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. Journal of General Virology. **71:**569-577.

Kibenge, F. S. B., P. K. McKenna, and J. K. Dybing. 1991. Genome cloning and analysis of the large RNA segment (segment A) of a naturally avirulent serotype 2 infectious bursal disease virus. Virology. **184:**437-440.

Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London). **312**:767-768.

Klein, T. M., and S. Fitzpatrick-McElligott. 1993. Particle bombardment: a universal approach for gene transfer to cells and tissues. Current Opinion in Biotechnology. **4:**583-590.

Knott, R. M., and A. L. S. Munro. 1986. The persistence of infectious pancreatic necrosis virus in Atlantic salmon. Veterinary Immunology and Immunopathology. **12:**359-364.

Koener, J. F., and J. C. Leong. 1990. Expression of the glycoprotein gene from a fish rhabdovirus by using baculovirus vectors. Journal of Virology. **64:**428-430.

Lachmi, B.-E., N. Glanville, S. Keranen, and K. L. 1975. Tryptic peptide analysis of nonstructural and structural precursor proteins from Semliki Forest virus mutant-infected cells. Journal of Virology. **16:**1615-1629.

Lachmi, B.-E., and L. Kaariainen. 1976. Sequential translation of nonstructural proteins in cells infected with Semliki Forest virus mutant. Proceedings of the National Academy of Sciences of the United States of America. **73:**1936-1940.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:**680-685.

Laine, R., M.-L. Kettunen, C. G. Gahmberg, L. Kaariainen, and O. Renkonen. 1972. Fatty chains of different lipid classes of Semliki Forest virus and host cell membranes. Journal of Virology. **10:**433-438.

Le Gouill, C., and C. V. Dery. 1991. A rapid procedure for the screening of recombinant plasmids. Nucleic Acids Research. **19:**6655.

Lecocq-Xhonneux, F., M. Thiry, I. Dheur, M. Rossius, N. Vanderheijden, J. Martial, and P. de Kinkelin. 1994. A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. Journal of General Virology. **75:**1579-1587.

Lehtovaara, P., I. Ulmanen, L. Kaariainen, S. Keranen, and Philipson. 1980. Synthesis and processing of Semliki Forest virus-specific non-structural proteins *in vivo* and *in vitro*. European Journal of Biochemistry. **112:**461-468.

Leong, J. 1992. Vaccine for immunizing fish against infectious pancreatic necrosis virus. State of Oregon Acting by and through the state board of higher education on behalf of Oregon state University, Eugene, Oregon., United States.

Levis, R., B. G. Weiss, M. Tsiang, H. Huang, and S. Schlesinger. 1986. Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell. **44:**137-145.

Liljestrom, P., and H. Garoff. 1991a. Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. Journal of Virology. **65:**147-154.

Liljestrom, P., and H. Garoff. 1991b. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Bio/Technology. **9:**1356-1361.

Liljestrom, P., S. Lusa, D. Huylebroeck, and H. Garoff. 1991. *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular weight membrane protein modulates virus release. Journal of Virology. **65:**4107-4113.

Lillehaug, A. 1989. A cost-effectiveness study of three different methods of vaccination against vibriosis in salmonids. Aquaculture. **83:**227-236.

Lobigs, M., and H. Garoff. 1990. Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein precursor p62. Journal of Virology. **64:**1233-1240.

Lobigs, M., Z. Hongxing, and H. Garoff. 1990a. Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal abolishes spike heterodimerisation and surface expression of E1. Journal of Virology. **64:**4346-4355.

Lobigs, M., J. M. Wahlberg, and H. Garoff. 1990b. Spike protein oligomerisation control of Semliki forest virus fusion. Journal of Virology. **64:**5214-5218.

Loewy, A., J. Smyth, C. H. von Bonsdorff., P. Liljestrom, and M. J. Schlesinger. 1995. The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. Journal of Virology. **69:**469-475

Loh, P. C., M. H. Lee, and R. K. Kelly. 1974. The polypeptides of infectious pancreatic necrosis virus. Journal of General Virology. **22:**421-423.

Lombardo, E., A. Maraver, J. R. Caston, J. Rivera, A. Fernandez-Arias, A. Serrano, J. L. Carrascosa, and J. F. Rodriguez. 1999. VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. Journal of Virology. **73:**6973-6983.

Lorenzen, N., E. Lorenzen, K. Einer-Jensen, J. Heppell, T. Wu, and H. Davis. 1998. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss,* Walbaum) following DNA vaccination. Fish and Shellfish Immunology. **8:**261-270.

Macdonald, R. D., and P. Dobos. 1981. Identification of the proteins encoded by each genome segment of infectious pancreatic necrosis virus. Virology. **114:**414-422.

Macdonald, R. D., and T. Yamamoto. 1977. The structure of infectious pancreatic necrosis virus RNA. Journal of General Virology. **34:**235-247.

Magyar, G., and P. Dobos. 1994b. Evidence for the detection of the infectious pancreatic necrosis virus polyprotein and the 17-kDa polypeptide in infected cells and of the NS protease in purified virus. Virology. **204:**580-589.

Magyar, G., and P. Dobos. 1994a. Expression of infectious pancreatic necrosis virus polyprotein and VP1 in insect cells and the detection of the polyprotein in purified virus. Virology. **198:**437-445.

Malsberger, R. G., and C. P. Cerini. 1965. Multiplication of infectious pancreatic necrosis virus. Ann. New York Academy of Science. **126:**320-327.

Manning, D. S., and J. C. Leong. 1990. Expression in *Escherichia coli* of the large genomic segment of infectious pancreatic necrosis virus. Virology. **179:**16-25.

Markestad, I. A., and K. Grave. 1997. Reduction of antibacterial drug use in Norwegian fish farming due to vaccination., p. 365-369. *In* R. Gudding and A.

Lillehaug and P. Midtlyng and F. Brown (ed.), Fish Vaccinology. Dev. Biol. Stand, vol. 90. International Association of Biological Standardisation, Basel.

Mattila, K. 1979. Separation of the integral membrane glycoproteins E1 and E2 of Semliki Forest virus by affinity chromatography on concanavalin A-sepharose. Biochemica et Biophysica Acta. **579:**62-72.

Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labelled DNA with base specific chemical cleavages. Methods in Enzymology. **65:**499-560.

McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson. 1986. Binding of HTLV-III/LAV to T4⁺Tcells by a complex of the 110K viral protein and the T4 molecule. Science. **231:**382-385

McGonigle, R. H. 1941. Acute catarrhal enteritis of salmonid fingerlings. Trans. Am. Fish. Soc. **70:**297.

McKnight, I. J., and R. J. Roberts. 1976. The pathology of infectious pancreatic necrosis. I. The sequential histopathology of the naturally occurring condidtion. British Veterinary Journal. **132:**76-85.

Melancon, P., and H. Garoff. 1987. Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. Journal of Virology. **61:**1301-1309.

Melby, H. P., P. Caswell-Reno, and K. Falk. 1994. Antigenic analysis of Norwegian aquatic birnavirus isolates using monoclonal antibodies - with special reference to fish species, age and health status. Journal of Fish Diseases. **17:**85-91.

Melby, H. P., and K. E. Christie. 1994. Antigenic analysis of reference strains and Norwegian field strains of aquatic birnaviruses by the use of six monoclonal antibodies produced against the infectious pancreatic necrosis virus N1 strain. Journal of Fish Diseases. **17:**409-415.

Mertens, P. P. C., and P. Dobos. 1982. Messenger RNA of infectious pancreatic necrosis virus is polycistronic. Nature. **297:**243-246.

Metiskko, K., and H. Garoff. 1990. Oligomers of the cytoplasmic domain of the p62/E2 membrane protein of Semliki Forest virus bind to the nucleocapsid *in vitro*. Journal of Virology. **64:**4678-4683.

Montgomery, D. L., J. J. Donnelly, J. W. Shiver, M. A. Liu, and J. Ulmer. 1994. Protein expression *in vivo* by injection of polynucleotides. Current Opinion in Biotechnology. **5:**505-510.

Moss, L. H., and M. Gravell. 1969. Ultrastructure and sequential development of infectious pancreatic necrosis virus. Journal of Virology. **3:**52-58.

Mossman, S. P., F. Bex, P. Berglund, J. Arthos, S. P. O'Neill, D. Riley, D. H. Maul, C. Bruck, P. Momin, A. Burny, P. N. Fultz, J. I. Mullins, P. Liljestrom, and E. A. Hoover. 1996. Protection against lethal simian immunodeficiency virus SIVsmmPBj14 disease by a recombinant Semliki Forest virus gp160 vaccine and by a gp120 subunit vaccine. Journal of Virology. **70:**1953-1960.

Muller, H., and H. Becht. 1982. Biosynthesis of virus-specific proteins in cells infected with infectious bursal disease virus and their significance as structural

elements for infectious virus and incomplete particles. Journal of Virology. **44**:384-392.

Muller, H., and R. Nitschke. 1987. The two segments of the infectious bursal disease virus are circularised by a 90,000 Da protein. Virology. **59**:174-177.

Nagy, E., R. Duncan, P. Krell, and P. Dobos. 1987. Mapping of the large RNA genome segment of infectious pancreatic necrosis virus by hybrid arrested translation. Virology. **158**:211-217.

Nicholson, B. L. 1971. Macromolecule synthesis in RTG-2 cells following infection with infectious pancreatic necrosis (IPN) virus. Journal of General Virology. **13**:369-372.

Novoa, B., A. Figueras, and C. J. Secombes. 1996. Effects of *in vitro* addition of infectious pancreatic necrosis virus (IPNV) on rainbow trout *Oncorhynchus mykiss* leucocyte responses. Veterinary Immunology and Immunopathology. **51**:365-376.

Okamoto, N., T. Sano, R. P. Hedrick, and J. L. Fryer. 1983. Antigenic relationships of selected strains of infectious pancreatic necrosis virus and European eel virus. Journal of Fish Diseases. **6**:19-25.

Oldstone, M. B. A., A. Tishon, F. J. Dutko, S. I. T. Kennedy, J. J. Holland, and P. W. Lampert. 1980. Does the major histocompatibility complex serve as a specific receptor for Semliki Forest virus? Journal of Virology. **34**:256-265.

Owen, K. E., and R. J. Kuhn. 1996. Identification of a region in the Sindbis virus nucleocapsid protein that is involved in specificity of RNA encapsidation. Journal of Virology. **70**:2757-2763.

Peranen, J., P. Laakkonen, M. Hyvonen, and L. Kaariainen. 1995. The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles. Virology. **208:**610-620.

Persson, R. H., and R. D. Macdonald. 1982. Evidence that infectious pancreatic necrosis virus has a genome-linked protein. Journal of Virology. **44:**437-443.

Press, C. M., and A. Lillehaug. 1995. Vaccination in European salmonid aquaculture: A review of practices and prospects. British Veterinary Journal. **151:**45-69.

Pryde, A., W. T. Melvin, and A. L. S. Munro. 1993. Nucleotide sequence analysis of the serotype-specific epitope of infectious pancreatic necrosis virus. Archives of Virology. **129:**287-293.

Pushko, P., M. Parker, G. V. Ludwig, H. L. Davis, R. E. Johnston, and J. F. Smith. 1997. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes *in vitro* and immunisation against heterologous pathogens *in vivo*. Virology. **239:**389-401.

Renkonen, O., L. Kaariainen, K. Simons, and C. G. Bahmbeg. 1971. The lipid class composition of Semliki Forest virus and plasma membranes of the host cells. Virology. **46:**318-326.

Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. Journal of Virology. **61:**3809-3819.

Riedel, H., H. Lehrach, and H. Garoff. 1982. Nucleotide sequence at the junction between the nonstructural and the structural genes of the Semliki Forest virus genome. Journal of Virology. **42:**725-729.

Ross, K., and A. L. S. Munro. 1995. Seological analysis of virulent infectious pancreatic necrosis virus strains. Journal of Fish Diseases. **18:**377-380.

Sadasiv, E. 1995. Immunological and pathological responses of salmonids to infectious pancreatic necrosis virus. Annual Review of Fish Diseases. **5:**209-223.

Salminen, A., J. M. Wahlberg, M. Lobigs, P. Liljestrom, and H. Garoff. 1992. Membrane fusion process of Semliki Forest virus II: cleavage-dependent reorganization of the spike protein complex controls virus entry. The Journal of Cell Biology. **116:**349-357.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.

Sariola, M., J. Saraste, and E. Kuismanen. 1995. Communication of post-Golgi elements with early endocytic pathway: regulation of endoproteolytic cleavage of Semliki Forest virus p62 precursor. Journal of Cell Science. **108:**2465-2475.

Sawicki, C. L., and P. J. Gomatos. 1976. Replication of Semliki Forest virus: polyadenylation in plus-strand RNA and polyuridylate in minus-strand RNA. Journal of Virology. **20:**446-464.

Sawicki, D. L., L. Kaariainen, C. Lambek, and P. J. Gomatos. 1978. Mechanism for control of synthesis of Semliki Forest virus 26S and 42S RNA. Journal of Virology. **25:**19-27.

Sawicki, D. L., and S. G. Sawicki. 1980. Short-lived minus strand polymerase for Semliki Forest virus. Journal of Virology. **34:**108-118.

Schnitzler, D., F. Bernstein, H. Muller, and H. Becht. 1993. The genetic basis for the antigenicity of the VP2 protein of the infectious bursal disease virus. Journal of General Virology. **74:**1563-1571.

Simons, K., and H. Garoff. 1980. The budding mechanisms of enveloped animal viruses. Journal of General Virology. **50:**1-21.

Singh, I., and A. Helenius. 1992. Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. Journal of Virology. **66:**7049-7058.

Sjoberg, E. M., and H. Garoff. 1996. The translation-enhancing region of the Semliki Forest virus subgenome is only functional in the virus-infected cell. Journal of General Virology. **77:**1323-1327.

Sjoberg, E. M., M. Suomalainen, and H. Garoff. 1994. A significantly improved Semliki Forest virus expression system based on translation enhancer segments from the viral capsid gene. Bio/Technology. **12:**1127-1131.

Smail, D. A., D. W. Bruno, G. Dear, L. A. McFarlane, and K. Ross. 1992. IPNV Sp serotype in farmed Atlantic salmon, *Salmo salar* L., post-smolts associated with mortality and clinical disease. Journal of Fish Diseases. **15:**77-83.

Smail, D. A., and A. L. S. Munro. 1985. Infectious pancreatic necrosis virus persistance in farmed Atlantic salmon (*Salmo salar*), p. 277-288. *In* A. E. Ellis (ed.), Fish and shellfish pathology. Academic press, London.

Smerdou, C., and P. Liljestrom. 1999. Two-helper RNA system for production of recombinant Semlki Forest virus particles. Journal of Virology. **73:**1092-1098.

Soderlund, H., and I. Ulmanen. 1977. Transient association of Semliki Forest virus capsid protein with ribosomes. Journal of Virology. **24:**907-909.

Soderlund, H., C.-H. von Bonsdorff, and I. Ulmanen. 1979. Comparison of the structural properties of Sindbis and Semliki Forest virus nucleocapsids. Journal of General Virology. **45:**15-26.

Spies, U., and H. Muller. 1990. Demonstration of enzyme activities required for cap structure formation in infectious bursal disease virus - a member of the birnavirus group. Journal of General Virology. **71:**977-981.

Stangeland, K., S. Hoie, and T. Taksdal. 1996. Experimental induction of infectious pancreatic necrosis in Atlantic salmon, *Salmo salar* L., post-smolts. Journal of Fish Diseases. **19:**323-327.

Strauss, J. H., and E. G. Strauss. 1994. The alphaviruses: gene expression, replication and evolution. Microbiological Reviews. **58:**491-562.

Suomalainen, M., P. Liljestrom, and H. Garoff. 1992. Spike protein-nucleocapsid interactions drive the budding of alphaviruses. Journal of Virology. **66:**4737-4747.

Takkinen, K. 1986. Complete nucleotide sequence of the nonstructural protein genes of Semliki Forest virus. Nucleic Acids Research. **14:**5667-5682.

Takkinen, K., J. Peranen, and L. Kaariainen. 1991. Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein. Journal of General Virology. **72:**1627-1633.

Taksdal, T., A. Ramstad, K. Stangeland, and B. H. Dannevig. 1998. Induction of infectious pancreatic necrosis in covertly infected Atlantic salmon, *Salmo salar* L., post-smolts by stress exposure, by injection of IPN virus and by cohabitation. Journal of Fish Diseases. **21:**193-204.

Taksdal, T., K. Stangeland, and B. H. Dannevig. 1997. Induction of infectious pancreatic necrosis (IPN) in Atlantic salmon *Salmo salar* and brook trout *Salvelinis fontinalis* by bath challenge of fry with infectious pancreatic necrosis virus (IPNV) serotype Sp. Diseases of Aquatic Organisms. **28:**39-44.

Tang, D.-C., M. DeVit, and S. A. Johnston. 1992. Genetic immunisation is a simple method for eliciting an immune response. Nature. **356:**152-154.

Tarrab, E., L. Berthiaume, S. Grothe, M. O'Connor-McCourt, J. Heppell, and J. Lecomte. 1995. Evidence of a major neutralisable conformational epitope region on VP2 of infectious pancreatic necrosis virus. Journal of General Virology. **76:**551-558.

Tarrab, E., L. Berthiaume, J. Heppell, M. Arella, and J. Lecomte. 1993. Antigenic characterisation of serogroup 'A' of infectious pancreatic necrosis virus with three panels monoclonal antibodies. Journal of General Virology. **74:**2025-2030.

Torrissen, O. J. 1995. Norwegian salmon culture 1 million tons in 2005? Aquaculture Europe. **19:**6-11.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America. **76:**4350-4354.

Tubulekas, I., P. Berglund, M. Fleeton, and P. Liljestrom. 1997. Alphavirus expression vectors and their use as recombinant vaccines: a minireview. Gene. **190:**191-195.

Ubol, S., and D. E. Griffin. 1991. Identification of a putative alphavirus receptor on mouse neural cells. Journal of Virology. **65:**6913-6921.

Underwood, B. O., C. J. Smale, F. Brown, and B. J. Hill. 1977. Relationship of a virus from *Tellina tenuis* to infectious pancreatic necrosis. Journal of General Virology. **36:**93-109.

Vaux, D. J. T., A. Helenius, and I. Mellman. 1988. Spike-nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. Nature. **336:**36-42.

von Bonsdorff, C.-H., and S. C. Harrison. 1978. Hexagonal glycoprotein arrays from Sindbis virus membranes. Journal of Virology. **28:**578-583.

von Bonsdorff, C.-H., and S. C. Harrison. 1975. Sindbis virus glycoproteins form a regular icosahedral surface lattice. Journal of Virology. **16:**141-145.

Wahlberg, J. M., W. A. M. Boere, and H. Garoff. 1989. The heteroodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. Journal of Virology. **63:**4991-4997.

Wahlberg, J. M., R. Bron, J. Wilschut, and H. Garoff. 1992. Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. Journal of Virology. **66:**7309-7318.

Weiss, B., H. Nitschko, I. Ghattas, R. Wright, and S. Schlesinger. 1989. Evidence for specificity in the encapsidation of Sindbis virus RNAs. Journal of Virology. **63:**5310-5318.

Welch, W. J., and B. M. Sefton. 1980. Characterisation of a small non-structural viral polypeptide present late during infection of BHK cells by Semliki Forest virus. Journal of Virology. **33:**230-237.

Welch, W. J., B. M. Sefton, and F. S. Esch. 1981. Amino-terminal sequence analysis of alphavirus polypeptides. Journal of Virology. **38:**968-972.

Wengler, G., and G. Wengler. 1976. Localization of the 26S RNA sequence on the viral genome type 42S RNA isolated from SFV-infected cells. Virology. **73:**190-199.

Weston, J. H., M. D. Welsh, M. F. McLoughlin, and D. Todd. 1999. Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar* L. Virology. **256:**188-195.

Wheeler, C. J., P. L. Felgner, Y. J. Tsai, J. Marshall, L. Sukhu, S. G. Doh, J. Hartikka, J. Nietupski, M. Manthorpe, m. Nichols, M. Plewe, X. Liang, J. Norman, A. Smith, and S. H. Cheng. 1996. A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. Proceedings of

the National Academy of Sciences of the United States of America. **93:**11454-11459.

White, C. L., M. Thomson, and N. J. Dimmock. 1998. Deletion analysis of a defective interfering Semliki Forest virus RNA genome defines a region that is required for efficient packaging of the genome into virus particles. Journal of Virology. **72:**4320-4326.

Wolf, K., S. F. Snieszko, C. E. Dunbar, and E. Pyle. 1960. Virus nature of infectious pancreatic necrosis in trout. Proceedings of the Society for Experimental Biology and Medicine. **104:**105-108.

Wolff, J. A., J. J. Ludtke, G. Ascadi, P. Williams, and A. Jani. 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Human Molecular Genetics. **1:**363-369.

Yao, J. S., E. G. Strauss, and J. H. Strauss. 1996. Interactions between PE2, E1 and 6K required for assembly of alphaviruses studied with chimeric viruses. Journal of Virology. **70:**7910-7920.

Yao, K., and V. N. Vakharia. 1998. Generation of infectious pancreatic necrosis virus from cloned cDNA. Journal of Virology. **72:**8913-8920.

Zapata, A. G., M. Torroba, A. Varas, and E. Jimenez. 1997. Immunity in fish larvae, p. 23-32. *In* R. Gudding and A. Lillehaug and P. Midtlyng and F. Brown (ed.), Fish Vaccinology. Dev. Biol. Stand, vol. 90. International Association of Biological Standardisation, Basel.

Zhou, X., P. Berglund, G. Rhodes, S. E. Parker, M. Jondal, and P. Liljestrom. 1994. Self-replicating Semliki Forest virus RNA as recombinant vaccine. Vaccine. **12:**1510-1514.

Zhou, X., P. Berglund, H. Zhao, P. Liljestrom, and M. Jondal. 1995. Generation of cytotoxic and humoral immune responses by nonreplicative recombinant Semliki Forest virus. Proceedings of the National Academy of Sciences of the United States of America. **92:**3009-3013.

Ziemiecki, A., H. Garoff, and K. Simons. 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. Journal of General Virology. **50:**111-123.