

ABSTRACT

Title of dissertation: FUNCTIONAL STUDIES OF INFECTIOUS
 PANCREATIC NECROSIS VIRUS PROTEINS AND
 MECHANISM OF VIRUS-INDUCED APOPTOSIS
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Infectious pancreatic necrosis virus (IPNV) encodes a 12 or 15-kDa nonstructural protein, known as VP5. To study the function of VP5, we generated three recombinant viruses rNVI15, rNVI15-15K, and rNVI15- Δ VP5, which could encode either 12-kDa VP5, 15-kDa VP5 or be deficient in VP5, respectively. VP5 was detected in rNVI15 and rNVI-15K infected cells but not in the cells infected with rNVI15- Δ VP5. However, the opal stop codon at nucleotide position 427 in rNVI15 virus was read-through, giving rise to a 15-kDa VP5 that is expressed poorly than rNVI15-15K virus-infected cells. All three recombinant viruses show similar replication kinetics in both Chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG-2) cells. Moreover, in Sp strains, IPNV segment A could encode a novel, putative 25-kDa protein from another ORF. This 25-

kDa protein could not be detected in virus-infected cells, however, we could recover a mutant virus lacking this ORF, indicating that it is not essential for virus replication.

To assess the molecular basis of virus adaptation in the cell culture, virulent rNVI15 was serially passaged in CHSE cells nine times to obtain a tissue-culture adapted virus, rNVI15TC. Comparison of the deduced amino acid sequences showed only one amino acid substitution at position 221 (Ala → Thr) in VP2. However, this adaptation mutation is only acquired in CHSE cells but not in RTG-2 cells. Two chimeric viruses, rNVI15 Δ VP2 and rNVI15-15K Δ VP2 were also generated, in which the residues at positions 217 and 247 in VP2 of the rNVI15 and rNVI15-15K viruses were replaced by the corresponding residues of an attenuated strain, Sp103. These two viruses have similar replication kinetics as Sp103, which replicates faster than rNVI15 *in vitro*, indicating that residues at positions 217 and 247 of VP2 may be the important markers for virus adaptation and attenuation *in vitro*. By generating a reassortant virus between rNVI15-15K and Sp103, we also demonstrate that VP1 is not involved in virus cell adaptation.

The signal pathways and nature of IPNV-induced apoptosis were investigated in RTG-2 cells. IPNV-induced apoptosis occurs at the late stage of viral life cycle. Caspase-3 is activated during virus infection, and inhibition of caspase-3 could partially inhibit virus-induced apoptosis. Moreover, NF- κ B activation is essential for IPNV-induced apoptosis, and it is involved in interferon-induced antiviral state. Both the NF- κ B inhibitor and the antioxidant could inhibit NF κ B activity and apoptosis induced by IPNV infection, but they do not affect viral replication.

FUNCTIONAL STUDIES OF INFECTIOUS PANCREATIC NECROSIS VIRUS
PROTEINS AND MECHANISM OF VIRUS-INDUCED APOPTOSIS

by

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LIST OF ABBREVIATIONS

AC-ELISA	antigen capture-enzyme linked immunosorbant assay
bp	basepairs
BSA	bovine serum albumin
° C	degrees Celsius
cDNA	complement DNA
CHSE	Chinook salmon embryo
CPE	cytopathic effect
dsRNA	double-stranded RNA
DXV	Drosophila X virus
ECL	enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
g	gram
hr	hour
IBDV	infectious bursal disease virus
IFA	immunofluorescence assay
IFN	interferon
IPN	infectious pancreatic necrosis
IPNV	infectious pancreatic necrosis virus
kDa	kilodaltons
L	liter
LCMV	lymphocytic choriomengitis virus
MAbs	monoclonal antibodies
mg	milligram
min	minute
ml	milliliter
MOI	multiplication of infection
NF-κB	nuclear transcription factor kappa B

NS	non-structural
nt	nucleotide
ORF	open reading frame
PBS	phosphate buffered saline
RdRp	RNA-dependent-RNA-polymerase
PEG	polyethylene glycol
PFU	plaque forming units
p. i.	post-infection
PKR	dsRNA activated protein kinase
ROS	reactive oxygen species
RTG	rainbow trout gonad
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
TBS	tris-buffered saline
TNT	transcription coupled translation
V	volts
VHS	viral hemorrhagic septicemia
VLPs	virus-like particles
VSV	vesicular stomatitis virus
WB	West Buxton
μL	microliter

LIST OF THREE AND ONE LETTER SYMBOLS FOR AMINO ACIDS

Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

Introduction

1.1 General introduction

In recent years, aquaculture has assumed great importance due to the growth of human population and the increasing demand for fish products. However, the rapid growth of aquaculture has provided opportunities for the emergence of diseases. Infectious pancreatic necrosis virus (IPNV) is the major causes of disease in juvenile rainbow and brook trout as well as Atlantic salmon, and it is responsible for huge economic losses in the aquaculture industry worldwide. Highly virulent strains of IPNV can cause greater than 90% mortality in hatchery stocks less than four months old, survivors of infections usually being persistently infected and serving as reservoirs of infection (Mangunwiryo and Aguis, 1988; Reno, Darley, and Savan, 1978). In Norway, a loss of about 60 million US dollars annually due to IPNV infection alone has been reported (Christie, 1997).

IPNV is a member of the *Birnaviridae* family, where genome consists of two segments of double-stranded (ds) RNA. Genome segment A (~ 3.1 kb) contains two overlapping open-reading frames (ORFs). A large ORF encodes a 106-kDa polyprotein (NH₂-pVP2-NS protease-VP3-COOH), which is cotranslationally cleaved by the protease to generate the major capsid proteins VP2 and VP3. A second small ORF, which overlaps the amino end of the large ORF, encodes a 17 kDa arginine-rich polypeptide (Dobos, 1995a). This nonstructural protein has been designated as VP5, in

correspondence with its positional homologue in infectious bursal disease virus (IBDV), another member of the *Birnaviridae* family. VP2 is the major outer capsid and the host-protective antigen, as it contains the antigenic region responsible for the induction of neutralizing antibodies and serotype specificity (Heppell et al., 1995b). VP3 is an internal capsid protein, which binds to dsRNA genome forming ribonucleoprotein core structure (Hjalmarsson, Carlemalm, and Everitt, 1999). Genome segment B (~ 2.8 kb) encodes a 94-kDa VP1, the putative virion-associated RNA-dependent RNA polymerase (RdRp). VP1 is present in the virions in two forms: as a free polypeptide and as a genome-linked protein or VPg (Calvert, 1991). There is extensive homology between the noncoding sequences of segment A and B. These sequences may be important for polymerase recognition, translation initiation, and possibly genome packaging (Dobos, 1995a).

IPNVs exhibit a high degree of antigenic heterogeneity and variation in biological properties, such as pathogenicity, host range, plaque size and temperature of replication. The vast majority of IPNVs are antigenically related and form a major serogroup A, relatively few antigenically unrelated IPNVs represent a second minor serogroup B (Hill and Way, 1995). Serogroup A contains 9 cross-reacting serotypes, A1-A9. Most IPNV isolates in the United States belong to the A1 serotype, like West Buxton (WB) strain, VR299 strain, Buhl strain and Reno strain (Blake et al., 2001). In this study, we chose Sp strain (A2 serotype), which is the major serotype in Norway, causing mortality in both Atlantic salmon (*Salmo salar* L.) fry and post-smolts (Jarp et al., 1994; Smail et al., 1995).

1.2 Functional study of IPNV nonstructural protein, VP5

The 17-kDa VP5 can be detected in infected cells, but until now no conclusive data has shown its presence in the virion (Magyar and Dobos, 1994a). Recently, it has been shown that VP5 is not essential for replication, but its function is still unknown (Weber et al., 2001). The genomic regions of VP5 from several IPNV strains have been sequenced. The deduced amino acid sequence of VP5 shows the presence of a potential phosphorylation site for tyrosine kinase and cAMP-dependent protein kinase. In addition, all VP5s are arginine-rich, have similar hydrophobicity profiles, and have an estimated PI between 9.9 and 11.0 (Heppell et al., 1995a). VP5 contains domains of BH1, BH2, BH3 and BH4 of a Bcl-2 homologue, but lacks the transmembrane region. Recently, Hong *et al.* (2002b) developed a stable Chinook salmon embryo (CHSE) cell line expressing VP5, and suggested that VP5 is a novel anti-apoptosis gene of the Bcl-2 family. Generation of a VP5-deficient mutant of IPNV would shed some light on the function of VP5. Earlier, our laboratory demonstrated that a mutant IBDV, which lacked the expression of the non-structural protein VP5, showed an attenuated phenotype and did not cause lesions after experimental infection of susceptible chickens, demonstrating that VP5 plays a key role in IBDV pathogenesis (Yao, Goodwin, and Vakharia, 1998). In this study, a virulent isolate, NVI15, which belongs to Sp serotype was cloned and sequenced. We wish to generate recombinant wild-type virus and VP5-deficient IPNV mutant using a reverse genetics system to study the function of VP5 both *in vitro* and *in vivo* (Yao and Vakharia, 1998).

1.3 Molecular determinants of virus cell adaptation and attenuation

For the purpose of developing a live attenuated viral vaccine, one requires the knowledge of the molecular determinants of virulence and attenuation, which may affect the host range and tissue tropism. A detailed understanding of these factors will facilitate efforts to ensure the safety of such vaccines (Vlaycheva and Chambers, 2002). Since RNA polymerases lack proofreading ability, the replication of RNA viruses is characterized by high mutation rates, which leads to rapid adaptation to their growth environment (Domingo, 1997). This has historically been exploited to generate live attenuated vaccines. On the other hand, unrecognized adaptive mutations occurring during propagation of viruses in the laboratory can be a source of misleading results and erroneous conclusions regarding the viral life cycle in the natural host. Therefore, it is of fundamental importance to understand the molecular processes of virus adaptation to particular host cells (Mandl et al., 2001). It is well known that IPNV loses virulence upon serial passage in cell culture (Dorson, Castric, and Torchy, 1978; McAllister and Owens, 1986), however, the molecular determinants of viral cell adaptation and attenuation have not been studied. Virulence is associated with segment A (Sano et al., 1992). Recently, Bruslind and Reno identified two amino acids differences within the VP2 region of a virulent and avirulent strains of IPNV (Bruslind and Reno, 2000). IPNV field isolates exist as quasispecies (Hsu, Chen, and Wu, 1995). Therefore, to study the molecular determinants of IPNV cell adaptation, a cloned virus generated by the reverse genetics approach would be ideal. Passage of recombinant virus in cell culture and characterization of its genomic sequence would allow one to identify markers of cell

adaptation. Moreover, by comparing the sequences of an attenuated field isolate (Sp103) and a virulent isolate (NVI15), we can make chimeric viruses by exchanging the corresponding coding region(s) of the two isolates, and identify putative amino acids involved in virulence of IPNV.

1.4 Possible mechanisms involved in apoptosis induced by IPNV in cell culture

Apoptosis is one of the cellular defense mechanism, which limits viral replication by shutting down the host translation process. On the other hand, apoptosis could help the virus to spread if it occurs at the late stages of viral infection (Razvi and Welsh, 1995). Hong et al., (1998) first demonstrated that IPNV causes both necrosis and apoptosis in infected CHSE cell-line, and apoptosis precedes necrosis. IPNV infection can up-regulate the host pro-apoptotic factors but down-regulate the anti-apoptotic factors (Hong, Hsu, and Wu, 1999; Hong and Wu, 2002). Apoptosis induced by IPNV infection has been detected in infected trout muscle cells but not in pancreatic cells (Eleouet et al., 2001). However, the role of apoptosis in IPNV infection and pathogenesis has not been well established. The study of the mechanism of IPNV-induced apoptosis will provide important information on viral pathogenesis and further facilitate control of this disease. In this study, we will investigate virus-induced apoptosis in rainbow trout gonad (RTG-2) cells, which have been shown to produce an interferon-like activity after treatment with dsRNA and/or virus infection (Garner, Joshi, and Jagus, 2003; Trobridge, Chiou, and Leong, 1997).

1.5 Objectives

The main goals of this research comprise of functional study of VP5 protein of IPNV; identification of the molecular determinants of virulence and cell adaptation of IPNV; and investigation of the mechanism of IPNV-induced apoptosis *in vitro*. The research objectives of my dissertation are to:

1. Clone and sequence the genomic segments A and B of a virulent Sp field isolate NVI15.
2. Generate recombinant and chimeric IPNVs using reverse genetics, and study their replication kinetics.
3. Characterize recombinant viruses *in vitro* and evaluate the virulence *in vivo*.
4. Identify potential signal transduction pathways of IPNV-induced apoptosis.

Chapter 2

Literature review

2.1 Infectious pancreatic necrosis

2.1.1 History of infectious pancreatic necrosis

Infectious pancreatic necrosis (IPN), also called acute catarrhal enteritis, is a highly contagious disease of trout and salmon. Historically, early North American fisheries linked mortality in hatchery trout fry to what appeared to be acute enteritis. Enteritis was presumably a result of very young fry that were unable to digest commercial food preparations. At the beginning, it was believed that a flagellate protozoan, *Hexamita* or *Octomitus* was the etiologic agent, because such protozoans were abundant in the gut of such fish (Wolf, 1988). McGonigle and coworkers found that this “enteritis” was accompanied by whirling behavior, yet death occurred in the absence of the protozoan (McGonigle, 1941). Fourteen years later, Wood named the disease as infectious pancreatic necrosis, based on the principal damage of pancreas after histopathologic examination (Wood, Snieszko, and Yasutake, 1955). The authors also postulated a viral etiology of this disease. After techniques for culturing fish cells and tissues were developed, Wolf and coworkers reported the isolation of IPNV, the first fish virus to be grown *in vitro* (Wolf, Dunbar, and Snieszko, 1958).

2.1.2 Clinical signs/pathology

Typical presentation of IPN is a sudden increase in mortality of fry or fingerling trout, with larger, more robust fish dying first. Clinical signs observed in diseased fish include: distended abdomen, aberrant swimming, trailing white feces, darkened pigmentation, exophthalmos, hemorrhage on the ventrum, and pale gills. In older fingerling trout, there may be many petechial hemorrhages in the viscera. In contrast, fry have pale viscera with few petechiae. Catarrhal exudates are often found in the stomach. Intestines may produce a mucoid, cohesive fecal pseudocast. Spleen, heart, liver and kidneys of fry are pale in color, and the digestive tract is always devoid of food (Wolf, 1988). The primary target of viral infection is the pancreatic acinar cell, and focal necrotic lesions found in exocrine pancreatic tissue are detectable by histopathological examination. Adjacent adipose tissue may be damaged. Another diagnostic feature is the presence of McKnight cells (epithelial cells of the pyloric ceca), which swell and develop fragmented nuclei; the eosinophilic cytoplasm is then shed into the lumen (McKnight and Roberts, 1976).

2.1.3 Epidemiology of IPN

IPNV usually causes high mortality in younger fish, whereas mortality is relatively rare in older fish. However, IPNV can cause significant mortality in Atlantic salmon postsmolts, especially after the stress of smoltification and introduction to saltwater. Five percent of the Atlantic salmon transferred to the sea are lost due to IPNV outbreaks (Mckenna et al., 2001). Mortality observed in outbreaks varies with fish age, species, temperature, and other environmental stresses (Frantsi and Savan, 1971;

McAllister and Owens, 1986; Silim, Elazhary, and Lagace, 1982; Wolf, 1988), but clinical signs appear typically on day 3 to 5 in fry or on day 8 to 10 in fingerlings, after exposure to the virus. Peak mortality usually occurs on day 12 to 18. The optimal temperature for disease development is between 10°C to 14°C (Noga, 1996). The virus is highly contagious in susceptible fish species. During outbreaks, lateral transmission results from contact and ingestion of infected tissue and feces. Vertical transmission occurs via transport in reproductive fluids and in the eggs. In lateral transmission, the digestive tract is presumably the prime site of infection, but the gills may be equally important because fry and fingerlings are easily infected by immersion (Wolf, 1988). Infection of IPNV can be persistent, where the surviving fish of an outbreak often become carriers and continue to shed viruses through urine and feces for two years. The IPN virus is one of the most environmentally persistent fish viruses, which can survive between outbreaks. It can survive for months in frozen viscera. In freshwater, it can survive for 5 days at 15°C, for 10 days in a 4°C stream, and for 3 months in sterile water. It survives air drying at 10°C for over 1 month (Toranzo and Hetrick, 1983).

Apart from salmonids, IPNV has also been isolated from fish representing at least 32 different families, 11 species of mollusks and four species of crustaceans (Hill and Way, 1995). However, most of the strains isolated from invertebrates are nonpathogenic to the host. The disease has been reported in North, Central and South America, Europe, Japan and Southeast Asia.

2.1.4 Diagnosis of IPNV

A definitive diagnosis of clinical IPN requires the isolation of high titers of virus from target tissues, with the appropriate clinical signs in susceptible species. The best tissue for isolation of virus is the posterior kidney. Presumptive diagnosis of IPN is based only on the presence of typical clinical signs and pathology in susceptible species, however, this could be misleading. Enzyme linked immunosorbant assay (ELISA), immunofluorescent assay and reverse transcription (RT)-PCR are used in confirmatory diagnosis (Cunningham, 2002). Isolation and identification of IPNV are usually straightforward, but IPNV may co-occur with other pathogens. Therefore, a complete and differential diagnosis is preferred.

2.1.4 Prevention and control of IPN

There is no reliable treatment for IPNV, and therefore, disinfection and quarantine is the only practical method of controlling an IPN epidemic. The virus can be readily inactivated by 40mg/l chlorine for 30 min, 20,000 ppm formalin for 5 min, 35 ppm iodine for 5 min, pH 12.5 for 10 min, or 90 ppm ozone for 0.5 to 10 min. However, ultraviolet light, sunlight, and drying seem ineffective in destroying the virus. Since the virus is environment stable, sanitary precautions should be strictly applied to prevent the spread of IPNV, although this may not be possible in many cases. Many watersheds have feral IPNV-infected salmonids. Other fish (e. g. striped bass) that are known to harbor IPNV may also transmit the virus to susceptible fish species. To avoid clinical IPN in such cases, young fish can be raised in a virus-free water source for the first 6 months, after

which the fish usually do not become sick after transfer to the IPNV-contaminated grow-out water (Noga, 1996). Vertical transmission of IPNV cannot be controlled using antiseptic egg baths, possibly because the virus is carried within the egg or is somehow sheltered on the egg's surface.

Vaccination is still the most common prevention method. Inactivated and recombinant vaccines against IPNV that are used commercially in Norway are administered by injection, but protection is not well documented (Christie, 1997). Live, attenuated vaccines potentially could be the most effective and inexpensive vaccine, since it replicates in fish, and can stimulate both humoral and cellular immune responses. If the vaccine strain is shed by vaccinated fish, effective dissemination of the antigen into unvaccinated populations would amplify the effects of the vaccine. Moreover, attenuated vaccines are inexpensive, easy to prepare, and require a low dose for administration into fish. However, there are risks of reversion to virulence and uncontrolled environmental spreading. Live, attenuated vaccines have only been allowed for field trial purposes in the catfish industry in the US (Gudding, Lillehaug, and Evensen, 1999). A better understanding of virulence factors, the mechanisms of virus attenuation and the determinants of host range and tissue tropism, will contribute to the development safe live attenuated vaccine in the future.

Fish can be vaccinated by injection (preferably intraperitoneally), immersion, or oral administration. Only injection and immersion delivery systems have been developed for routine use by the aquaculture industry. For oral vaccination, the major problem to be

resolved is how to protect antigens from being digested and decomposed during passage through the stomach and anterior gut. These methods have different advantages and disadvantages with regards to the level of protection, side effects, practicality and cost-efficiency (Gudding, Lillehaug, and Evensen, 1999). Immersion and oral administration cause less stress for the fish, and less time is required to vaccinate fish fry. Injection, although impractical for immunizing large numbers of fry and the associated high labor costs, is widely used because of the high levels of protection obtained for the duration of the growth cycle after a single administration (Horne, 1997).

2.2 Infectious pancreatic necrosis virus

2.2.1 Nomenclature and classification

Infectious pancreatic necrosis virus (IPNV) is the prototype of the family *Birnaviridae* and belongs to the *Aquabirnavirus* genus. *Birna* implies the most important features of these viruses: *bi* indicates the genome consists of two-segments, as well as its double-strandedness; *rna* signifies the viral nucleic acid is RNA. The family contains two other genera, genus *Avibirnavirus* (type species: Infectious bursal disease virus, IBDV), and genus *Entomobirnavirus* (type species: Drosophila X virus, DXV) (Dobos, 1995a). The genus *Aquabirnavirus* includes IPNV, blotched snakehead virus (BSNV), marine birnavirus, oyster virus, tellina virus, and sandgoby virus. These viruses, which are isolated from fish and other aquatic animals, exhibit the largest, most antigenically diverse group within the family *Birnaviridae* (Hill and Way, 1995).

There are two distinct serogroups of aquatic birnaviruses, designated serogroups A and B. Serogroup A comprises the vast majority of antigenically related IPNVs, and are usually pathogenic to fish. Relatively few antigenically unrelated IPNV represent a second minor serogroup B (Hill and Way, 1995). Serogroup A contains 9 cross-reacting serotypes: A1 (type strain West Buxton), A2 (type strain Sp), A3 (type strain Ab), A4 (type strain He), A5 (type strain Te), A6 (type strain Canada 1), A7 (type strain Canada2), A8 (type strain Canada 3), and A9 (type strain Jasper-ATCC VR1325). Serogroup B consists of one serotype isolated from mollusks. Most IPNVs in United States belong to the A1 serotype. Serotypes A6, A7, A8, A9 occur in Canada, A2-A5 in Europe. And A1, A2 and A3 have been found in Asia and South America (Blake et al., 2001).

2.2.2 Virus virion

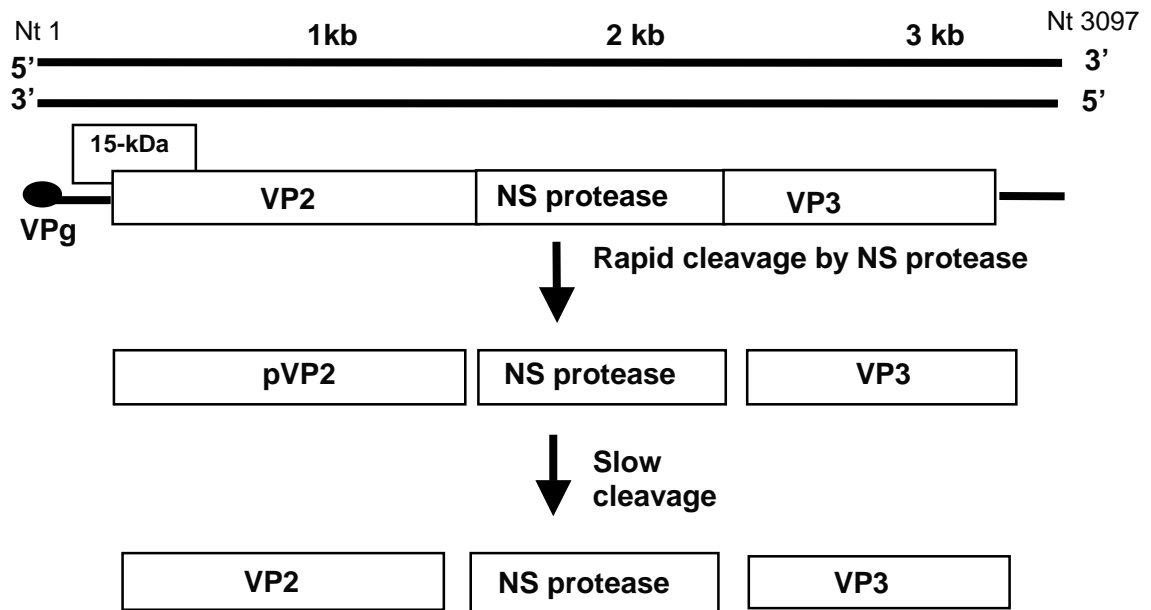
The virion of IPNV is non-enveloped, single-shelled icosahedron of 60 nm in diameter (Dobos et al., 1979). The capsid consists of 132 capsomeres with a skewed (left) icosahedral pattern of T=13 (Özel and Gelderblom, 1985). The buoyant density of IPNV is 1.33 g cm⁻³ in CsCl and that of the empty capsid is 1.29 g cm⁻³ (Dobos et al., 1977). The molecular weight of virion has been calculated to be 55 x 10⁶ and the genome has a molecular weight of 3.77 x 10⁶, thereby contributing 6.9% of the mass of the virion (Dobos, 1995a).

2.2.3 IPNV genome structure and organization

The dsRNA genome of IPNV has two segments, segment A and segment B (Fig. 2-1). Larger segment A of Jasper (3097 bp) contains two partially overlapping open reading frames (ORFs), the larger ORF encodes a 106-kDa precursor protein, which is cotranslationally cleaved by the viral encoded protease (VP4) to generate major capsid polypeptide pVP2, and VP3 (Dobos, 1995a; Duncan et al., 1987). The pVP2 (62-kDa) is further cleaved by cellular protease to VP2 (54-kDa) during virus maturation (Dobos, 1977). Segment A also encodes an arginine-rich minor 17-kDa protein (also called as VP5) from a small ORF, which precedes and partly overlaps the large ORF (Duncan et al., 1987). Segment B (2777 bp) encodes VP1, a 94-kDa protein, which is the virion associated RNA-dependent RNA polymerase (Dobos, 1995b; Duncan et al., 1991). This protein is found both as free polypeptide and covalently linked to the 5' ends of the genomic RNA segments (Calvert, 1991).

Noncoding regions (NCR) in both segments A and segment B have been completely sequenced in different strains, and comparisons were made between segments, serotypes and among different strains (Duncan et al., 1991; Weber et al., 2001; Yao and Vakharia, 1998). There is extensive homology between the noncoding sequences of A and B segment; 32 of 50 nucleotides are conserved at the 5' terminal region and 29 of 50 nucleotides at the 3' terminus. In addition, the inverted terminal repeats in both segments are conserved among different serotypes. These inverted terminal repeats may form a panhandle structure, which may be important for polymerase recognition; a similar sequence has also been reported in IBDV NCR (Dobos, 1995a).

Segment A



Segment B

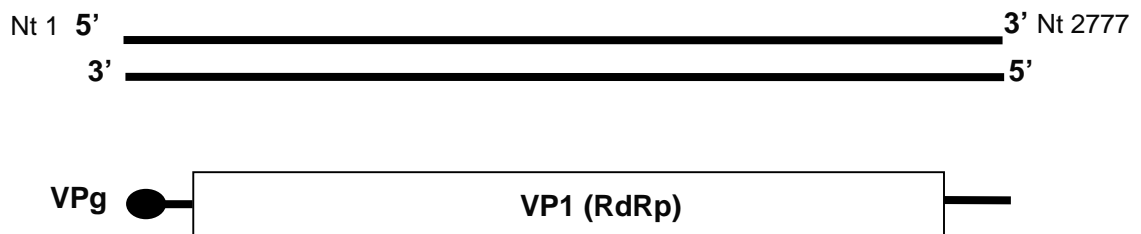


Fig. 2-1. Organization and expression of IPNV genome. Segment A produces mature viral proteins VP2, NS protease and VP3 after processing of polyprotein, and a 17-kDa protein from the overlapping minor ORF. Segment B produces the viral RNA-dependent-RNA-polymerase (RdRp). Viral polymerase is also found attached to both the segments at the 5' ends in the form of VPg.

2.2.4 Viral proteins

Segment B encodes a 94-kDa polypeptide, designated VP1, which is viral encoded RNA depended RNA polymerase (RdRp). VP1 has a low copy number and forms 4% of the total virion protein. There is a debate as to whether VP1 of both IBDV and IPNV function as guanylyl transferases or not. Although birnavirus VP1 can be guanylylated *in vitro*, neither the reversibility of the reaction nor the transfer of the GMP to an acceptor has been demonstrated, indicating that VP1 is not a guanylyl transferase or capping enzyme (Dobos, 1995b). Like RdRps of other RNA viruses, birnavirus VP1 has several conserved motifs. However birnavirus RdRps possess some features that distinguish them from most RdRps. First, they are found in both free and genome-linked forms. It is present as VPg linked to the 5'-end of both genome segments by a serine-5' GMP phosphodiester bond, and serves as the primer using a hydroxyl groups of amino acid residues within the protein itself (Calvert, 1991). In contrast to reovirus, the *in vitro* synthesis of IPNV mRNA follow a semiconservative strand displacement mechanism (Dobos, 1995b). More importantly, the RdRps of IPNV and IBDV lack the GDD sequence in the motif VI, the hallmark feature of this enzyme family. In IBDV, VP1 contains a LDD motif in place of the GDD, which possibly performs the catalytic activity, but in IPNV and DXV, the GDD motif is completely absent from the region corresponding to the presumptive motif VI of IBDV. One explanation for the lack of GDD in the IPNV RdRp sequence is that a downstream LDD motif (residue 653-655) might function as a catalytic site (Duncan et al., 1991; Shwed et al., 2002).

VP2 is the major outer capsid protein, representing 60% of the virion protein (Dobos, 1995a). In infected cells, the polyprotein is rapidly cleaved into pVP2, VP4 and VP3. The further processing of pVP2 to VP2 is brought about by fish cell proteases rather than the virus encoded protease, VP4 (Dobos et al., 1977; Magyar and Dobos, 1994b). Azad and coworkers showed that the maturation of VP2 in IBDV involves the cleavage of pVP2 at the carboxy end (Azad et al., 1987). There has been some controversy about whether VP2 is glycosylated (Estay et al., 1990; Perez, Chiou, and Leong, 1996). However, recent data suggest that VP2 is glycosylated freely in the cytoplasm and it is an O-glycosylated protein (Espinoza and Kuznar, 2002; Hjalmarsson, Carlemalm, and Everitt, 1999).

Since VP2 elicits neutralizing antibodies and carries serotype-specific and group-specific antigenic determinants, this protein is important for the development of a subunit vaccine, diagnostics and serological typing of different isolates. The only viral subunit vaccine that is commercially available in Norway is derived from VP2 expressed in *E. coli* (Christie, 1997).

The second major structural protein, VP3, is the most abundant polypeptide, with 672 molecules per virion, as compared to 544 molecules of VP2. However, due to the smaller size (31-kDa), it forms only 34% of the total virion mass (Dobos, 1995a). VP3 is known to be an internal protein, with a likely exposure of some epitopes on the surface of the virion (Caswell-Reno, Reno, and Nicholson, 1986; Tarrab et al., 1993). A truncated form of VP3, called VP3a (29-kDa), is also detected in varying amounts in purified

virions. It has been shown that VP3 binds with dsRNA to form a unique threadlike ribonucleoprotein complex (Hjalmarsson, Carlemalm, and Everitt, 1999). In IBDV, Lombardo and coworkers found that the interaction between VP1 and VP3 results in the encapsidation of VP1 into virus-like particles (VLPs) in the absence of the IBDV genome. This indicates that VP3 functions as a connector between VP1 and VPX/2 during the assembly of IBDV virions (Lombardo et al., 1999). Furthermore, the interaction motif in VP3 was narrowed down to its basic carboxyl-terminal domain (Maraver et al., 2003; Tacken et al., 2000). Like IPNV, VP3 of IBDV has been shown to interact with dsRNA of both viral genomes (Tacken et al., 2002). Thus, birnavirus VP3 is possibly a pivotal organizer of virus structure and may be important for viral assembly and replication.

The polyprotein encoded by segment A is cotranslationally processed and cleaved at the pVP2-VP4 and VP4-VP3 junctions by VP4, a viral encoded protease (Fig. 2-1). It has been shown that birnavirus VP4 forms a non-canonical RNA viral Lon protease, even though it lacks an ATPase domain (Birghan, Mundt, and Gorbalenya, 2000). Using site-directed mutagenesis, Petit and coworkers identified two residues (serine 633 and lysine 674) in IPNV VP4 that are critical for its cleavage activity (Petit et al., 2000). These residues are conserved across the Lon/VP4 family and form a Ser-Lys catalytic dyad that performs cleavage function (Birghan, Mundt, and Gorbalenya, 2000). They also determined putative cleavage sites for pVP2-VP4 at amino acid position 508-509, and for VP4-VP3, at position 734-735. All the cleavage sites have the (Ser/Thr)-X-Ala↓

(Ser/Ala)-Gly motif, a target sequence with similarities to cleavage sites of bacterial leader peptidases and herpesvirus proteases.

As mentioned earlier, segment A encodes a 17-kDa nonstructural protein, called VP5, which is detected in IPNV-infected cells (Magyar and Dobos, 1994a). Using reverse genetics, it was shown that VP5 is not required for replication and the VP5-deficient mutant virus has similar replication kinetics as the wild-type virus (Weber et al., 2001). However, the function of VP5 *in vivo* is not known. In IBDV, segment A also encodes a 17-kDa NS protein (from a small ORF), which is found in IBDV-infected cells, but not in the virion (Mundt, Beyer, and Muller, 1995). This NS protein, also called VP5 in IBDV, is not required for viral replication but plays an important role in viral pathogenesis (Yao, Goodwin, and Vakharia, 1998). The NS-deficient virus was attenuated in *in vivo* studies. In cell culture, it displayed delayed replication kinetics and had one log lower titer after infection than the wild-type virus. Apoptosis can also be induced by transient expression of IBDV VP5 protein in chicken cell lines (Yao and Vakharia, 2001). IBDV VP5 has a putative transmembrane domain and the expression of VP5 induces cell lysis, which may play an important role in the release of IBDV progeny (Lombardo et al., 2000).

2. 2. 5 Viral replication

The replication cycle of IPNV has not been completely elucidated. The entire process consists of several steps. Attachment to the host cells is the first step, followed by

entry into the host cell. Once inside the cell, virion particles are disassembled and the nucleic acids are released. Subsequent steps include replication, transcription and translation. Finally, viral particles are assembled, and the matured virions are released from the host cell (Marsh and Helenius, 1989).

The replication of IPNV takes place in the cytoplasm and a single cycle of replication takes 16-20 hours at 22 °C *in vitro*. Little is known about the early steps of virus-cell interaction. IPNV attaches specifically and non-specifically to CHSE cells; only the specific binding leads to a productive infection (Kuznar et al., 1995). VP2 appears to be the cell-attachment protein (Dobos, 1995a). Like other non-enveloped animal viruses, IPNV seems to be internalized by receptor-mediated endocytosis (Granzow et al., 1997; Kuznar et al., 1995). However, the receptor for virus binding has not been identified. Imajoh and coworkers recently found that a marine birnavirus could bind to a 250-kDa protein, which may serve as the common receptor that allows the virus to enter different fish cell lines (Imajoh, Yagyu, and Oshima, 2003).

After cell entry, birnavirus may directly proceed to initiate transcription and replication without uncoating, since the RdRp remains transcriptionally active without any proteolytic pretreatment or degradation of the capsid of the virus particles (Cohen, 1975; Spies, Muller, and Becht, 1987). However, in reovirus, which also contains a dsRNA-dependent RNA polymerase, partial uncoating to form a core-like structure is required to activate the virion transcriptase, which then allows the *in vitro* synthesis of viral mRNAs (Skehel and Joklik, 1969). Pulse labeling experiments of viral RNA

synthesis in infected CHSE cells revealed that the rate of virus specific RNA synthesis was maximal at 8-10 hr after infection and completely diminished by 14 hr (Somogyi and Dobos, 1980). Three forms of RNA, the 14-16S transcription intermediate, 24S ssRNA (viral mRNA), and 14S dsRNA (virion RNA) has been detected. Segment A specific mRNA was synthesized 2-3 times more than segment B mRNA, reflecting the relative abundancy of major virion polypeptides. Viral mRNA contains no poly A tract and no subgenomic mRNA species were found in virus-infected cells (Dobos, 1995a).

Using monoclonal antibodies, Espinoza and coworkers detected VP3 synthesis as early as 6 hr post-infection (p.i.). At 8 hr p.i., the first strong signals of VP2 appeared. During early infection, both VP2 and VP3 were colocalized in the cytosol. Later (10-12 hr p.i.), VP2 was visualized as inclusion bodies in perinuclear region of cells. Sometimes, it was found in elongated tubular structures that might correspond to type I tubules seen in IBDV infected cells. These results suggest that early in the infection, both VP2 and VP3 are synthesized in free polyribosomes, and after the bulk of viral RNA has been synthesized, assembly of IPNV begins to take place near the nucleus of the cells (Espinoza et al., 2000).

There may exist translational control of VP1 expression in infected cells. VP1 is a minor protein *in vivo*, which is produced in much low amounts than would be expected by the relative abundance of the viral 24S mRNAs. It is possible that the VP1 gene is translationally repressed by the products of segment A, since there is an abundant

production of VP1 in insect cells infected by recombinant baculoviruses carrying only the B segment of IPNV cDNA (Magyar and Dobos, 1994b).

Little is known about IPNV assembly and release from the host cells. In IPNV, virus-induced apoptosis has been observed in CHSE cells (Hong et al., 1998), which may provide the mechanism for virus release from the cells.

2.3 Molecular basis for antigenic variation, cell adaptation and virulence in IPNV

2.3.1 Molecular basis of antigenic variation in IPNV

Since RNA polymerases lack proofreading ability, the replication of RNA viruses is characterized by high mutation rates. In addition, RNA viruses have large population sizes, high replication rates and short generation times. All these properties are responsible for the extremely high genetic variability of RNA virus populations (Moya et al., 2000). This results in the emergence of new populations that are more adaptable to the environment, and could escape the surveillance of host immune system.

Aquatic birnaviruses are the largest and most diverse group of viruses within the family *Birnaviridae*. They have been isolated from at least 32 different fish families, 11 species of mollusks and four species of crustaceans (Hill and Way, 1995). These viruses exhibit a high degree of antigenic heterogeneity and variation in biological properties such as pathogenicity, host range, and temperature of replication. Antigenic variation

among different strains of IPNV has been reported by many laboratories (Berthiaume et al., 1992; Caswell-Reno, Reno, and Nicholson, 1986; Lee et al., 1996). Based on reciprocal neutralization tests with polyclonal antisera and enzyme immunoassays with monoclonal antibodies, serogroup A was divided into 9 subtypes (Hill and Way, 1995).

Most of the information available on the antigenic variation of birnavirus proteins comes from studies on IBDV. These studies revealed that there is a central variable region within VP2, containing conformational epitopes recognized by neutralizing mAbs (Vakharia et al., 1994). Similar molecular determinants of antigenic variation have been determined in IPNV VP2. Using monoclonal antibody mapping epitopes, Frost et al. (1995) discovered that the variable epitopes depended on the region between amino acid 204-330 in VP2 (Frost et al., 1995). After sequencing the VP2 region of five IPNV strains, Heppell et al. (1995) found the central variable domain is between amino acids 183 and 335, which encompasses two hydrophilic hypervariable segments (Heppell et al., 1995b). Sequences comparison of 28 IPNV strains revealed that the most of the changes in the amino acid sequence between different strains reside in the region between residues 243 and 335 (Blake et al., 2001). One would expect the hypervariable region to be located in VP2, since VP2 is the major outer capsid protein, and comprises all the neutralizing epitopes and cell attachment sites that determine host/cell range. The unique signature amino acid for distinguishing different strains was also identified within the VP2 region.

2.3.2 Molecular determinants of virulence and cell adaptation

Viral virulence is the relative ability of a virus to produce disease or lesions in a host. It is dependent on various host and virus factors, including changes in viral tissue tropism and alteration in the level of viral replication and transcription. Changes in viral virulence resulting from mutations in viral gene products have been observed in many viral systems. Many viral genome characteristics can influence viral pathogenesis and virulence, such as: viral envelope and capsid proteins; core, matrix, and nonstructural proteins; and noncoding regions of the viral genome (Tyler and Fields, 1996).

It is not surprising that the alteration of capsid and envelope proteins can dramatically change viral tropism and pathogenicity, since they frequently function as viral cell binding and receptor recognition proteins. For example, poliovirus (a naked icosahedral virus) has mutations in its outer capsid proteins that are responsible for virus attenuation. In reovirus, the S1 gene, which encodes the cell recognition protein $\sigma 1$, determines its central nervous system (CNS) tropism, and the outer capsid protein $\mu 1$ encoded by the M2 gene is the important determinant of neurovirulence for T3 strains (Tyler and Fields, 1996). For enveloped viruses, glycoproteins play an important role in host cell binding and reorganization. In Sindbis virus, the prototype member of the genus *Alphavirus* in the family *Togaviridae*, single mutation in the E2 glycoprotein is rapidly acquired during replication in the nervous system and plays a critical role in the acquisition of neurovirulence (Lustig et al., 1988).

Polymerases, nonstructural proteins and noncoding regions can also be involved in virulence. In lymphocytic choriomeningitis virus (LCMV), a single amino acid change

in the viral polymerase (residue 1079) is a major determinant of macrophage tropism and virulence (Matloubian et al., 1993). Murine rotavirus encodes a nonstructural protein, NS53, which is a determinant of virulence and capacity of spread in the infected mouse (Broome, Vo, and Ward, 1993). Noncoding regions of poliovirus and Sindbis virus are proved to be important for virulence and pathogenicity, which is presumably due to their important effects on gene replication and transcription (Kawamura et al., 1989; Kuhn et al., 1992).

Most of the information about birnavirus virulence determinants has again been obtained from IBDV. It has been shown that VP2 carries the determinants of virulence and the residues responsible for cell culture adaptation (Boot et al., 2000; Brandt et al., 2001; Lim et al., 1999). The IBDV noncoding region (NCR) is not involved in virulence, since a chimeric virus, which contains the NCR of serotype II and the coding region of serotype I virus, does not lose its pathogenicity in chickens, even though the serotype II virus is not pathogenic to chickens (Schroder et al., 2000). The involvement of VP3 in IBDV virulence is uncertain. Brandt et al. (2001) demonstrated that VP3 and VP4 do not contain virulence markers. However, Boot and coworkers found that the exchange of the C-terminal part of VP3 from the very virulent virus with serotype II resulted in an attenuated phenotype (Boot et al., 2002). In addition, the nonstructural protein, VP5, may play an important role in viral virulence, even though the precise function remains undetermined (Lombardo et al., 2000; Yao, Goodwin, and Vakharia, 1998). VP1 of the very virulent strain has one unique amino acid substitution, which may be involved in virulence of IBDV (Islam, Zierenberg, and Muller, 2001). Moreover, there

is evidence suggesting that VP1 may be involved in cell tropism of IBDV (Brandt et al., 2001).

Presence of IPNV is necessary, but not sufficient, to develop IPN disease, which also depends upon viral strain, environment, fish species and age of fish (Frantsi and Savan, 1971; Jarp et al., 1994; Ozaki et al., 2001; Smail et al., 1995). Virulence markers for IPNV have not been extensively studied. By making reassortant viruses between two serotypes, VR299 and Ab, Sano and coworkers demonstrated that virulence of IPNV is associated with genomic segment A (Sano et al., 1992). Using the same approach, they found that plaque size is dependent on segment A and is not associated with virulence (Sano, Okamoto, and Sano, 1994). Within the same serotype, there are differences in virulence and pathogenicity among different isolates, although they share the extreme homology sequences (Bruslind and Reno, 2000; Hill, 1982; Silim, Elazhary, and Lagace, 1982). The variation of virulence among isolates may be a reflection of the complex nature of this disease. Therefore, comprising the sequences of two closely related isolates, which exhibit markedly different virulence levels, could pinpoint the amino acid(s) that affect viral performance. Bruslind and Reno (2000) identified two amino acid differences at positions 217 and 286 in VP2, which may distinguish the virulent isolates from the attenuated isolates. After sequencing different isolates that belong to serotype Sp, our laboratory has identified residues at positions 217, 221, 247, 500 in VP2 that may be involved in virulence of IPNV (Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). However, using a reverse genetic approach, it is possible to

generate recombinant viruses that would allow us to pinpoint the important residue(s) involved in virulence.

It has been known that IPNV will lose virulence with serial passages *in vitro* (Dorson, Castric, and Torchy, 1978; McAllister and Owens, 1986). Dorson et al., (1978) observed that a virulent Sp strain, which lost its virulence after passaged 13 times in RTG-2 cells, produced larger plaques, and grew more rapidly in RTG-2 cells. Moreover, this attenuated virus could be neutralized by normal trout serum, whereas, the virulent one could not. However, the molecular basis for this attenuation was not known. High mutation rates of IPNV field isolates have been observed by Hsu, et al. (1995), who also demonstrated that IPNV field isolates exist as quasispecies.

2.4 Reverse genetics system

The first reverse genetics system for IPNV was established by Yao and Vakharia (1998). In this system, both genomic segments of IPNV WB strain were cloned and placed behind a T7 promoter. IPNV was rescued by the delivery of the synthetic positive-sense RNA transcripts into CHSE cells (Fig. 2-2). Using this technique, we could easily manipulate the viral genome and generate knockout or mutant viruses that will allow us to study the functions of viral proteins, or identify the residues involved in virulence of IPNV. After identifying the molecular determinants of virulence and persistence, we can use this reverse genetics approach to generate safe and stable vaccine stocks, and finally control this disease.

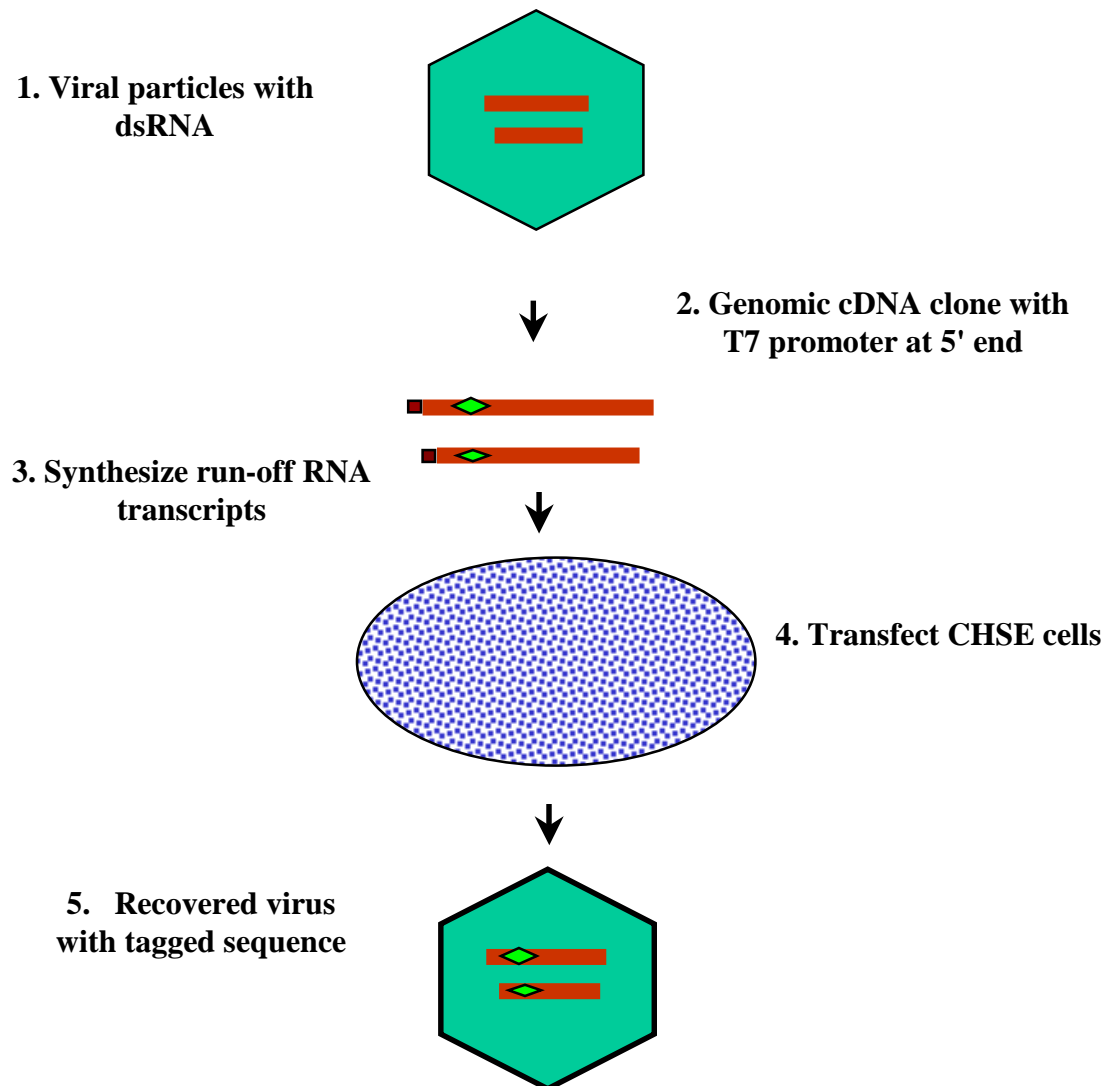


Fig. 2-2. The reverse genetics system developed in our laboratories for recovering recombinant IPNVs from clone-derived transcripts. The T7 promoter sequence is placed in from of the 5'-end of genomic cDNA clones.

2.5 Apoptosis and interferon

2.5.1 Apoptosis and virus

Apoptosis, also called programmed cell death, is considered to be a physiological process involved in normal tissue turnover, which occurs during embryogenesis, aging, and tumor regression. However, pathological stimuli, such as cell damage, irradiation, serum starvation, LPS (lipopolysaccharide), and viral infection can also trigger the apoptotic process. Apoptosis is defined by some typical morphological changes that are distinct from necrosis, including membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and formation of apoptotic bodies (Hengartner, 2000). There is no release of cellular contents or induction of inflammation around apoptotic dead cells. In the case of virus infection, since apoptotic cells are phagocytosed by normal cells without being attacked by the host immune system, progeny virus can easily and rapidly spread to neighboring cells (Razvi and Welsh, 1995). On the other hand, apoptosis is a defense mechanism that helps the host cell against virus infection, because it curtails the infection cycle and prevents neighboring cells from being infected. Some viruses encode anti-apoptotic molecules to suppress or delay apoptosis, consequently keeping host cells alive for the production of sufficient quantities of progeny. For example, the adenoviral E1B gene and the BHRF1 gene of Epstein-Barr Virus (EBV) have been shown to possess anti-apoptotic functions (Boyed et al., 1994; Henderson et al., 1993)

2.5.2 Apoptosis signal pathways

A set of cysteine proteases, called caspases, play a central role in the execution of apoptosis. Upon being activated, caspase will selectively cleave a restricted set of target proteins (Thornberry and Lazebnik, 1998). There are close to 100 caspase substrates have been reported over the years. Among them, the most distinct substrate is DNA ladder nuclease (now known as caspase-activated Dnase, or CAD), which cuts genomic DNA between nucleosomes to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs (Enari et al., 1998; Liu et al., 1997). Therefore, DNA laddering has been used extensively as a marker of apoptotic cell death. Other features of apoptosis, such as nuclear shrinking and budding, loss of overall cell shape, and membrane blebbing, can also be explained as the consequences of caspase-mediated cleavage of specific substrates (Buendia, Santa-Maria, and Courvalin, 1999; Rao, Perez, and White, 1996). However, several of the key apoptotic subprogrammes, such as cell shrinking and the emission of pro-engulfment signals, are still poorly understood (Thornberry and Lazebnik, 1998).

All the apoptotic caspases exist in normal cells as enzymatically inert zymogens. When cells undergo apoptosis, these caspases become activated by sequential proteolytic events that cleave the single peptide precursor into large and small fragments that constitute the active enzyme (Thornberry and Lazebnik, 1998). Caspases can be functionally divided into initiator caspases, such as caspases-8 and 9, and effector caspases, such as caspase-3, 6 and 7. There are two well studied pathways of caspase activation cascades that regulate apoptosis: one is initiated from the cell surface death receptor and the other is triggered by changes in the mitochondrial pathway (Fig. 2-3)

(Hengartner, 2000). The death-receptor pathway is triggered by the death-receptor superfamily (such as Fas and tumor necrosis factor receptor). These receptors share a conserved cysteine-rich repeat in their extracellular domains. The binding of Fas ligand to Fas (also known as CD95L and CD95) induces receptor trimerization and formation of a death-inducing signaling complex. Multiple procaspase-8 are recruited to this complex through the adaptor molecule FADD (Fas-associated death domain protein). Procaspase-8 oligomerization results in its proteolytic autoactivation and subsequent activation. The activated caspase-8 will then activate downstream effector caspase-3, 6, 7 and also caspase-9.

The mitochondrial pathway is used extensively in response to both extracellular cues and internal stress. These diverse response pathways converge on mitochondria by the activation of pro-apoptotic member of the Bcl-2 family. There are pro- and anti-apoptotic proteins in the Bcl-2 family; they compete to regulate cytochrome c discharge from mitochondria. If the pro-apoptotic members win, cytochrome c exits from mitochondria compartments. The released cytochrome c associates with Apaf-1 and then procaspase-9 to form the apoptosome, where caspase-9 is activated. The activated caspase-9 will also cleave and activate downstream effector caspase-3, 6 and 7. Thus, the death-receptor and mitochondrial pathways converge at the level of effector caspase activation. Additional death-inducing pathways must exist, since developmental apoptosis proceeds normally in caspase-8 and 9 defective mice (Wang and Lenardo, 2000).

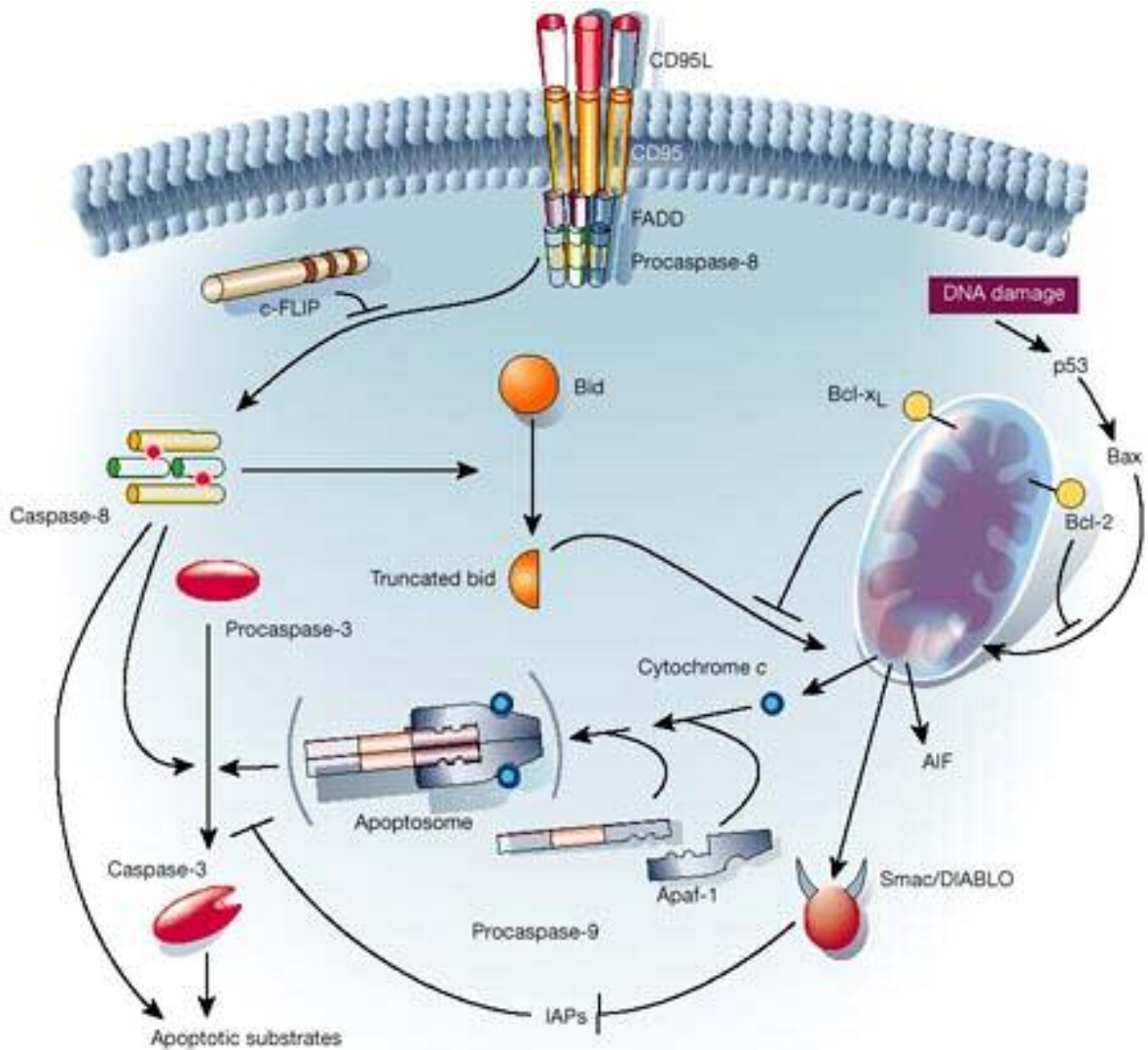


Fig. 2-3. Two major apoptotic pathways in mammalian cells, death receptor (CD95) mediated pathway and mitochondrial mediated pathway. “ —| “, direct inhibitory modification; “ —▶ “, direct stimulatory modification. The figure is adapted from Hengartner, 2000.

The nuclear transcriptional factor kappa B (NF- κ B) is thought to be a central player in apoptosis regulatory pathways, and it has both anti-apoptotic and pro-apoptotic functions. NF- κ B is normally kept in a quiescent state in the cytoplasm by association with its inhibitor, I κ B (Baeuerle and Baltimore, 1988). Upon receipt of various stress signals, such as lipopolysaccharide, tumor necrosis factor, interleukin (IL)-1 and viral dsRNA, I κ B is phosphorylated by an I κ B kinase. The phosphorylated I κ B becomes ubiquitinated by an E3 ubiquitin ligase and is subsequently degraded by proteasomes. Once the inhibitory I κ B is destroyed, NF- κ B is free and can enter the nucleus, where it activates transcription of target genes, which regulate inflammation and immune responses, viral replication, nitric oxide production, cell-cell interactions, apoptosis, and proliferation (Janssen-Heininger, Poynter, and Baeuerle, 2000). Exposure to dsRNA activates NF- κ B via the dsRNA-dependent protein kinase R (PKR) (Maran et al., 1994; Yang et al., 1995). NF- κ B confers a survival signal to many cells, such as in cells treated with TNF- α (Beg and Baltimore, 1996). The NF- κ B-dependent survival signal requires transcriptional activation of genes that include anti-apoptotic factors (Bcl-2, Bcl-X) (Tamatani et al., 1999) and inhibitors of apoptosis genes (c-IAP-1, c-IAP-2 etc.) (Wang et al., 1998). NF- κ B is also reported to potentiate apoptosis (Connolly et al., 2000; Grimm et al., 1996; Lin et al., 1995). Some proapoptotic proteins, such as p53, caspase-1, and Fas L, are regulated by NF- κ B and contain NF- κ B response elements in their promoters (Connolly et al., 2000). However, the oxidative signaling always triggers the activation of NF- κ B during apoptosis (Baeuerle and Henkel, 1994).

2.5.3 Interferon and its antiviral action

Interferons (IFNs) are a large family of multifunctional cytokines involved in antiviral defense, cell growth regulation, and immune activation. The IFNs are commonly classified into two distinct types. Type I IFNs, including IFN α and IFN β , are produced in direct response to virus infection. IFN α is predominantly secreted by leukocytes, and IFN β by fibroblasts. Type II IFNs are also known as immune IFN (IFN γ), which is only synthesized by certain cell types of the immune system, such as activated T lymphocytes and natural killer cells (Vilcek and Sen, 1996).

IFN α/β are among the first line of host defenses against viral infection. The induction of IFN- β is generally assumed to be elicited by viral dsRNA, and it occurs primarily at the level of transcription initiation, in which NF- κ B is the key transcription factor (Lenardo et al., 1989). Once IFN α/β is synthesized, it functions in both autocrine and paracrine fashions to prevent the replication and spread of viruses (Barber, 2001). Extracellular IFN α/β bind to cell surface receptors to trigger the activation of signal transduction pathways, through a phosphorylation cascade, and then induce the expression of IFN-responsive genes. Three IFN-inducible gene products have been shown to have antiviral functions, namely, Mx proteins, the 2'-5'-oligoadenylate synthetase system (2-5A synthetase), and the dsRNA-activated protein kinase (PKR) (Sen, 2001). Mx proteins are a family of related GTPase that inhibits viral polymerase activity (Pavlovic and Staeheli, 1991). Both 2-5 A synthetase and PKR antiviral pathways play a key role in the intracellular regulation of protein synthesis, a powerful

countermeasure of the host cell against viral infection. Expression of these enzymes is induced by IFNs, but they are latent until after activation of dsRNA. The activated 2-5 A synthetase catalyzes the synthesis of short oligonucleotides with a general structure ppp(A 2'p5')nA., which will bind and activate RNase L to degrade both cellular and viral RNAs, thus preventing protein synthesis (Player and Torrence, 1998). Activated PKR can phosphorylate eIF-2 α , which switch off host cell protein synthesis (Gale, Tan, and Katze, 2000).

Because of the importance of IFN α/β in host antiviral response, many viruses have evolved different strategies to subvert the IFN system (Samuel, 2001). Both DNA and RNA viruses encode inhibitors of IFN signaling pathway, such as a soluble IFN receptor homologues (vIFN-Rc) of poxvirus (Smith, Symons, and Alcami., 1998), the E1A protein of adenovirus (Samuel, 2001), the C proteins of Sendai virus (Garcin, Latorre, and Kolakofsky, 1999), and the NS1 protein of influenza A virus (Wang et al., 2000). Some viruses encode proteins or viral RNA to counteract the function of interferon-induced gene products. The best-characterized factors encoded by viruses are those that block the function of PKR. For example, E3L and K3L of poxvirus, NS1 of influenza virus, VAI RNA of simian virus (SV40), and NS5A of hepatitis C virus (HCV) are capable of blocking PKR activity (Samuel, 2001).

IFN α/β are essential mediators of apoptosis. IFNs can establish an antiviral state that may result in either cell death or cell surviving, depending on the stimulus, such as the viral species involved (Barber, 2001). For example, with VSV infection, IFN inhibits

viral replication and translation at very early step, which block virus-induced apoptosis. When host cells were treated by dsRNA or influenza virus, IFN appears to sensitize cells to apoptosis predominantly via the FADD/caspase-8 signaling pathway. IFN alone does not induce apoptosis unless it is combined with dsRNA. PKR and 2-5 A-dependant RNase L play key roles as effectors of apoptosis. Induction of apoptosis by PKR involves eIF-2 α and NF- κ B (Samuel, 2001). However, IFN alone is able to induce caspase-1, 3, 8, subsequently enhancing the sensitivity of cells to virus-induced apoptosis (Goodbourn, Didcock, and Randall, 2000).

2.5.4 Interferon and apoptosis in fish

The study of interferon in fish is still in its infancy. The production of IFN-like activity have been elicited in fish cells treated with poly (I)-poly (C) or virus infection (Garner, Joshi, and Jagus, 2003; Nygaard et al., 2000; Trobridge et al., 1997). Recently, a cDNA encoding interferon has been cloned from zebrafish. It has 15% and 14% identity, over entire sequence, to human IFN- α and IFN- β , respectively, but 25 and 24% identity to human IFN- α and IFN- β , respectively, when only compared with conserved domain (Altmann et al., 2003). cDNAs encoding fish PKRs have not yet been identified, but its substrate, eIF-2 α , was cloned from zebrafish and rainbow trout, which are 93% and 91% identical to the human eIF2 α , respectively (Garner, Joshi, and Jagus, 2003). Rainbow trout cells show increasing eIF-2 α phosphorylation after IPNV infection, which implies that the interferon/eIF2 α /PKR response may be involved in lower vertebrates to protect against virus infection. Schlezinger and coworkers first cloned NF- κ B in fish, which

binds the consensus binding sequence of human NF- κ B and is activated by aryl hydrocarbon receptor agonists (Schlezing et al., 2000).

The apoptosis pathway in fish cells appears to be similar to that in mammalian cells. cDNA encodes caspase-3 of zebrafish has been cloned and shown to have approximately 60% identity with caspase-3 from *Xenopus*, chicken and mammals (Yabu et al., 2001). Overexpression of zebrafish caspase-3 induces apoptosis in fish fathead minnow tailbud cells and in zebrafish embryos. Similarly, greasy grouper nervous necrosis virus (GGNNV) induces apoptosis in sea bass (SB) cells, which requires both caspase-3 and caspase-8 activities (Guo et al., 2003).

2.5.5 IPNV-induced apoptosis

Little research has been done to determine if IPNV induces apoptosis in infected fish. Eleouet and coworkers found that IPNV induced apoptosis in rainbow trout muscle cells, but not in pancreatic cells (Eleouet et al., 2001). Whether IPNV-induced apoptosis play a role in viral pathogenesis has not been studied. Most information about IPNV-induced apoptosis has been obtained *in vitro*. IPNV can cause both necrosis and apoptosis in the CHSE cell line, and apoptosis precedes necrosis (Hong et al., 1998). Mcl-2, a member of the Bcl-2 gene family having the capacity to promote cell viability, was found to decrease markedly in cells undergoing apoptosis after IPNV infection (Hong, Hsu, and Wu, 1999). Bad, a pro-apoptotic factor also belonging to Bcl-2 family, is induced by IPNV infection in CHSE cells (Hong and Wu, 2002). These results suggest

that IPNV infection may involve the Bcl-2 family regulated apoptosis pathway. VP5 of IPNV contains domains of BH1, BH2, BH3 and BH4 of Bcl-2 homologue, but lacks the transmembrane region. Recently, Hong and coworkers developed a stable CHSE cell line expressing VP5, which shows increased cell viability after virus infection, suggesting that VP5 is a novel anti-apoptosis gene of the Bcl-2 family (Hong, Gong, and Wu, 2002).

2.6 Current status of research and questions that remain

Although IPN disease is a serious problem in the aquaculture industry, little is known about the molecular basis of virulence and persistence in IPNV. As more genetic information of IPNV becomes available, it become clear that VP2 contains that the determinants of virulence. However, which residues of VP2 are pertinent and whether other proteins besides VP2 are involved in virulence or persistence remain unknown. An additional question is how IPNV loses virulence after serial passage in cell culture. Is it because the substitution of residues, which favor virus growth in cell culture, impairs its ability to infect fish?

There are many basic questions that remain to be answered. What is the nature of virus receptor that triggers host entry? It is likely that common receptors are shared among different fish species, since IPNV can infect large number of fish species. However, specific receptors for virus entry should also exist because same strain of IPNV can infect and cause disease in some fish species but not the others. Is it due to cell tropism? Which factors are involved in persistent infection? What is the function of VP5

in vivo? What is nature of IPNV-induced apoptosis, and does it play a role in viral pathogenesis? The answer to all these questions would facilitate our understanding of IPN disease, and finally control of this disease.

Chapter 3

Characterization of two putative non-structural proteins in Infectious Pancreatic Necrosis virus

ABSTRACT

Infectious pancreatic necrosis virus (IPNV), a member of the *Birnaviridae* family, encodes an arginine-rich basic, 15-kDa protein, known as VP5, which is not essential for virus replication *in vitro*. Previously, we have shown that in the Sp strains, there is a presence of a premature in-frame stop codon (UGA) at nucleotide position (nt) 427, preceding the 15-kDa VP5 stop codon at nt 511 (Shivappa, Song, and Vakharia, 2003). In order to characterize the different forms of VP5, we used the reverse genetic approach to generate the recombinant viruses rNVI15, rNVI15-15K and rNVI15-ΔVP5, which could encode either 12-kDa VP5, 15-kDa VP5, or be deficient in VP5, respectively. On the basis of Western blot analysis and immunofluorescence assay, VP5 was detected in rNVI15 and rNVI15-15K infected cells but not in the cells infected with rNVI15-ΔVP5. Surprisingly, both rNVI15 and rNVI15-15K infected cell lysates yielded identical 15-kDa bands, but lower amounts of this 15-kDa protein were observed in rNVI15-infected cells in comparison to rNVI15-15K infected cells, suggesting that the opal stop codon at nt 427 in rNVI15 could be read through. *In vitro* transcription and translation of these 15-kDa and 12-kDa (keeping the nt 511 position stop codon) open reading frames (ORF) under control of a T7 promoter revealed the expression of the 15-kDa protein band by both constructs. This result further confirmed the suppression of the 12-kDa VP5 stop

codon at nt 427 in rNVI15. All three recombinant viruses show similar replication kinetics in both Chinook salmon embryo (CHSE-214) cells and rainbow trout gonad (RTG-2) cells. Moreover, in the Sp strains, IPNV segment A could encode a novel, putative 25-kDa protein from another ORF between the VP2 and VP4 coding regions. We could not detect the 25-kDa protein in virus-infected cells, however, we could recover a mutant virus, rSp122- Δ 25K, lacking the 25-kDa ORF. The mutant virus exhibits similar replication kinetic as wild type Sp122, indicating that whether or not the 25-kDa protein is made by the virus, it is not essential for virus replication.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is the prototype virus of the family *Birnaviridae* and belongs to the *Aquabirnavirus* genus (Dobos, 1995a). It is the major cause of infectious pancreatic necrosis (IPN) disease in juvenile rainbow and brook trout, as well as Atlantic salmon. Highly virulent strains of IPNV can cause greater than 90% mortality in hatchery stocks less than 4 month old. Survivors of infection can remain persistently infected and serve as reservoirs of infection (McAllister, Owens, and Ruppenthal, 1987). The IPNV genome consists of two segments of double-stranded RNA that are surrounded by a single-shelled icosahedral capsid, 60 nm in diameter (Dobos, 1976). The smaller genomic segment B encodes VP1, a 94-kDa minor internal protein, which is the putative RNA-dependent RNA polymerase (Duncan et al., 1991). In virions, VP1 is present as a free polypeptide, as well as a genome-linked protein, VPg (Calvert, 1991). Segment A encodes a 106-kDa precursor protein in a single large open

reading frame (ORF), which is cotranslationally cleaved by the viral nonstructural (NS) protein, VP4, to generate mature VP2 and VP3 structural proteins (Dobos, 1977). The active residues responsible for VP4 protease activity have been identified, and the cleavage sites of the polyprotein have been located (Petit et al., 2000).

Segment A also encodes an arginine-rich, 15-kDa protein, called VP5, from a small ORF, which precedes and partially overlaps the major polyprotein ORF. This 15-kDa protein has been detected in IPNV-infected cells (Magyar and Dobos, 1994a). Weber and coworkers generated a VP5-deficient mutant virus using reverse genetics and demonstrated that this protein is not essential for viral replication (Weber et al., 2001). The function of VP5 in infected fish is not known, but it has shown that VP5 of IPNV contains Bcl-2 homologue domains and confers anti-apoptotic function when overexpressed in CHSE-214 cells (Hong, Gong, and Wu, 2002). Similarly, in infectious bursal disease virus (IBDV), another member of the *Birnaviridae* family, segment A encodes a 17-kDa NS protein (from a small ORF), which is found in IBDV-infected cells (Mundt, Beyer, and Muller, 1995). It was shown that this NS protein is not required for viral replication but plays an important role in viral pathogenesis and virus-induced apoptosis *in vitro* (Yao, Goodwin, and Vakharia, 1998; Yao and Vakharia, 2001). The expression of VP5 induces cell lysis and may play an important role in the release of IBDV progeny (Lombardo et al., 2000).

There are two distinct serogroups of IPNV, designated serogroups A and B. Serogroup A comprises nine serotypes that are pathogenic to fish, whereas, serogroup B

comprises one serotype isolated from mollusks (Hill and Way, 1995). Recently, our laboratory has cloned and sequenced several field isolates belonging to the Sp serotype (Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). Some of these isolates encode the 15-kDa VP5, whereas, other isolates have a premature stop codon at nt 427 that could encode a putative 12-kDa VP5, lacking the BH2 domain of Bcl-2 homologue (Hong, Gong, and Wu, 2002). Sequence data also revealed a putative 25-kDa ORF, which is present only in Sp serotype and has 12.7% identity and 44.7% similarity to a *Drosophila* X-virus (DXV) 27-kDa protein (Shivappa, Song, and Vakharia, 2003). This putative protein contains two basic, arginine-rich, bipartite nuclear targeting sequence motifs. However, it is not known whether this protein is expressed in the infected cells or if this ORF is required for viral replication.

Therefore, in order to characterize the expression of these two nonstructural proteins and determine their role in viral replication, we prepared mono-specific antisera against these proteins and constructed full-length cDNA clones of segments A and B of IPNV serotype Sp strains, NVI15 and Sp122. Using the cRNA-based reverse genetics system, we recovered the parental wild-type IPNV and three mutant viruses, including the ones that lack the expression of either 15-kDa or putative 25-kDa nonstructural protein. In this report, we describe whether the 12- or 15-kDa VP5 protein and putative 25-kDa nonstructural protein are synthesized in infected cells and investigate the replication kinetics of the recovered IPNVs in both Chinook salmon embryo (CHSE) and rainbow trout gonad (RTG-2) cells.

MATERIALS AND METHODS

Cells, viruses and sera. Chinook salmon embryo cells (CHSE-214 ATCC CRL-1681) were maintained at 15°C in minimal essential medium containing Hanks' salts and supplemented with 10% fetal bovine serum (FBS) and used for propagation of the field isolates and recombinant viruses. Rainbow trout gonad (RTG-2) cells (ATCC CCL-55) were grown in L-15 medium supplemented with 10% FBS at 15°C. Field isolates, Sp122, NVI15 and NVI20, which belong to Sp serotype were used in this study. Sp122 were obtained from field outbreaks of IPN in northwestern Norway in 1998, and the latter two isolates in 2000. Sp122 can cause 70% cumulative mortality in Atlantic salmon post-smolts (Shivappa, Song, and Vakharia, 2003). Both NVI15 and NVI20 isolate are highly virulent and cause as much as 90% mortality in challenged fry (Santi, Vakharia, and Evensen, 2003). The genomic segments A and B of these viruses have been completely sequenced; Sp122 and NVI15 could encode a 12-kDa VP5 protein, whereas, NVI20 encodes a 15-kDa VP5 protein (Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). The viruses were propagated in CHSE or RTG cells, and viral double stranded RNA was extracted as described (Yao and Vakharia, 1998).

The polyclonal rabbit anti-IPNV Sp serotype serum was prepared by repeated injections of the purified virus particles to the rabbit. To prepare mono-specific antisera against the 15-kDa and 25-kDa nonstructural proteins, peptides corresponding to N-terminal of 15-kDa protein (NH₂-²⁰RDWTSKHPGRHNGETHLKT³⁸-COOH), and C-terminal of putative 25-kDa protein (NH₂-¹⁴⁷RVGHRRGHTLRGRQHVLC¹⁶⁵-COOH) were

custom synthesized, and used for repeated immunization of individual rabbits to obtain anti-VP5 and anti-25K polyclonal antibodies, respectively (Bio-Synthesis).

Construction of full-length cDNA clones. Construction of full-length cDNA clones containing the entire coding and noncoding regions of IPNV, RNA segments A and B were prepared by standard cloning procedures as described previously (Yao and Vakharia, 1998). On the basis of the published IPNV sequence of the Sp strains, several primer pairs were synthesized and employed in RT-PCR amplifications (Table 3-1). To generate cDNA clones of segment A of NVI15, two primer pairs (A-A5'NC plus A-KpnR, and A-KpnF plus A-3'NC) were used for RT-PCR amplification (Table 3-1). Using genomic RNA as a template, desired overlapping cDNA fragments of segment A were synthesized and amplified in accordance with the supplier's protocol (Perkin-Elmer). Amplified fragments were cloned into the *EcoRI* site of the pCR2.1 vector (Invitrogen Corp.) to obtain plasmids pCR15A5' and pCR15A3', which were used for sequence analysis. Then, plasmids pCR15A5' and pCR15A3' were double digested with restriction enzyme pairs *XbaI* plus *KpnI* and *KpnI* plus *PstI* to release 5'-end and 3'-end fragments. These fragments were then cloned between the *XbaI* and *PstI* sites of pUC19 vector to obtain plasmid pUC19NVI15A. This plasmid contains a full-length copy of segment A, which encodes all the structural proteins (VP2-VP3), NS protease (VP4), 12-kDa polypeptide (VP5), and putative 25-kDa ORF (Fig. 3-1.). A similar approach was used to construct the full-length cDNA clone of Sp122 segment A, designated pUC19Sp122A.

Table 3-1. Oligonucleotides used for construction of full-length cDNA clones of IPNV serotype Sp genomic segments A and B*.

Nucleotide sequence	Orientation	Designation	Nucleotide no.
<i>TAATACGACTCACTATAGGAAAGAGAGTTTCAACG</i>	+	A-A5'NC	1-18
GGCCATGGAGTGGTACCTTC	-	A-SpKpnR	1584-1603
GAAGGTACCACTCCATGGCC	+	A-SpKpnF	1584-1603
<i>AAAGCTTCTGCAGGGGGCCCCCTGGGGGGC</i>	-	A-SpPstR	3079-3097
CCATATGCGGTGTGAAATACCG	+	pUC19- pUCNdeF	482-503
CAATCTATATCAATACAAGATGAA	+	A-VP5ΔF	100-123
GTTTCATCTTGTATTGATATAGATTG	-	A-VP5ΔR	123-100
CTCCTTTGGTCACCAGCT	-	A-BstER	582-599
GTCAACAACCAGCTGGTGACC	+	A-BstEF	572-592
GAATTCCTCTAACTATGTCTCTCCAGCCCCAGGCCTTTG	-	A-25Kdelta EcoRR	1437-1475
<i>TAATACGACTCACTATAGGAAACAGTGGGTCAACG</i>	+	B-B5'NC	1-18
GTTGATCCCCGTCTTTGCTTCG	-	B-SpBIR	1643-1622
CTTCCTCAACAACCATCTCATG	+	B-SpBIF	1529-1550
AAGATCTGGGGTCCCTGGCGGAAC	-	B-Bgl3'NC	2766-2783

* Composition and locations of the oligonucleotide primers used for RT-PCR and cloning are shown. T7 promoter sequences are italicized, the virus-specific sequences are underlined, and the restrictions sites are in bold. The positions where the primers bind (nucleotide number) are according to the known sequences of IPNV Sp serotype sequenced previously (Shivappa, Song, and Vakharia, 2003). Orientation of the virus-specific sequences of the primer is shown as sense (+) or antisense (-).

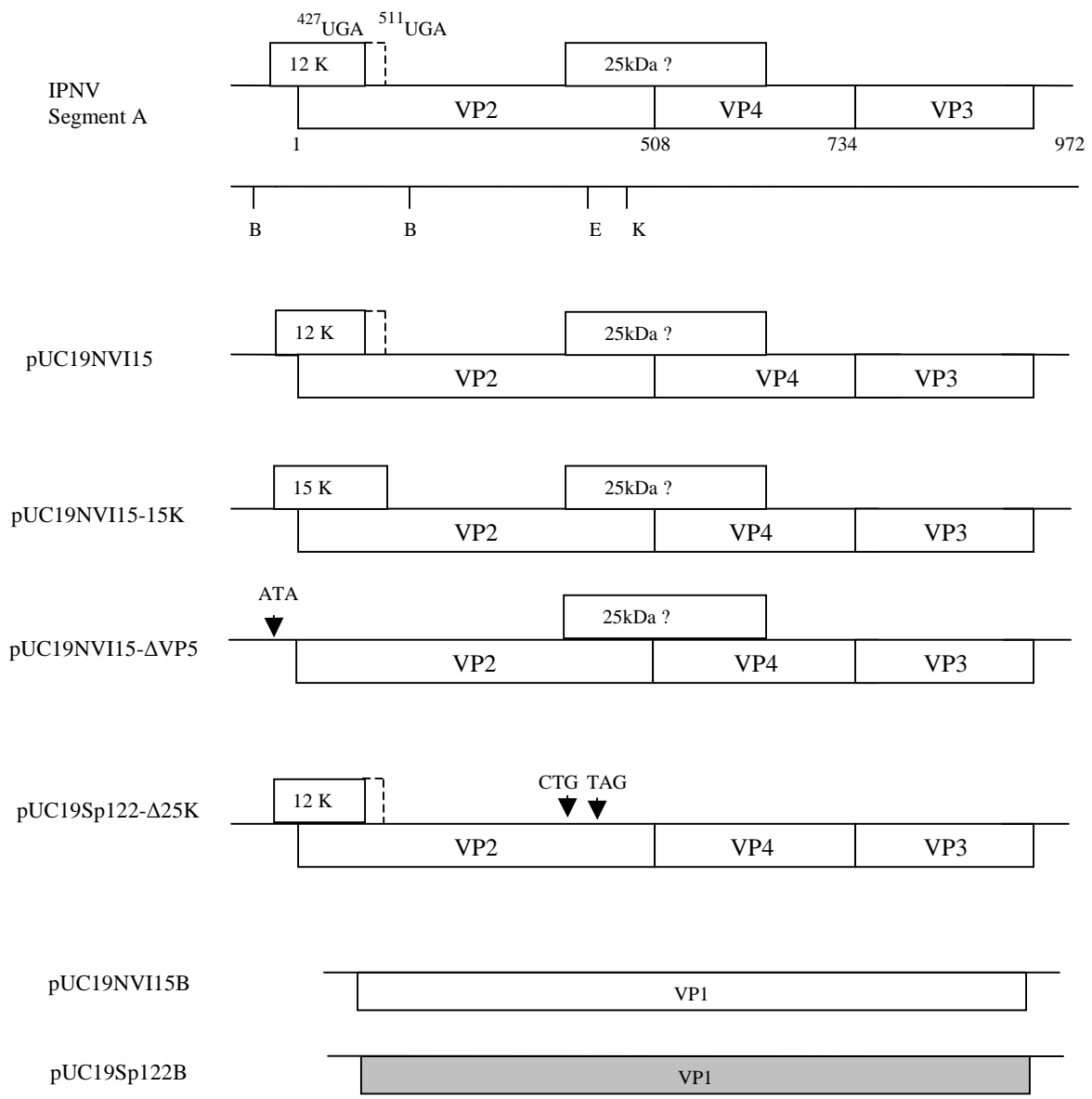


Fig. 3-1. Schematic presentation of IPNV cDNA constructs for the generation of plus-sense RNA transcripts using T7 RNA polymerase. Plasmid pUC19 NVI15 encodes the polyprotein (VP2-VP4-VP3), a 12-kDa protein and a putative 25-kDa polypeptide of IPNV Sp serotype, NVI15. Plasmid pUC19NVI15-15K encodes a 15-kDa protein instead of 12-kDa. In plasmid pUC19NVI15- Δ VP5, the initiation codon of a 15-kDa gene is mutated to ATA. In plasmid pUC19Sp122- Δ 25K, the initiation codon of the 25-kDa gene is replaced by CTG, and a stop codon (TAG) is introduced 19-nucleotides downstream of the CTG. Plasmids pUC19NVI15B and pUC19Sp122B encode the RNA-dependent RNA polymerase (VP1) of strains NVI15 and Sp122, respectively. All the plasmids contain a T7 promoter sequence at their 5'ends.

To construct a cDNA clone of IPNV segment B, two pair of primers B-B5'NC plus B-BIR and B-BIF plus B-Bgl3'NC were synthesized and used for RT-PCR amplification (Table 3-1). The amplified fragments were cloned into pCR2.1 vector as described above to obtain plasmids pCR15B5' and pCR15B3'. To construct full-length cDNA clone of this segment, the 5'-end fragment of IPNV (from plasmid pCR15B5' fragment) was first cloned into *EcoRI* site of pUC19 vector to obtain pUC15B5'. Then the 3'-end fragment of IPNV (from plasmid pCR15B3') was inserted between the unique *MfeI* and *SphI* sites of plasmid pUC15B5' to obtain plasmid pUC19NVI15B, which encodes VP1 protein (Fig. 3-1). A similar approach was used to construct full-length clone of Sp122 segment B, pUC19Sp122B.

The 5'-end of NVI20 segment A was also amplified by RT-PCR as described above, and the amplified fragment was cloned into pCR2.1 to obtain plasmid pCR20A5'. Plasmid pUC19NVI15-15K was prepared by replacing a *BstEII* fragment in plasmid pUC19NVI15A with the respective *BstEII* fragment derived from plasmid pCR20A5'.

Two primer pairs (pUCNdeF plus VP5ΔR and VP5ΔF plus A-BstER) were designed to construct a mutant cDNA clone of segment A lacking the initiation codon of the VP5. These primers were used for PCR amplification of the parent plasmid pUC19NVI15A, which yielded DNA fragments of 353 bp and 463 bp, respectively. These fragments were combined and subsequently amplified by PCR, using the flanking primers (pUCNdeF and A-BstER) to produce an 816 bp fragment. This fragment was cloned into pCR2.1 vector to obtain plasmid pCRΔVP5. This plasmid was digested with

*Nde*I and *Bst*E II, and the resulting fragment was cloned into appropriately cleaved pUC19NVII15A. The final mutant clone of segment A was designated pUC19NVII15-ΔVP5 (Fig.3-1).

To construct a mutant cDNA clone of segment A lacking the initiation codon of the 25-kDa polypeptide, a pair of primers (A-BstEF plus A-25KdeltaEcoRR) was synthesized and used for PCR amplification of the parent plasmid pUC19Sp122 A (Table 3-1). In primer A-25K delta EcoRR, the initiation codon of 25-kDa polypeptide was replaced by CTG, and a stop codon (TAG) was introduced 19 nucleotides downstream of the CTG. The PCR product was cloned into the pCR2.1 vector to produce pCRΔ25K. This plasmid was digested with *Bst*E II and *Eco*RI enzymes and the released fragment was inserted into plasmid pUC19Sp122A, to obtain pUC19Sp122-Δ25K (Fig. 3-1).

DNA from the above-mentioned plasmids was sequenced by the dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystem), and the sequence data were analyzed using PC/Gene (Intelligenetics) software. The integrity of the full-length constructs was tested by an in vitro transcription and translation coupled reticulocyte lysate system using T7 RNA polymerase (Promega Corp.).

Transcription and transfection of synthetic RNAs. Plasmids pUC19NVII15A, pUC19NVII15-15K, pUC19NVII15-ΔVP5 and pUC19Sp122-Δ25K were linearized by *Pst*I, whereas, pUC19NVII15B and pUC19Sp122B was digested by *Bgl*III. Further

treatments were carried out as described previously (Yao and Vakharia, 1998). The linearized DNA was used to produce *in vitro* transcripts with T7 mMessage mMachine™ kit (Ambion) according to manufacturer's instructions. Briefly, approximately 3µg linearized DNA template was added to the transcription reaction mixture (20µl), containing 40mM Tris-HCl (pH 7.9), 10mM NaCl, 6mM MgCl₂, 2mM spermidine, 0.5 mM ATP, CTP and UTP each, 0.1 mM GTP, 0.25 mM cap analog [m⁷G (5') ppp (5') G], 120 units of RNasin, 150 units T7 RNA polymerase, and incubated at 37°C for 90 min.

CHSE-214 cells grown to 90% confluency in a T-25 flask were transfected with cRNA of both segments as described previously (Yao and Vakharia, 1998). Briefly, cells were washed once with phosphate-buffered saline (PBS). Three milliliters of OPTI-MEM I (GIBCO/BRL) were added to the monolayer, and the cells were incubated at room temperature (RT) for 1 hr. Simultaneously, 0.15 ml of OPTI-MEM I was incubated with 12.5 µg of lipofectin reagent for 45 min in a polystyrene tube at RT. Equimolar amounts of RNA transcripts of segments A and B (≈ 8µg each), resuspended in 0.15 ml of diethyl pyrocarbonate-treated water, were added to the OPTI-MEM/Lipofectin mixture, mixed gently, and incubated on ice for 5 min. After removing the OPTI-MEM I from the monolayers in T-25 flask and replacing it with a fresh 1.5 ml of OPTI-MEM, the nucleic acid-containing mixture was added drop-wise to the CHSE cells and swirled gently. After 3 hr of incubation at RT, the mixture was replaced with minimal essential medium containing Hanks' salts and 10% FBS (without rinsing the cells). The cultures were incubated at 15°C for 5 days, and the cell supernatant was harvested by freeze-thawing twice, and passaged onto fresh CHSE monolayers. Cytopathic effect (CPE) usually was

visualized 4 days after the second pass. All the recombinant viruses were then passaged into CHSE once more, the cells were freeze-thawed twice, and the cell-free supernatants were stored at -70 °C for use as virus stocks in this work. The identities of recovered viruses were verified by RT-PCR, using specific primers for IPNV segment A.

Immunofluorescence. Infection of CHSE cells by the recombinant viruses were analyzed by immunofluorescence assay (IFA) using rabbit anti-VP5 specific serum. Briefly, CHSE cells were infected with IPNV viruses at an MOI of 1 and incubated at 15°C. At 16 hr postinfection, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at RT. Cells were permeabilized with 0.1% Triton-100 for 10 min at RT, then incubated with anti-VP5 diluted 1:100 in PBS containing 2% BSA for 1 hr. After being washed with PBS, the cells were treated with fluorescein-labeled goat anti-rabbit antibody at 1:1000 dilution (Kirkegaard & Perry laboratories) and examined by fluorescence microscopy.

Growth curve and plaque assay. To analyze the growth characteristics of IPNV, confluent CHSE or RTG-2 cells (in 35-mm dish) were infected with the recombinant viruses at MOI of 1 (for rNVI15, rNVI15-15K and rNVI15-ΔVP5) or MOI of 0.01 (rSp122-Δ25K and Sp122). Aliquots collected at various time points were stored at -70°C. Supernatants were centrifuged and titrated on CHSE cells by plaque assay. Briefly, confluent monolayers of CHSE cells grown in six-well plates were infected with serially diluted supernatants from virus stock. After 1 hr incubation at 15°C, cell was washed once with PBS and overlaid with 0.6% SeaPlaque Agrose (Difco) in Eagles

MEM medium containing 5% FBS and 1% L-glutamine. After 3 days of incubation at 15°C, the overlays were removed and the cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at RT. After rinsing the cells with distilled water, the plaques were counted.

Immunoblot Analysis. To analyze viral protein synthesis during single round replication, RTG-2 cells grown in 6-well plates were infected with the wild type or recombinant IPNVs at MOI of 10. At indicated time intervals, the cells were scraped off the plates, and sedimented by centrifugation. The cells were washed twice with ice-cold PBS, resuspended in 30µl of PBS and mixed with an equal volume of 2 X SDS sample loading buffer (100mM Tris-Cl pH 6.8, 200mM DTT, 4% SDS, 0.1% bromophenol blue, 10% glycerol). The samples were then boiled for 5 min, and centrifuged at 13,000g for 3 min. The supernatants were stored at -70°C. For immunoblotting, total protein from the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 5% stacking and 12.5% resolution gels, and proteins were transferred to a nitrocellulose membrane for immunoblot analysis. After electrotransfer, the membranes were blocked in the blocking buffer, 5% nonfat dry milk in Tris-buffered saline (TBS) (150mM NaCl, 10mM Tris-Cl, pH 7.5) for 90 min at RT. The membranes were incubated for 60 min in 10 ml of a 1:400 dilution of a rabbit anti-IPNV polyclonal antibody or 1:200 dilution of anti-VP5 in blocking solution, and then washed for 3 X 15min in 1 X TBS (0.1% Tween in TBS buffer). The membranes were then incubated with a 1:25000 dilution of a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) for 60 min and then washed 3 X 15min as above. The

detection of IPNV structural proteins or VP5 was performed by enhanced chemiluminescence (ECL) western blot detection system (Amersham Pharmacia Biotech Inc.). The membrane was stripped in 0.1M glycine buffer (pH 3.0) for 30 min and blocked again as described. The membranes were subsequently probed for actin using an anti-actin polyclonal antibody (Sigma). The protein was quantified by radiograph densitometry of the VP3 protein band using NIH image software.

***In vitro* expression and protein labeling.** To prepare a recombinant expression plasmid, the VP5 coding regions in plasmid pUC19NV115A and pUCNV115-15K were amplified by PCR using a pair of oligonucleotide primers:

SpNSHindF (5'-GAAGCTTATGCAAGATGAACAC-3') plus SpNSBamR (5'-AGGATCCTCAGACAGACTGCC-3'). The PCR products were cloned into a pCR2.1 vector and sequenced. The fragments were further cloned into the *Hind*III and *Bam*HI cloning sites of the eukaryotic expression vector pcDNA3 (Invitrogen), downstream of the T7 promoter and human cytomegalovirus (HCMV) promoter, to produce pcDNA-15KVP5 and pcDNA-12KVP5 respectively. A similar approach was used to generate pcDNA-12KVP5(-), in which 12-kDa VP5 ORF was inserted into pcDNA3 in an opposite orientation to obtain pcDNA-12KVP5. *In vitro* transcription and translation was performed using the TNT Quick Coupled Transcription/Translation System as described by the manufacturer (Promega). Briefly, the DNA template (2µg) was added to a tube containing TNT buffer, ³⁵S labeled methionine, amino acid mix (minus methionine), water, reticulocyte lysate, and T7 polymerase. The mixture was then incubated at 30°C for 90 min. After incubation, aliquots of 10µl were submitted to SDS-PAGE. The gel was

fixed with a mixture of 30% methanol and 8% acetic acid for 30 min followed by EN³HANCE solution (Biotechnology Systems, NEN Research Products) for 45 min, dried and exposed to film overnight at -70°C for autoradiography. The film was developed using an automated film processor (Kodak XR-2).

RESULTS

Construction of full-length cDNA clones. To determine the role of two minor ORFs in replication, we constructed the full-length cDNA clones of segments A and B of IPNV strains NVI15 and Sp122, as shown in Fig. 3-1. Using parental clone pUC19NVI15A as a backbone, two mutant cDNA clones of NVI15 segment A were prepared. In plasmid pUC19NVI15-15K, a *Bst*EII-fragment (nucleotides 24 to 586) of the parental plasmid were replaced with a similar *Bst*EII-fragment of IPNV strain NVI20, which does not contain a premature stop codon at nt 427 (TGA→CGA). Thus, this chimeric clone encodes a 15-kDa VP5, but does not contain any additional mutation in either VP2 or VP5. Previously we have shown that one of the field isolates, NVI10, does not encode VP5 protein as it contains ATA instead of ATG – the initiation codon of VP5 (Santi, et al., 2003). Using site-directed mutagenesis, we prepared plasmid pUC19NVI15-ΔVP5, which lacks the initiation codon for VP5 expression. To determine whether the putative 25-kDa ORF is essential for viral replication, we constructed another clone, pUC19Sp122-Δ25K, in which the first and only initiation codon of 25-kDa protein was mutated to CTG, and a stop codon (TAG) was introduced 19 nucleotides downstream of the CTG. The functionality of all these clones was tested by in vitro transcription-

coupled translation reactions, which yielded protein products that comigrated with the marker IPNV proteins after separation on SDS-PAGE and autoradiography (data not shown).

Transfection and recovery of mutant viruses. In order to recover the recombinant viruses, we transfected CHSE cells with combined plus-sense transcripts derived from various plasmids as described previously (Yao and Vakharia, 1998). As expected, rNVI15, rNVI15-15K and rNVI15- Δ VP5 were rescued from the transcripts derived from wild-type or modified segment A and NVI15 segment B. A 25-kDa-deficient mutant virus, rSp122- Δ 25K, was also generated by transfection with the transcripts derived from pUC19Sp122- Δ 25K and pUC19Sp122B. To verify all the mutant viruses are indeed recovered from the cRNAs, the genomic RNA was isolated and analyzed by RT-PCR using a primer pair specific for segment A. Sequence analysis of the cloned PCR products confirmed the expected nucleotide mutations from the mutant viruses. To detect expression of 15-kDa protein, CHSE cells were infected with the recovered viruses and analyzed by immunofluorescence assay (IFA) using 15-kDa-specific antiserum. Cells infected with rNVI15 and rNVI15-15K gave positive immunofluorescence signals that confirmed the expression of 15-kDa protein. The fluorescence of the 15-kDa protein was seen in the cytoplasm, mainly within the prenuclear region (Fig. 3-2b, 3-2c). Cells infected with rNVI15- Δ VP5 did not give any fluorescence signal, nor did mock-infected cells (Fig. 3-2d, 3-2a). Similarly, to detect the expression of putative 25-kDa protein,

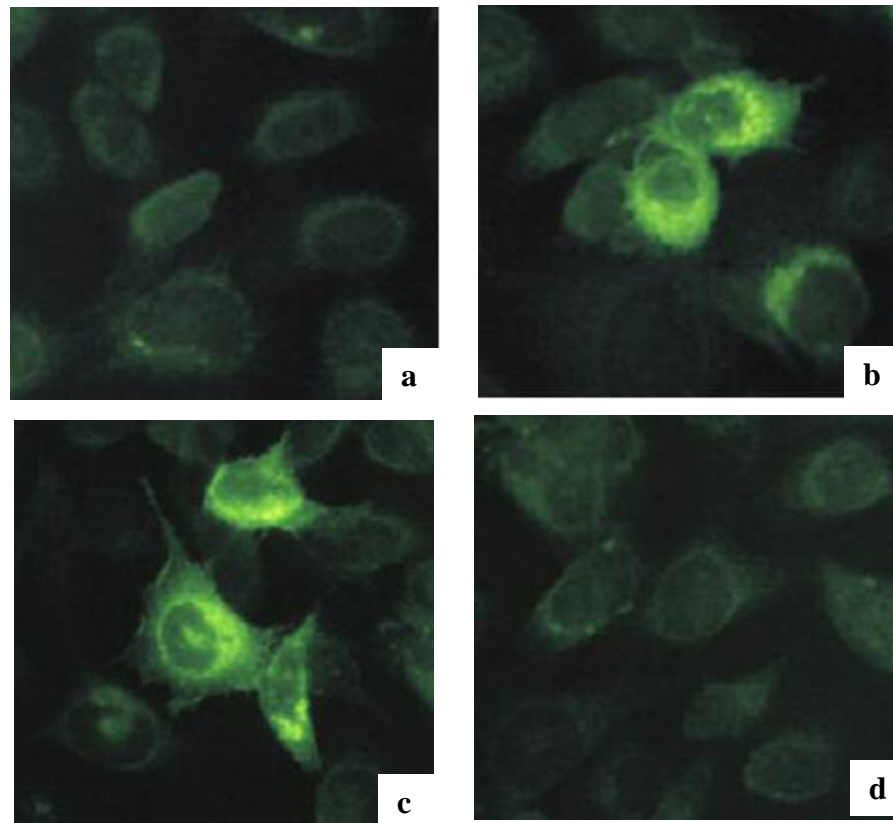
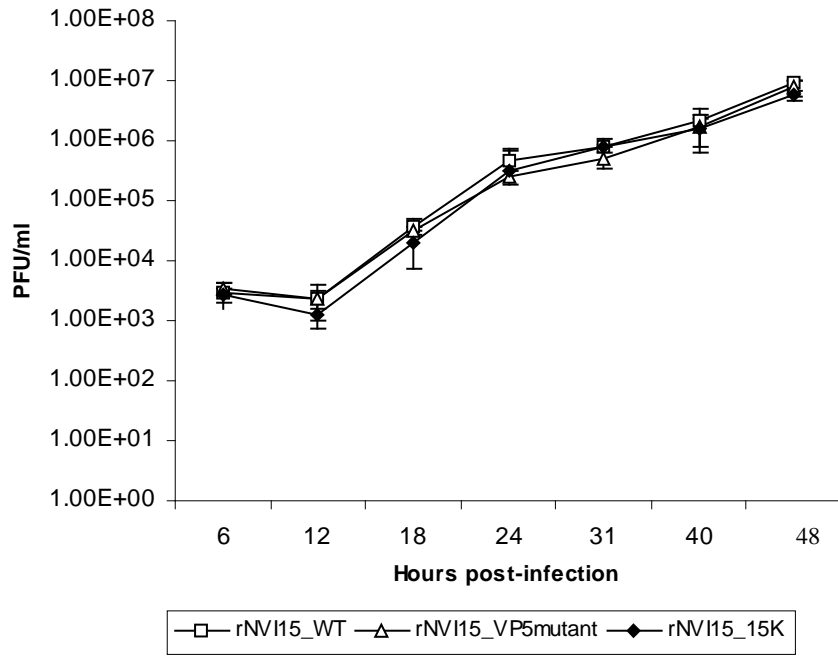


Fig. 3-2. Immunofluorescence staining of IPNV-infected cells to detect the expression of the 15-kDa protein. CHSE cells were infected with rNVI15 (b), rNVI15-15K (c), rNVI15- Δ VP5 (d) virus stock at an MOI of 1. Uninfected CHSE cells were used as negative control (a). After 16 hr post infection, the cells were fixed, permeabilized and analyzed by immunofluorescence staining with rabbit anti-15-kDa protein-specific serum.

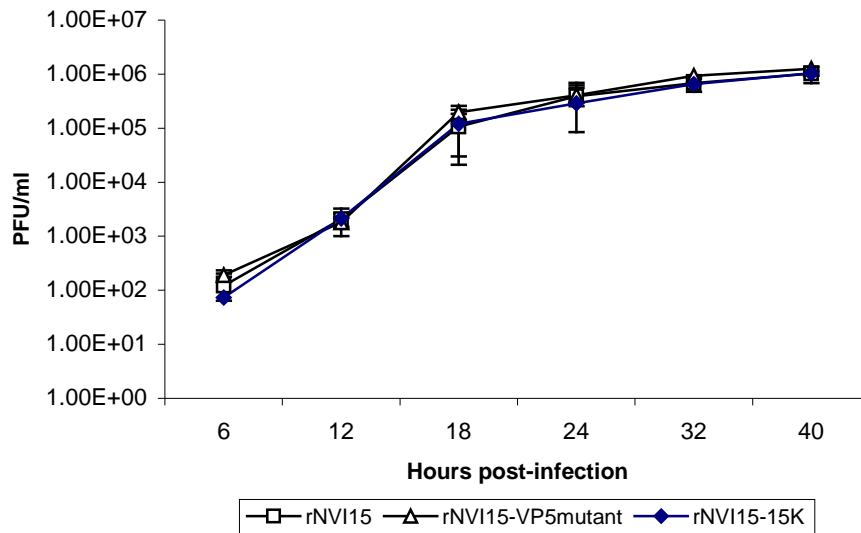
CHSE cells were infected with the parental or recovered IPNVs and analyzed by IFA using 25-kDa-specific antiserum. No immunofluorescence signal was obtained in virus-infected cells, although this antibody is capable of detecting *E. coli* expressed 25-kDa polypeptide by Western blot (data not shown). These results suggest that the 25-kDa protein is not synthesized in virus-infected cells, or is expressed below the detection level of the antibody.

Characterization of recombinant viruses *in vitro*. To compare the replication kinetics of the recovered viruses, both CHSE and RTG-2 cells were infected by these viruses at MOI of 1. At the indicated time points, the supernatants from infected fish cells were collected and titrated in CHSE cells by plaque assay. Figure 3 depicts the growth curves of rNVI15, rNVI15-15K and rNVI15- Δ VP5 in CHSE (Fig. 3-3A) and RTG-2 cells (Fig. 3-3B), respectively. These viruses grew to similar titer in CHSE cells, which is in accord with the results of Weber and coworkers, who could not detect any difference in growth among the recombinant VP5-deficient mutants and wild type IPNV (Weber et al., 2001). These viruses showed a slight delay in growth in CHSE cells as compared to RTG-2 cells. Cytopathic effects (CPE) appeared earlier in RTG-2 cells than in CHSE cells, but the final titers of all viruses were one log higher in CHSE cells than in RTG-2 cells. In order to determine if the 25-kDa ORF can interfere with virus growth, CHSE cells were infected with both wild type Sp122 and rSp122 - Δ 25K at MOI of 0.1. The mutant virus showed no difference in replication kinetics when compared with wild type virus at any time point (Fig. 3-3C), indicating that this putative 25-kDa ORF is dispensable for virus replication.

A.



B.



C

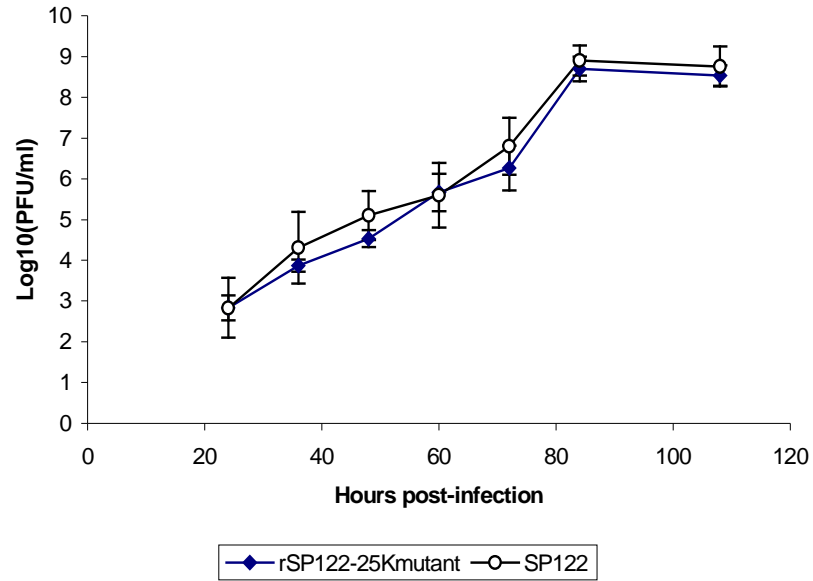


Fig 3-3. Growth curves of the recombinant and wild-type IPNVs. Monolayers of CHSE cells (A and C) or RTG-2 (B) cells were infected with various recombinant viruses at a MOI of 1(A and B) or at MOI of 0.01 (C), harvested at the indicated time points, and virus titers were determined by plaque assay. Average titers and standard deviation (error bars) from three independent experiments are shown.

Expression of viral proteins in infected RTG-2 cells. Since all the recombinant viruses show delayed growth in CHSE cells, single round replication of IPNV in RTG 2 cells was carried out to study the synthesis of viral proteins. RTG-2 cells grown in 6-well plates were infected by IPNVs at MOI of 10, and cell lysates were collected at the indicated time points and subjected to SDS-PAGE. Figure 3-4A shows the results of a Western blot analysis indicating the expression of 15-kDa protein at 4-hour time intervals postinfection (p.i.). As expected, the 15-kDa polypeptide was present in rNVI15-15K infected cells. However, in rNVI15-infected cells, instead of a 12-kDa band, a 15-kDa band was detected by the rabbit anti-15-kDa serum and its expression level was lower than the rNVI15-15K infected cells, indicating that the stop codon (UGA) at nt 427 is read-through. Of course, in rNVI15- Δ VP5 infected cells, there were no 15-kDa or 12-kDa protein bands detected. To further characterize the synthesis of 15-kDa protein, the early time points postinfection were selected at 2-hr time intervals (Fig. 3-4B). The results indicate that the synthesis of 15-kDa protein in rNVI15-15K infected cells started at 6 hr p.i., reached its peak rate of synthesis around 12 hr p.i., and decreased thereafter. The read-through product, 15-kDa polypeptide of rNVI15, appeared at 8 hr p.i., two hour later than that of rNVI15K-15K, but reached its peak expression at same time as rNVI15K-15K, around 12 hr p.i., and decreased thereafter. Compared with their expression level at peak expression time point, 12 p.i., the amount of 15-kDa protein synthesized in rNVI15 infected cells is eight-fold less than in rNVI15-15K, suggesting that the read-through efficiency at this 427 nt stop codon is about 10-20%. The expression of VP3 in all the viruses was similar and it initiated at 4 hr p.i., which is consistent with the result of growth curve shown above.

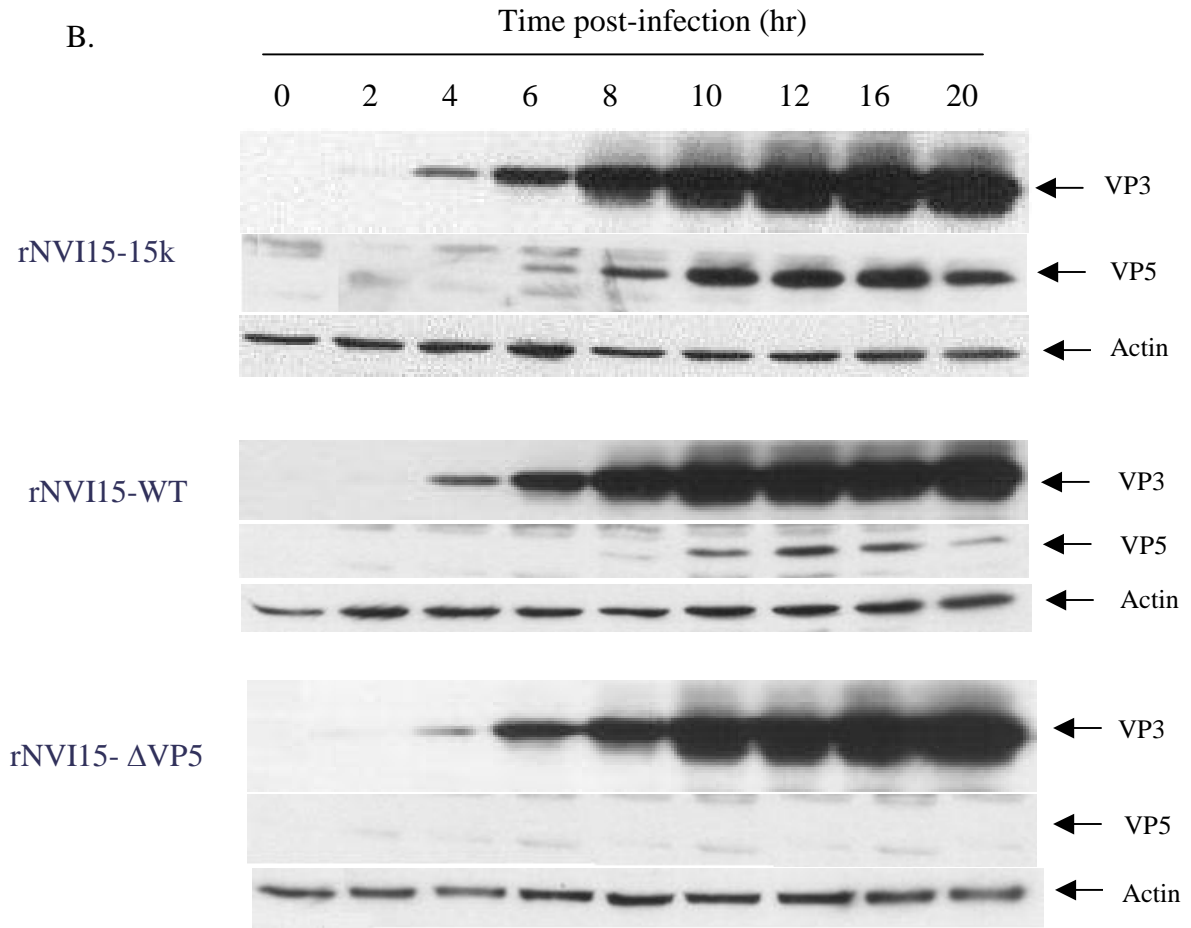
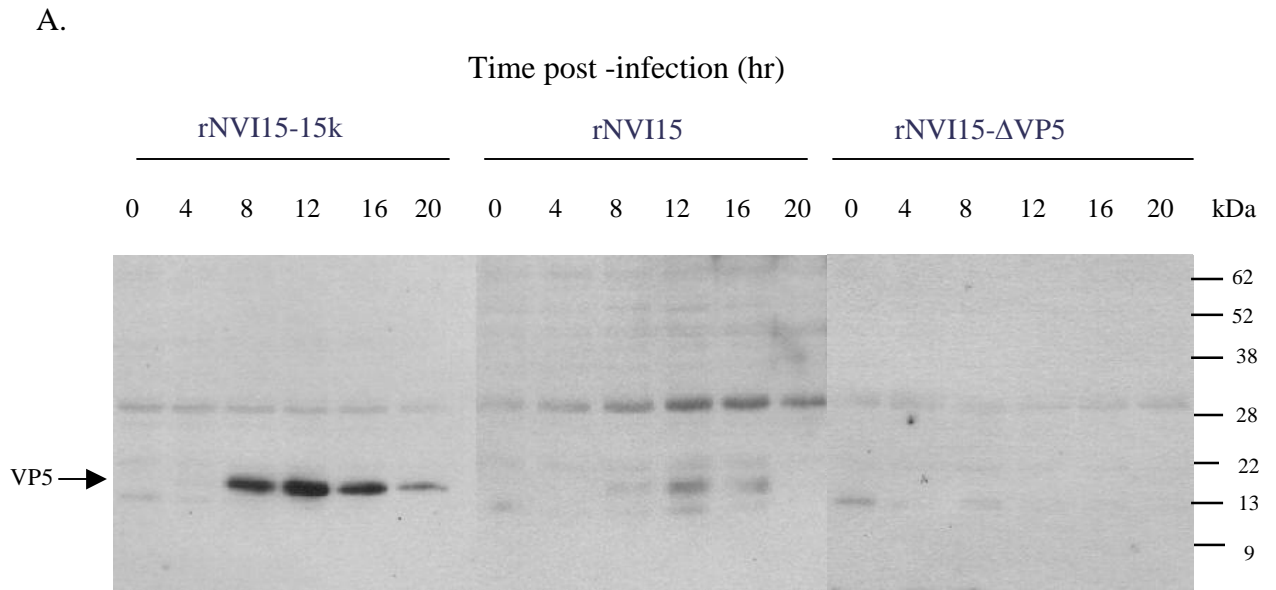


Fig. 3-4. Western blot analysis of IPNV-infected cells. RTG-2 cells were infected with the recombinant viruses at a MOI of 1. At indicated time interval, cell lysates were collected and the proteins were separated by SDS-PAGE. (A) The membrane was incubated with anti-15-kDa-specific serum. (B) Membrane was cut in half along the marker of 22-kDa, the upper part was incubated with anti-IPNV polyclonal antibodies, and the lower part was incubated with anti-15-kDa antibody. The upper part membrane was stripped and restained with anti-actin polyclonal antibodies to determine the expression level of actin, and used as a control.

Using polyclonal anti-25-kDa-specific antibody, we were unable to detect the expression of 25-kDa protein either in CHSE or RTG cells (data not shown).

***In vitro* VP5 expression in cell free lysates.** To further demonstrate that the stop codon at nt 427 is read-through, we constructed two clones, pcDNA-15KVP5 and pcDNA-12KVP5, in which the 15 -kDa ORF was inserted immediately after the T7 and CMV promoters. The former clone has only one stop codon at nt 511, while the latter has both stop codons at nt 427 and nt 511. The negative control, pcDNA-12KVP5 (-) has the 12-kDa ORF inserted into pCDNA3 in the opposite direction. After *in vitro* transcription and translation of these three clones, the radiolabeled proteins were fractionated by SDS-PAGE. Both pcDNA3-15KVP5 and pcDNA3-12KVP5 could express 15-kDa protein, but the density of the autoradiograph band is about five-fold greater in pcDNA3-15KVP5 than in pcDNA3-12KVP5 (Fig. 3-5). These results demonstrate that the stop codon at nt 427 can be read-through in a cell-free rabbit reticulocyte translation system. As in the virus infected cell lysate, we could not detect the specific 12-kDa protein band.

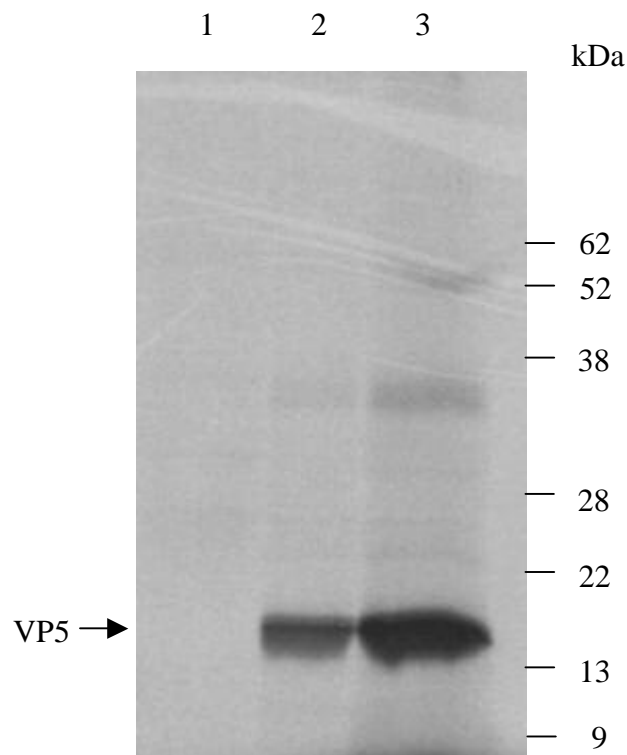


Fig. 3-5. Fractionation of the [^{35}S] -labeled 15-kDa protein by a 12.5% SDS-PAGE. The ORFs of 15-kDa and 12-kDa proteins (keeping the nt 511 stop codon) were cloned into pcDNA3 vector, under control of a T7 promoter to obtain pcDNA-15KVP5 and pcDNA-12KVP5, respectively. Plasmid pcDNA-12KVP5 (-) was produced by inserting the 12-kDa ORF in pcDNA3 in an opposite direction. The plasmids were transcribed and translated in a rabbit reticulocyte lysate system, and the translated protein products were subjected to SDS-PAGE and autoradiography. Lane 1 = protein produced by pcDNA-12KVP5 (-) (negative control); Lane2 = protein derived from pcDNA-12KVP5; Lane 3 = protein product obtained from pcDNA-15KVP5.

Confirmation of genetic stability of recombinant IPNVs. To determine the genetic stability of recovered IPNVs *in vitro*, the viruses were serially passaged in RTG-2 cells (up to ten times). A 15-kDa polypeptide could be detected in rNVI15 and rNVI15-15K, but not in rNVI15- Δ VP5 infected cells when examined by immunostaining. The 15-kDa nonstructural gene was amplified by RT-PCR and the sequence analysis of the cloned PCR product confirmed the expected nucleotide mutations in NS gene of the mutant viruses, whereas, no mutation was detected in the PCR product of rNVI15, which indicated that all the viruses are genetically stable even after ten passages in cell culture.

DISCUSSION

We have developed the reverse genetics system for Sp serotype of IPNV and have characterized two ORFs, encoding a 15-kDa polypeptide and a putative 25-kDa ORF, which overlap the main ORF. Our results demonstrate that the stop codon at nt 427 of IPNV nonstructural protein (VP5) is read-through to produce a 15-kDa protein, rather than a truncated 12-kDa protein. The 25-kDa ORF, which is unique for Sp serotype, is not expressed in the infected cells and is nonessential for viral replication.

Previous studies have shown that the expression of VP5 in strain VR299 is initiated at the second in-frame start codon at nt position 113, yielding a 15-kDa protein (Weber et al., 2001). However, results from our laboratories indicate that the avirulent strains of Sp serotype encode a 15-kDa protein, whereas, the virulent strains contain a truncated form VP5 protein gene (Santi, Vakharia, and Evensen, 2003; Shivappa, Song,

and Vakharia, 2003). In order to characterize the expression of this 12-15-kDa VP5 protein, we constructed infectious cDNAs clones of the virulent IPNV strain, NVI15, and generated VP5 mutant viruses. The rescue of the recombinant IPNVs was verified by RT-PCR, and by immunofluorescence using anti-15-kDa-specific antibody. Growth kinetics of the VP5 mutant viruses in CHSE and RTG-2 cells was similar, which confirmed that VP5 does not affect the rate of replication and it is dispensable for viral replication. These results are agreement with Weber and coworkers, who also reported similar findings in CHSE cells (Weber et al., 2001).

A similar growth curve in RTG-2 cells indicates that VP5 is unlikely to play a role in virus anti-interferon activity. It is known that RTG-2 cells can produce interferon-like activities when treated with dsRNA (Trobridge, Chiou, and Leong, 1997). RTG-2 cells responds to IPNV infection and poly I:C treatment by increasing eIF2alpha phosphorylation, which implies that RTG-2 cells express the interferon-induced eIF2alpha-kinase, PKR (Garner, Joshi, and Jagus, 2003). All three viruses show a similar level of induction of eIF2alpha phosphorylation after infection in RTG-2 cells (data not shown), contributing further evidence that VP5 does not play a role in anti-interferon activity *in vitro*.

The expression of a 15-kDa rather than a 12-kDa form of VP5 in rNVI15 virus was unexpected. We propose that this opal codon at nt position 427 is read-through. To verify this, we used *in vitro* transcription and translation system to express the ORF of both 15-kDa and 12-kDa proteins; we can only detect the expression of a 15-kDa protein

but not the 12-kDa protein. This result confirmed that the stop codon at nt 427 is a very weak stop codon, which can be read-through by the ribosome. However, it is not clear why we could not detect the 12-kDa protein in both virus-infected cells or in cell-free translation system.

Read-through of a stop codon can occur by the misincorporation of an amino acid at the termination codon by natural tRNA, the most common amino acid encoded by the read-through of UGA is tryptophan, and of UAG is glutamine (Harrell, Melcher, and Atkins, 2002). Compared with the other two stop codons, UGA is less likely to provide proper translation termination (MacBeath and Kast, 1998). It is well known that the immediate upstream and downstream sequences surrounding the termination sequence are important for read-through efficiency. In mouse cells, an A in the -1 position is always associated with the highest read-through level, whereas, a U is associated with the lowest (Cassan and Rousset, 2001). The 3' termination contexts of the form CAR-YYA confer leakiness was also identified in tobacco mosaic virus (TMV) for read-through to synthesize a replicase (Skuzeski et al., 1991). The internal UGAC is a relatively poor stop signal in *E. coli* (Poole, Brown, and Tate, 1995); similarly in yeast, the residue at +4 position of UGA and UAA stop codons were found to be important in termination efficiency. Cytosine has lowest termination efficiency and guanine has the highest (Bonetti et al., 1995). It was also shown that the signal for Sindbis virus read-through UGA stop codon involves a C at the +4 position (Li and Rice, 1993). Comparing the two stop codons in VP5 ORF, UGA at nt 427 appears to be a weak termination codon because it has A at -1 , C and A at +4 and +6 respectively, whereas, the UGA at nt 511 would be

an optimal stop codon because it has a C at -1 and a G at +4, and does not have the consensus CAR-YYA immediately distal to the termination codon (Fig. 3-6). To our knowledge, this is the first report suggesting that in fish cells a stop codon read-through mechanism is conserved, similar to other prokaryocytes and eukaryotes.

Because of the limitations in genomic size, viruses often use the read-through of internal termination codons to regulate the synthesis of two related polypeptides, which are needed in different amounts. The suppression of leaky UAG in TMV results in replicase synthesis (Skuzeski et al., 1991). For animal viruses, alphavirus RNA polymerase is expressed by UGA read-through (Strauss, Rice, and Strauss, 1984). In IPNV serotype Sp, the expression level of VP5 is regulated due to the presence of the premature stop codon at nt 427. Whether the different VP5 expression is involved in virus pathogenesis need to be further investigated. However, these three recombinant viruses cause similar cumulative mortality in Atlantic salmon smolts, indicating that VP5 is not involved in virulence (Santi, unpublished data). Study is under way to determine if VP5 is involved in viral persistent infection.

A putative ORF in segment A of Sp strain encodes a 25-kDa polypeptide. However, we were unable to detect this 25-kDa protein either by IFA or immunoblotting using specific antibody. The success of rescuing the mutant virus lacking this 25-kDa ORF indicates that it is not required for virus replication. Further work is required to determine whether this protein is present in virus-infected fish, and if it has a function in viral pathogenesis *in vivo*.

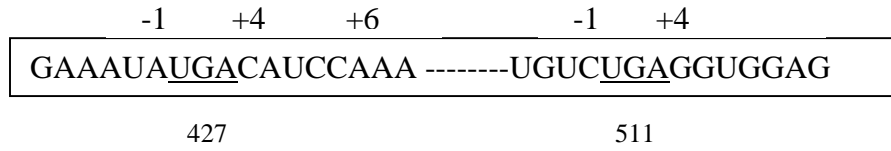


Fig. 3-6. Sequence contexts of two stop codons in 15-kDa-protein gene. The position of first nucleotide in UGA stop codon is designated as + 1, the position immediately upstream of the stop codon is -1, and the position distal to the UGA is + 4.

Chapter 4

Molecular determinants of cell adaptation in Infectious Pancreatic Necrosis Virus

ABSTRACT

Infectious pancreatic necrosis virus (IPNV) loses its virulence when passaged in cell culture. However, the molecular basis of virulence in IPNV and its adaptation to cell culture is not known. In previous study, using the cRNA based reverse genetics system developed for IPNV, we recovered the rNVI15, which shows more than 90% cumulative mortality in Atlantic salmon. The recombinant virus was serially passaged in CHSE cells nine times to obtain a tissue-culture adapted virus, rNVI15TC. Complete nucleotide and deduced amino acid sequences of this virus revealed only one amino acid substitution at position 221 (Ala to Thr) of the VP2 protein. Comparison of the replication kinetics of rNVI15 and rNVI15TC viruses in Chinook salmon embryo (CHSE) cells shows that the cell culture adapted virus had two log higher titer and produced larger plaques than rNVI15. However, when rNVI15 virus was passaged nine times in rainbow trout gonad (RTG-2) cells, no amino acid changes were detected in both segments A and B. IPNV strain Sp103 is an attenuated field isolate, which is completely sequenced in previous study (Shivappa, Song, and Vakharia, 2003). Compared with rNVI15, Sp103 exhibited more rapid growth kinetics and grew to a higher titer in both CHSE and RTG-2 cells. To determine the amino acid residues responsible for virulence and virus attenuation in cell culture, two chimeric viruses, rNVI15 Δ VP2 and rNVI15-15K Δ VP2 were generated, in

which the residues at position 217 and 247 in VP2 region of the virulent rNVI15 and rNVI15-15K viruses were replaced by the corresponding residues of the attenuated strain Sp103, respectively. These two viruses have similar replication kinetics as Sp103, indicating that residues at 217 and 247 of VP2 may be the important markers for virus adaptation and attenuation *in vitro*. A reassortant virus rNVI15-15KΔVP1, which contains rNVI15-15K segment A and Sp103 segment B, exhibited similar replication kinetics as the virulent strain, suggesting that VP1 is not involved in virus cell adaptation.

INTRODUCTION

Infectious pancreatic necrosis virus is the prototype virus of the *Birnaviridae* family. The genome of IPNV consists of two segments of double-stranded RNA, which are packaged in a nonenveloped icosahedral shell of 60nm in diameter. Segment A encodes a 106-kDa precursor protein in a single large open reading frame (ORF), which is cotranslationally cleaved by the viral encoded protease (VP4) to generate major capsid polypeptide pVP2, and VP3 (Dobos, 1995a; Duncan et al., 1987). The pVP2 (62-kDa) is further cleaved to VP2 (54-kDa) during virus maturation (Dobos, 1977). VP2 is the major outer capsid protein and is responsible for the production of type-specific neutralizing antibody (Dobos, 1995a; Nicholson, 1993). VP3 is an internal capsid protein, which binds to the virus RNA forming ribonucleoprotein core structure (Hjalmarsson, Carlemalm, and Everitt, 1999). Segment A also encodes an arginine-rich minor 17-kDa NS protein (also called VP5) from a small ORF, which precedes and partly overlaps the large ORF (Duncan et al., 1987). This protein has been detected in infected cells (Magyar and Dobos, 1994a).

Recently, Weber and coworkers have shown that VP5 is not essential for virus replication, however, its function is still unknown (Weber et al., 2001). Segment B encodes VP1, a 94-kDa protein, which is the virion associated RNA-dependent RNA polymerase (Dobos, 1995b; Duncan et al., 1991). This protein is found both as free polypeptide and covalently linked to the 5' ends of the genomic RNA segments (Calvert, 1991).

IPNV is a pathogen that causes acute, contagious disease in young salmonid fish as well as trout (Wolf, 1988). IPNVs exhibit wide host ranges, and apart from salmonids, they have been isolated from fish belonging to at least 32 different families, 11 species of mollusks and four species of crustaceans (Hill and Way, 1995). There are two distinct serogroups of IPNV. Serogroup A comprises of 9 serotypes that are pathogenic to fish, whereas serogroup B comprise one serotype, which is avirulent for fish (Hill and Way, 1995). Within the same serotype, virulence varies among the strains (Bruslind and Reno, 2000; Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). Recent studies in our laboratory have identified amino acids at positions 199, 217, 221, 247 and 500 of VP2 to be involved in virulence of IPNV. It was further narrowed down to residues 217 and 221 by comparing complete deduced amino acids sequences of nine Sp isolates that exhibit different mortality in Atlantic salmon fry (Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). However, to date, the use of chimeric viruses generated by reverse genetics to pinpoint the residues responsible for virulence has not been done.

Earlier studies have shown that virulent IPNV lose their virulence potential after serial passage in fish cells (Dorson, Castric, and Torchy, 1978; McAllister and Owens, 1986). However, the molecular basis for cell adaptation and attenuation is not known, because none of these viruses were cloned and characterized by sequencing. In a recent study, Santi and coworkers cloned and sequenced an IPNV field isolate from infected fish before and after propagation in cell cultures and observed a substitution of residues at position 221 (Ala to Thr), suggesting that this residue may be responsible for virus cell adaptation (Santi, Vakharia, and Evensen, 2003). Since IPNV field isolates exist as quasispecies, we believe it is possible that this substitution would allow this virus, which may be a minor population, to grow efficiently in cell culture (Hsu, Chen, and Wu, 1995). Therefore, to study the molecular determinants of IPNV cell adaptation, a cloned virus generated by the reverse genetic approach would be an ideal candidate. Passage of recombinant virus in cell culture and characterization of its genomic sequence would allow one to identify markers of cell adaptation.

In this study, we serially passaged a recovered rNVI15 virus, derived from cloned DNA, in cell cultures and identified residues involved in cell adaptation during passage in CHSE cell. Furthermore, we generated chimeric viruses between the attenuated strain Sp103 and virulent strains rNVI15 and rNVI15-15K, by substituting two residues in rNVI15 or rNVI15-15K with corresponding residues of Sp103 VP2 or generating a reassortant virus containing the VP1 protein of Sp103. By characterizing the recombinant viruses in two different cell lines, we identified specific residues involved in efficient viral replication, plaque phenotype and possible attenuation in cell culture.

MATERIALS AND METHODS

Cells and viruses. Chinook salmon embryo cells (CHSE-214 ATCC CRL-1681) were maintained at 15°C in minimal essential medium containing Hanks' salts and supplemented with 10% fetal bovine serum (FBS) and used for propagation of the viruses. Rainbow trout gonad cells (RTG-2 ATCC CCL-55) were grown in L-15 medium supplemented with 10% FBS at 15°C. Generation of rNV115, rNV115-15K and rNV115-ΔVP5 viruses has been described in previous study (Chapter 3). Sp103 is an attenuated strain of the IPNV-Sp serotype, which had been sequenced earlier (Shivappa, Song, and Vakharia, 2003). The virus was subjected to two-rounds of plaque purification before propagation once in CHSE cells to obtain viral stock. The nucleic acid of the virus was sequenced completely to ensure no inadvertent mutation occurred due to cell adaptation.

Construction of full-length cDNA clones. All manipulations of DNAs were performed according to standard protocols (Sambrook, Fritsch, and Maniatis, 1989). Construction of full-length cDNA clones of pUC19NV115A or pUC19NV115-15K was described earlier (Chapter 3). It encodes all of the structural proteins (VP2, VP3 and VP4) and VP5 (Fig. 4-1). The 5' - end of Sp segment A was cloned by RT-PCR as described previously, the amplified fragment were cloned into pCR2.1 to obtain the plasmid pCRSp103A5'. Plasmid pUC19NV115-15KΔVP2 and pUC19NV115ΔVP2 was prepared by replacing a *BstEII-KpnI* fragment in plasmid pUC19NV115-15K and pUC19NV115A respectively, with the respective *BstEII-KpnI* fragments derived from plasmids pCRSp103A5'.

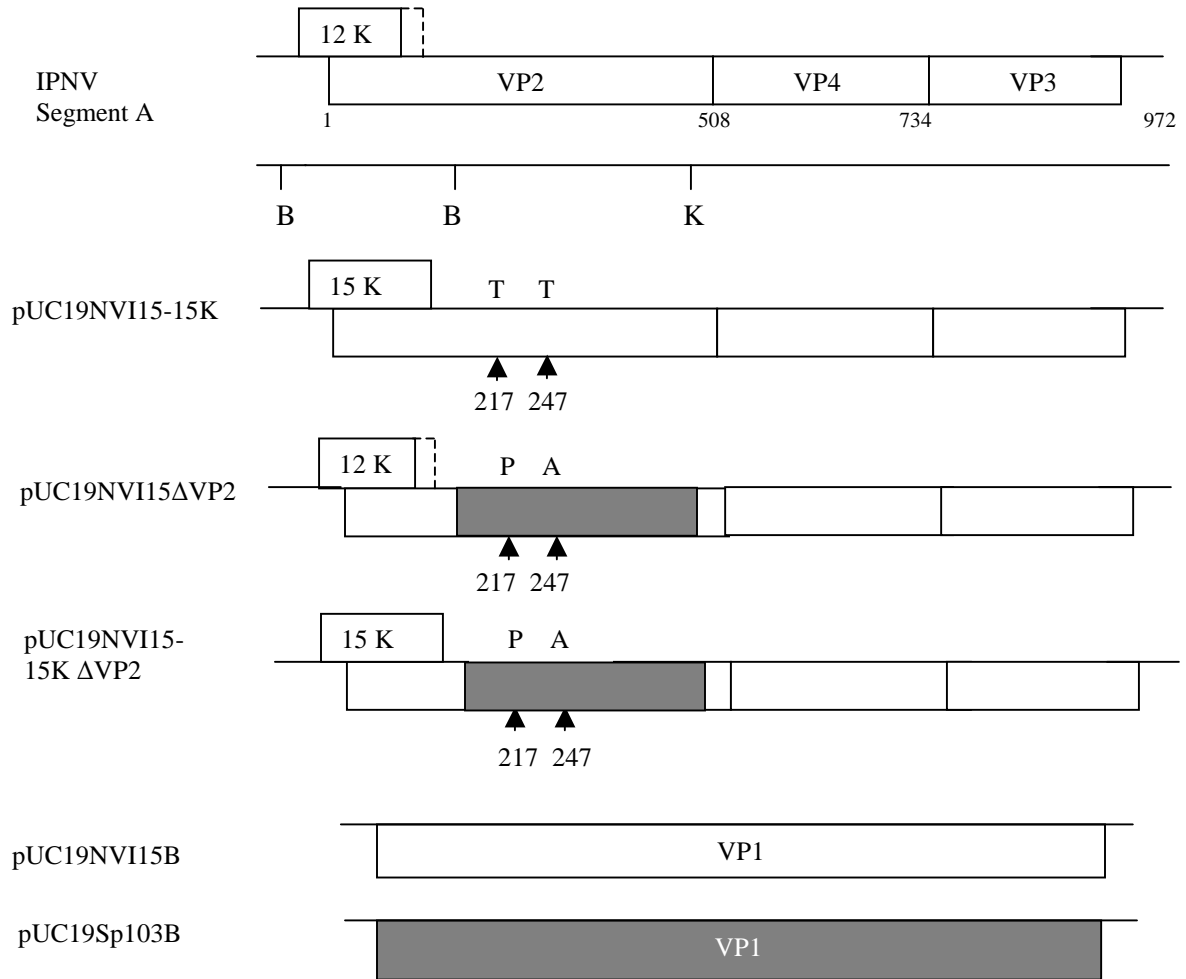


Fig. 4-1. Schematic presentation of IPNV cDNA constructs for the generation of plus-sense RNA transcripts using T7 RNA polymerase. A map of the IPNV genome segments A and B, with its coding capacity, is shown at the top. The open boxes depict the coding region of NVI15 (with 12-kDa VP5), whereas the solid boxes represent the coding regions of the Sp103 strain. Selected restriction sites, important for the construction of chimeric cDNA clones of segment A, are shown. B, *BstEII*, K, *KpnI*. All of the constructs contain a T7 polymerase promoter sequence at the 5' end.

Plasmids pUC19NVI15B and pUC19Sp103B, which comprises the full-length segment B of NVI15 and Sp103 respectively, were obtained as described before (Chapter 3).

DNA from the above-mentioned plasmids was sequenced by the dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystem), and the sequence data were analyzed using PC/Gene (Intelligenetics) software. The integrity of the full-length constructs was tested by an *in vitro* transcription and translation coupled reticulocyte lysated system using T7 RNA polymerase (Promega Corp.).

Transcription and transfection of synthetic RNAs. Plasmid pUC19NVI15-ΔVP2, pUC19NVI15-15KΔVP2 and pUC19NVI15-15K were linearized by *Pst*I, whereas pUCNVI15B and pUCSp103B was digested by *Bgl*III. Further treatment was followed as described previously (Yao and Vakharia, 1998). The linearized DNA was used to produce *in vitro* transcripts with T7 mMessage mMachine kit (Ambion) according to manufacturer's instructions. Briefly, approximately 3μg linearized DNA template was added to the transcription reaction mixture (20μl), containing 40mM Tris-HCl (pH 7.9), 10mM NaCl, 6mM MgCl₂, 2mM spermidine, 0.5 mM ATP, CTP and UTP each, 0.1 mM GTP, 0.25 mM cap analog [m7G (5') ppp (5') G], 120 units of RNasin, 150 units T7 RNA polymerase, and incubated at 37°C for 90 minutes.

CHSE-214 cells grown to 90% confluency in T-25 flask were transfected with cRNA of both segments as described previously (Yao and Vakharia, 1998). Briefly, cells were washed once with phosphate-buffered saline (PBS). Three milliliters of OPTI-MEM

I (GIBCO/BRL) were added to the monolayer, and the cells were incubated at room temperature for 1 hr. Simultaneously, 0.15ml of OPTI-MEM I was incubated with 12.5 µg of lipofectin reagent for 45 min in a polystyrene tube at room temperature. Equimolar amounts of RNA transcripts of segments A and B ($\approx 8\mu\text{g}$ each) were resuspended in 0.15 ml of diethyl pyrocarbonate-treated water, were added to the OPTI-MEM/Lipofectin mixture, mixed gently, and incubated on ice for 5 min. After removing the OPTI-MEM I from the monolayers in T-25 flask and replacing it with a fresh 1.5 ml of OPTI-MEM, the nucleic acid-containing mixture was added drop-wise to the CHSE cells and swirled gently. After 3 hr of incubation at room temperature, the mixture was replaced with minimal essential medium containing Hanks' salts and 10% FBC (without rinsing the cells). The cultures were incubated at 15°C for 5 days, and the cell supernatant was harvested by freeze-thawing twice, and passaged onto fresh CHSE monolayers. Cytopathic effect (CPE) usually was visualized at 4 days p.i. The flasks were freeze-thawed twice, the supernatant were kept in -70 °C as virus stock used in this work. Recovered viruses were verified by RT-PCR, using the specific primers for IPNV segment A.

Characterization of recovered IPNV. To determine the specificity of the recovered viruses, the CHSE cells were infected with the recovered viruses and the infected cells were analyzed by immunofluorescence assay with rabbit anti-IPNV polyclonal serum, as described (Yao and Vakharia, 1998). To examine structural protein expression by the recovered chimeric viruses, the viruses were purified by sucrose gradient centrifugation

and analyzed by Western blotting. To further characterize the recovered virus, RT-PCR was performed on the chimeric virus with the appropriate primer pair, used to produce the original clone. The resulting PCR product was directly sequenced as described above using one of the primer pair specific for IPNV segment A.

Sequence of serially passed recombinant viruses. Viruses were serially passaged in CHSE or RTG-cells at MOI of 0.001. At different passages, the viral RNA was extracted and subjected to RT-PCR. The PCR product was directly sequenced as described earlier. After nine passages in CHSE cells, the cell-adapted rNVI15 virus was plaque purified twice, before passage in CHSE one more time to make a stock. Entire segments A and B of the cell-adapted virus were sequenced, using methods and primers mentioned in previous study (Chapter 3). For other recombinant viruses, only VP2 region was sequenced.

Growth curve and plaque assay. To analyze the growth characteristics of IPNVs, confluent CHSE cells or RTG-2 cells (in 35-mm dish) were infected with the recombinant virus stocks at MOI of 1. Infected cell cultures were removed and stored at -70°C at different time intervals; the supernatants were centrifuged and titrated on CHSE cells by plaque assay. Briefly, the confluent monolayers of CHSE cells, grown in six-well plates, were infected with serially diluted supernatants from virus stock. After 1hr incubation at 15°C, the cells were washed once by PBS and overlaid with 0.6% SeaPlaque Agrose (Difco) in Eagle MEM medium containing 5% FBS and 1% L-glutamine. After 3 days of incubation at 15 °C, the overlays were removed and the cells

were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature. After rinsing the cells with distilled water, the plaques were counted.

RESULTS

Sequence of cell-adapted IPNV variants. To determine the molecular basis of virus adaptation in cell culture, and the possible mechanism for virus attenuation, a recombinant virus rNVI15, which caused more than 90% mortality in Atlantic salmon smolts, was serially passaged in CHSE cells. At the indicated passage, viral RNAs were extracted, amplified by RT-PCR and their products were directly sequenced. Comparison of the deduced amino acid sequences in VP2 region of virus at different passages revealed substitution of a single amino acid residue at position 221 from Ala to Thr, due to a point mutation at nucleotide position 779 (G to A) in segment A (Fig. 4-2). After the fourth passage, about half of the virus population has Thr at position 221, whereas after the ninth passage all the virus population has Thr at that position. After two rounds of plaque purification of the ninth passage virus, we obtained the cell-adapted virus, rNVI15TC, which has a Thr at 221 position in VP2. Complete deduced amino acid sequence analyses of segments A and B of rNVI15 and rNVI15TC exhibited only one amino acid substitution at position 221 of the polyprotein (encoded by segment A), suggesting an important role of this residue in cell culture adaptation to CHSE cells. Not

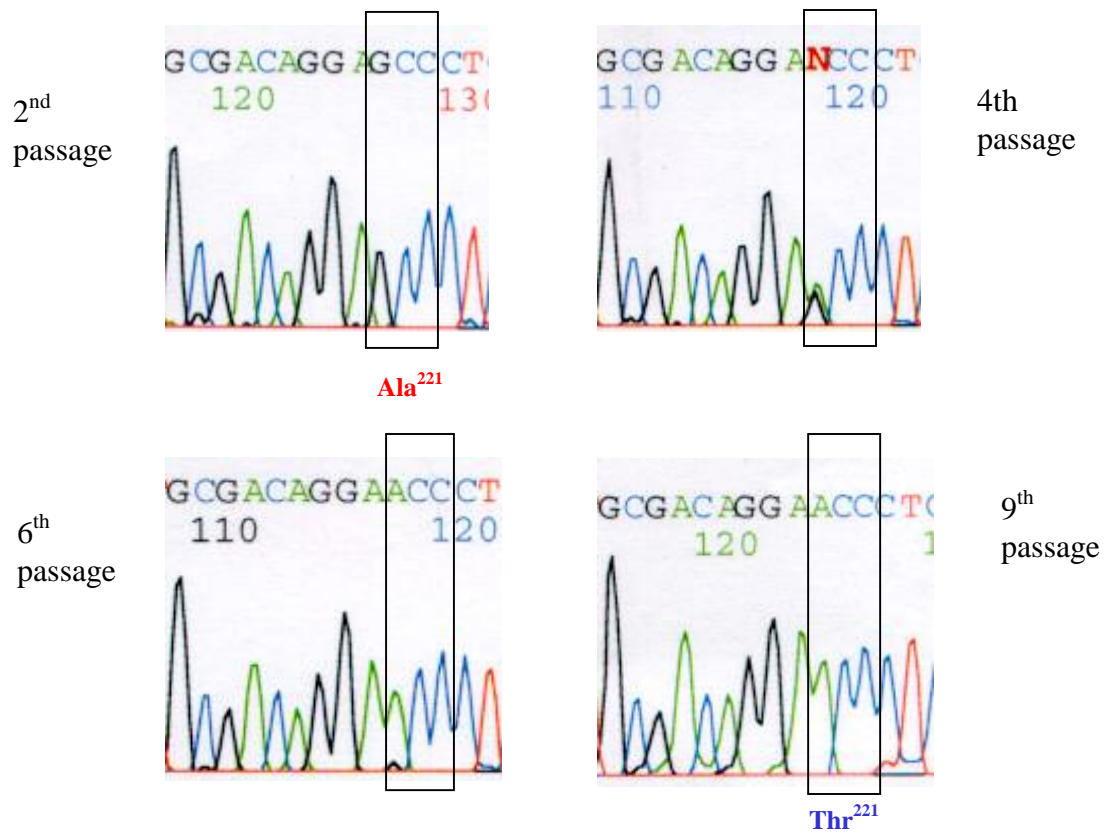


Fig. 4-2. Chromatographs showing DNA sequences of RT-PCR products obtained from different IPNVs. Recombinant rNVI15 virus was serially passaged in CHSE cells. At indicated passage, the virus was harvested, viral dsRNA extracted, and subjected to RT-PCR using a pair of VP2-specific primer. The RT-PCR products were purified and directly sequenced.

a single nucleotide substitution was detected in the 5' - and 3' - non-coding regions of both segments A and B.

Similarly, rNVI15 virus was serially passaged in RTG-2 cells. However, the deduced amino acid sequence analyses of the genomic segments A and B of the ninth passage virus did not show any amino acid substitution. To further confirm these findings, other two cloned viruses, rNVI15-15K and rNVI15- Δ VP5, were also serially passaged in CHSE or RTG-2 cells. Both of these viruses gradually acquired the amino acid substitution at position 221 (Ala to Thr) during passage in CHSE cells, but not in RTG-2 cells. These results indicate that amino acid substitution at position 221 (Ala to Thr) occurs during virus adaptation to CHSE cells but not in RTG-2 cells, and the selection of this mutation is not affected by the presence or absence of VP5.

One-step growth curve of cell-adapted IPNV variants. To determine if there are differences in the growth properties of the parental rNVI15 virus and the cell-adapted virus, a one-step growth curve analysis was carried out in both CHSE and RTG-2 cells. rNVI15TC caused CPE in CHSE cells at 18 hr post-infection (p.i.), and reached maximal virus production (1×10^8 PFU/ml) at 40 hr p.i. (Fig. 4-3 A), whereas, rNVI15-infected CHSE cells developed CPE at 40 hr p.i., and virus had a titer of 3×10^6 PFU/ml at 48 hr p.i.. Both rNVI15 and rNVI15TC viruses did not exhibit any difference in their replication kinetics in RTG-2 cells (Fig 4-3B).

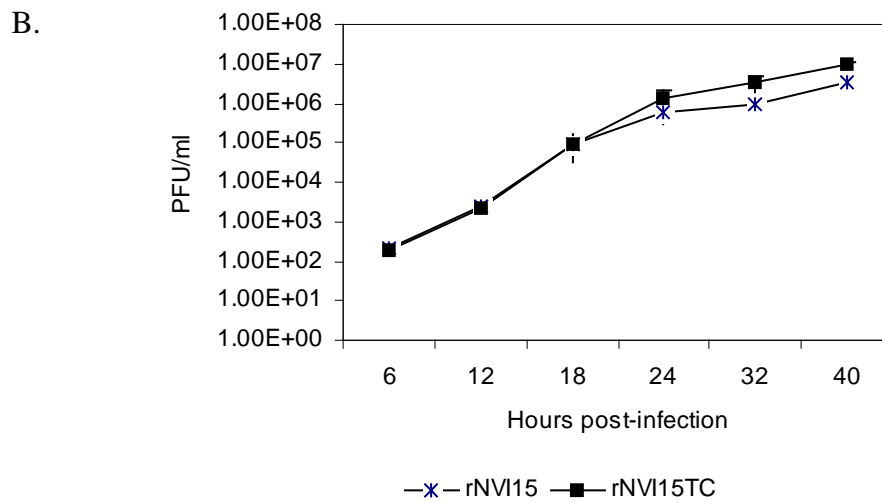
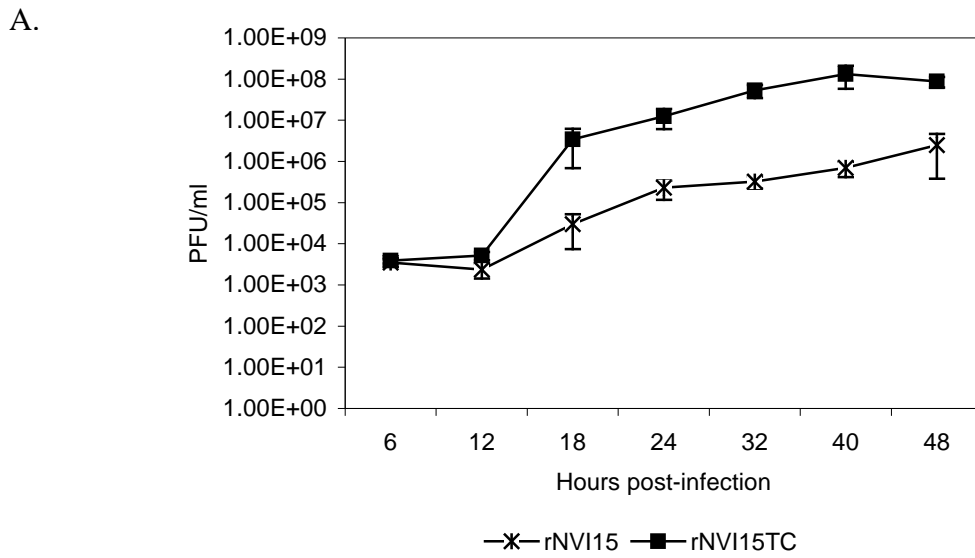


Fig. 4-3. Replication kinetics of recombinant rNVI15 and rNVI15TC viruses. Monolayers of CHSE cells (A) or RTG-2 cells (B) were infected with the indicated viruses at MOI of 1 and harvested at the indicated time points and infectious titers were determined by plaque assay.

Plaque phenotype of IPNV variants. To determine the role of this single amino acid substitution in virus plaque phenotype, rNVI15 and rNVI15 TC viruses were subjected to plaque assay on both CHSE and RTG-2 cells, and analyzed 3 days post-infection. As shown in Fig. 4-4, rNVI15TC virus produces larger plaques than its parental virus (rNVI15), which correlates with the enhanced growth kinetics of this virus in CHSE cells. To confirm that the large-sized plaques are formed due to cell adaptation in CHSE cells, we evaluated the plaque size of rNVI15 virus after different passages in CHSE cells. Our results indicated that after fourth passage, large-plaque variants begin to emerge and become predominant after six passages (data not shown), which corresponds to the sequence data presented earlier in Fig. 4-2. However, both viruses produce similar sized plaques on RTG-2 cells (Fig 4-4).

Construction of chimeric and reassortant viruses. Unlike rNVI15, Sp103 is an attenuated strain of IPNV, which contains Ala at position 221 of VP2, and it encodes a 15-kDa VP5 protein instead of a 12-kDa VP5. Comparison of the deduced amino acid sequences of Sp103 and NVI15 revealed only four amino acid substitutions at positions 217 (Thr to Pro), 247 (Thr to Ala), 500 (Trp to His) and 802 (Arg to His) in polyprotein, and two residues at positions 125 (Ile to Met) and 240 (Arg to His) in VP1. In order to pinpoint the residues involved in virus attenuation, we constructed two chimeric viruses rNVI15 Δ VP2 and rNVI15-15K Δ VP2, in which the residues at positions 217 and 247 were substituted by Thr to Pro and Thr to Ala, respectively. The only difference between these two chimeric viruses is that rNVI15 Δ VP2 encodes a 12-kDa VP5 ORF, whereas rNVI15-15K Δ VP2 encodes a 15-kDa VP5 ORF, as in Sp103. To assess the possible

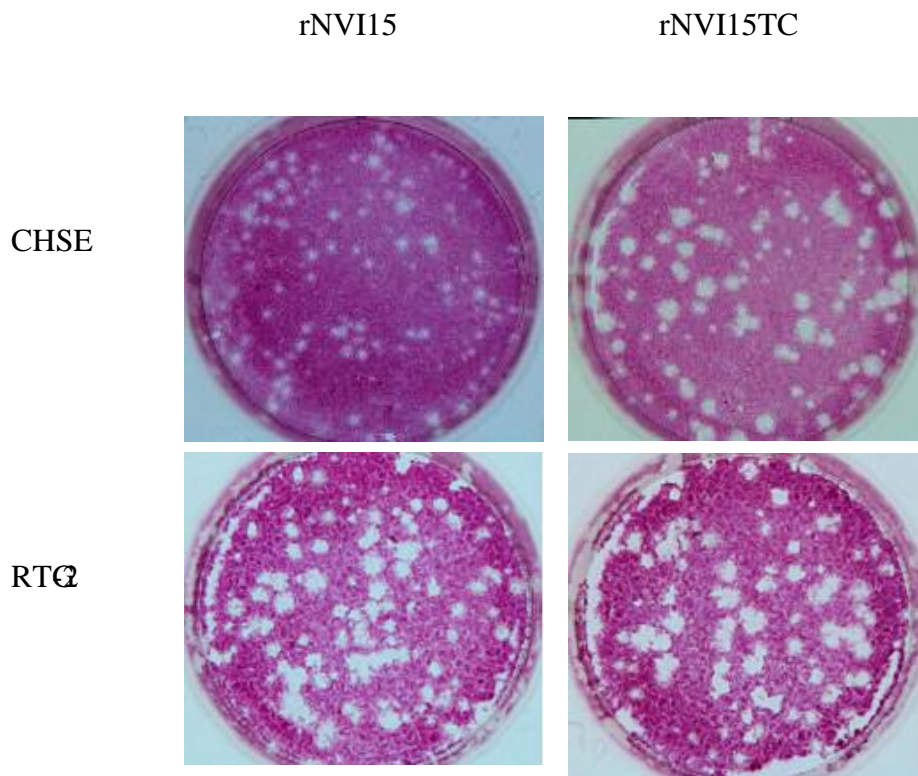
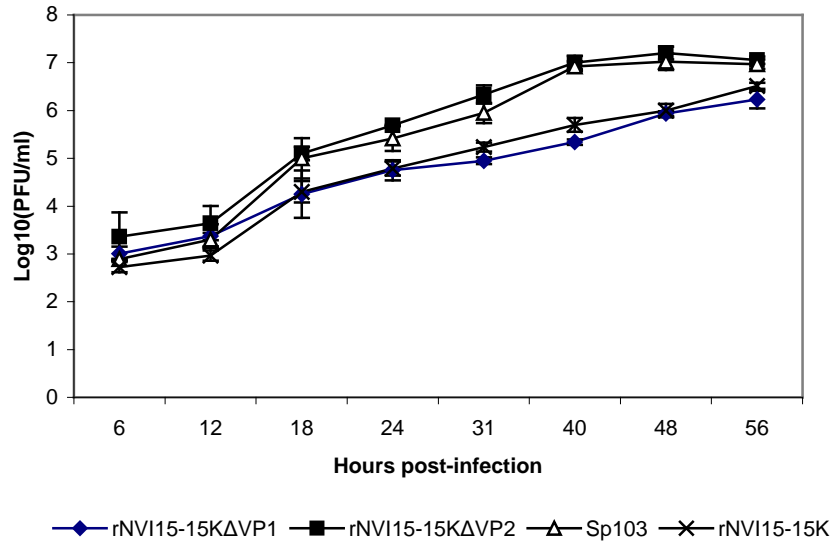


Fig. 4-4. Plaque morphology of recombinant rNVI15 and rNVI15TC viruses in different cell-lines. CHSE or RTG-2 cells were infected with either rNVI15 or rNVI15TC viruses by absorption for 1 hr. Cells were rinsed and immobilized with overlay medium containing 0.6% agarose. Three days post infection, the cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature, followed by rinsing with distilled water.

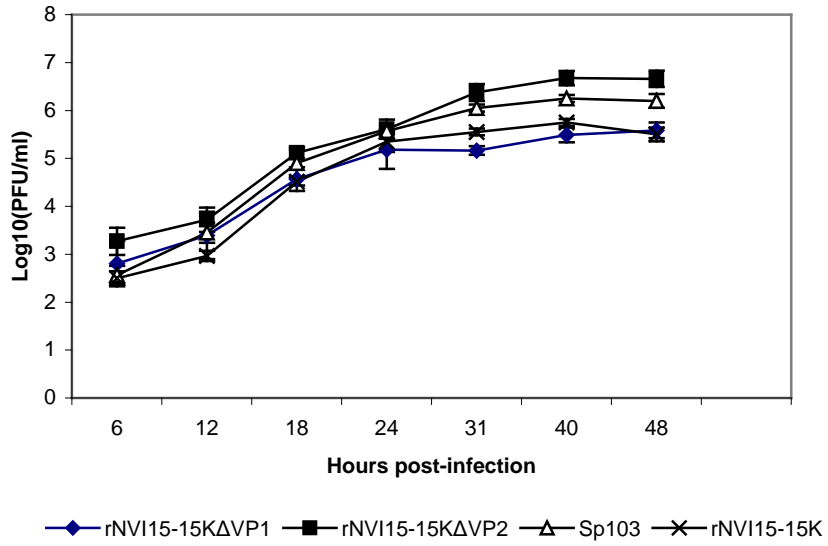
involvement of VP1 in virulence, a reassortant virus (rNV115-15KΔVP1) was generated by transfection of CHSE cells with combined plus-sense transcripts derived from pUC19NV115-15K and pUC19Sp103B (Fig 4-1). The genomic RNAs of the recovered viruses were analyzed after RT-PCR amplification and the sequence analysis of the RT-PCR products confirmed the expected mutations in the VP2 and VP1 regions of the chimeric and reassortant viruses. Furthermore, complete nucleotide sequences of segment A of rNV115ΔVP2 and rNV115-15KΔVP2 were determined, which did not exhibit any other nucleotide substitutions.

Characterization of chimeric IPNVs *in vitro*. To investigate whether VP2 and VP1 proteins are involved in the efficiency of viral replication *in vitro*, CHSE cells and RTG-2 cells were infected with each virus at MOI of 1, and the virus titer was analyzed by plaque assay. Figure 4-5A depicts the growth curve of each virus in CHSE cells at different time points post-infection. The results indicate that Sp103 virus replicated faster and had a titer that is one log higher than rNV115-15K. Recombinant rNV115-15KΔVP2 virus grew to a similar titer as the Sp103 virus, whereas rNV115-15KΔVP1 virus exhibited similar replication kinetics as the rNV115-15K. In RTG-2 cells, both rNV115-15KΔVP2 and Sp103 viruses grew to higher titer than rNV115-15KΔVP1 and rNV115-15K viruses (Fig. 4-5B). Moreover, rNV115ΔVP2 virus replicated faster and grew to a higher titer than the parental rNV115 virus in both cell lines (Fig. 4-5C, D). These results demonstrate that the amino acid substitution at positions 217 (Thr to Pro) and 247 (Thr to Ala) improves viral growth in both cell lines.

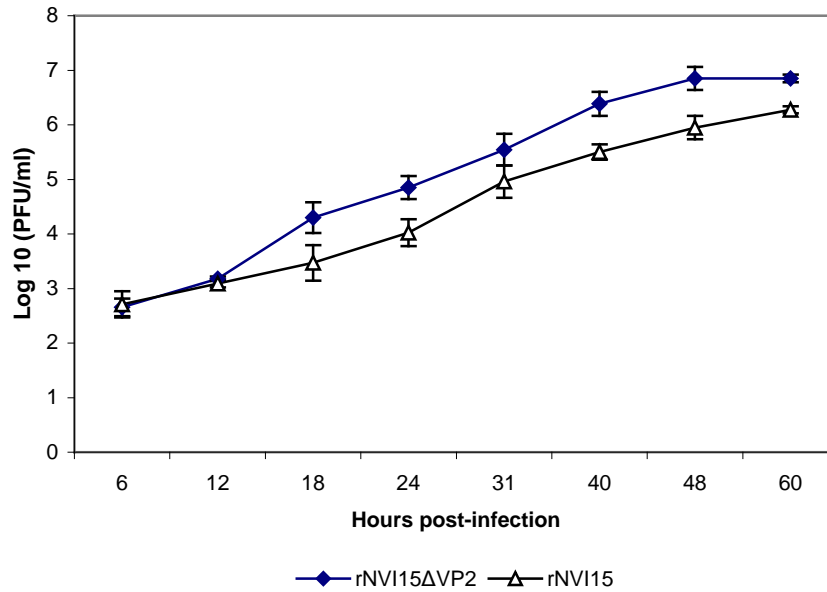
A.



B.



C.



D.

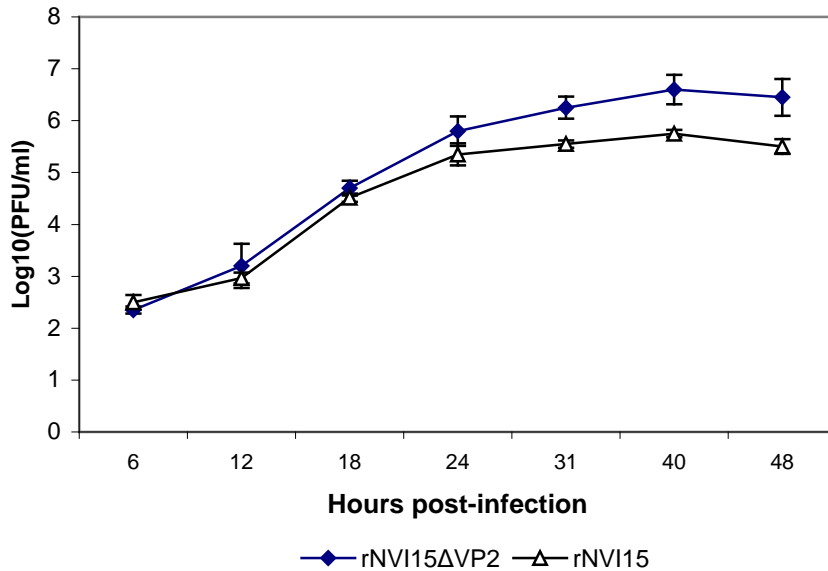


Fig. 4-5. Replication kinetics of various recombinant IPNVs in cell culture. Monolayers of CHSE cells (A, C) or RTG-2 cells (B, D) were infected with the indicated viruses at MOI of 1 and harvested at the indicated time points and infectious titers were determined by plaque assay.

Identification of the residues after serial passage of chimeric viruses in cell culture.

To identify possible amino acid substitutions involved in cell adaptation, the chimeric viruses were serially passaged in either CHSE or RTG-2 cells at an MOI=0.001. Viral RNAs were extracted from these viruses, amplified by RT-PCR and their products were directly sequenced using a pair of primers specific for VP2 hypervariable region. Our results indicate that there are no adaptation mutations in this region, except for substitution of a residue from Ala to Thr at position 221. The occurrence of this substitution in the virus population is shown in Table 4-1. In rNVI15-15K Δ VP1 virus, there is a rapid substitution of a residue at position 221 (Ala to Thr) after the fourth passages, which is similar to what was observed for rNVI15 and rNVI15-15K viruses. In rNVI15 Δ VP2 and rNVI15-15K Δ VP2 viruses, the substitution at position 221 (Ala to Thr) could not be detected until the ninth passage, and almost half of virus population still had Ala at that position. However, when these cloned viruses were serially passaged into RTG-2 cells, not a single amino acid substitution was detected in the hypervariable region.

DISCUSSION

Since RNA polymerases lack proofreading ability, the replication of RNA viruses is characterized by high mutation rates, which leads to rapid adaptation to their growth environment. This has historically been exploited to generate live attenuated vaccines. On the other hand, full understanding of the molecular processes of virus adaptation to particular host cells would ensure that no unrecognized adaptive mutations occur during

Table 4-1. Presence of amino acid residue at position 221 in VP2 region of IPNV after serial passage in CHSE cells.

Viruses \ Passages	2	*4	6	9
rNVI15	Ala	Ala/Thr	Thr/Ala	Thr
rNVI15-15K	Ala	Ala/Thr	Thr/Ala	Thr
rNVI15 Δ VP2	Ala	Ala	Ala	Thr/Ala
rNVI15-15K Δ VP2	Ala	Ala	Ala	Thr/Ala
rNVI15-15K Δ VP1	Ala	Ala/Thr	Thr/Ala	Thr

* The chromatograms from sequences obtained after indicated passages of IPNV in CHSE cells often have a double peak at nucleotide position 779, representing a mixture of two residues (see Fig. 4-2). The dominant amino acid is named first in this table.

propagation of virus in cell culture, which could be a source of misleading results and erroneous conclusions regarding the viral life cycle in the natural host (Mandl et al., 2001).

Previous studies has shown that IPNV field isolates tend to lose their virulence after serial passage in cell culture (McAllister and Owens, 1986). However, there is no sequence data to reveal the molecular basis of this process. Comparison of the deduced amino acid sequences of the virulent and attenuated Sp strains revealed specific amino acid substitutions at positions 219, 217, 221, 247 in the hypervariable region of VP2 protein (Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). However, there are additional amino acid substitutions that are scattered in VP2, VP3 and VP1 proteins. Therefore, the precise residues involved in virulence and cell tropism could not be identified. In this study, we used recombinant viruses generated by reverse genetics approach to study the mechanism of IPNV adaptation in cell culture. We demonstrate that specific residues in VP2 region are involved in viral adaptation to cell culture, and these residues are also responsible for the plaque phenotype.

RNA viruses often have the ability to adapt quickly to their environment. Alterations at vial entry steps, including receptor recognition, virus attachment and/or penetration, membrane fusion, could affect virus growth and tissue tropism, which are the major determinants of virus pathogenesis (Lee et al., 2002). Therefore, it is not surprising to know that for enveloped virus, mutation acquired during tissue adaptation is often located in the envelope proteins. For example, McMinn and coworkers found that adaptation mutations in E protein of Murray Valley encephalitis virus were selected by

serial passage in cell culture, which are proposed to affect virus binding to target cells (McMinn, Marshall, and Dalgarno, 1995). Similarly, mutations in tick-borne encephalitis virus E protein were acquired during cell culture adaptation, which confer increased binding to cell surface heparan sulfate but results in reduced virulence in animal (Mandl et al., 2001). In nonenveloped viruses of eukaryotes, adaptation mutation usually occurs in outer capsid proteins, which function as viral cell binding and receptor recognition proteins. For example, a mutation in VP1 protein of chicken anemia virus was selected after extensive passage in cell culture (Todd et al., 2002), and in poliovirus, molecular determinants of mouse adaptation were identified to be on the virion surface structure (Jia et al., 2001).

In our study, the adaptation mutation was present in the VP2 protein. There are two lines of evidence, which indicate that substitution of a residue at position 221 (Ala to Thr) is responsible for rapid adaptation of the viruses to CHSE cells. First, the adaptation mutation at position 221 (Ala to Thr) was present in three independent CHSE cell-passaged IPNV viruses, rNVI15, rNVI15-15K and rNVI15- Δ VP5. The cell-adapted virus rNVI15TC replicates much faster and produces larger plaques than the parental virus, rNVI15. Secondly, the recombinant viruses rNVI15 Δ VP2, rNVI15-15K Δ VP2, and rNVI15-15K Δ VP1, which possess Ala at position 221, also acquired this adaptation mutation after several passage in CHSE cells. Adaptation mutation at position 221 was rapidly acquired by rNVI15, rNVI15-15K, rNVI15-15K Δ VP1 viruses, rather than rNVI15 Δ VP2 and rNVI15-15K Δ VP2 viruses, indicating that in order to obtain optimal growth in CHSE cells, the virus needs to acquire this specific mutation, however, the

occurrence of the adaptation mutation is delayed in the later two viruses that have better fitness in CHSE cells. The substitution of Ala to Thr may facilitate efficient binding of the virus to receptor for CHSE cells. Like other non-enveloped animal viruses, IPNV is internalized by receptor-mediated endocytosis, and VP2 appears to be the cell-attachment protein (Dobos, 1995a; Granzow et al., 1997; Kuznar et al., 1995). For IBDV, it was recently shown that cell culture adaptation mutations also reside in the VP2 region, which facilitates virus entry to the cells (Liu, 2003).

It is interesting to know that when the recombinant viruses were serially passaged nine times in RTG-2 cells, not a single amino acid substitution was detected in both segments A and B. However, extensive passage of the viruses in RTG-2 cells could yield attenuated mutants, as reported earlier by other investigators (Dorson, Castric, and Torchy, 1978). CHSE cell adapted virus rNVI15TC grew to a similar titer as its parental viruses in RTG-2 cells, indicating that Ala at position 221 does not enhance virus growth in RTG-2 cells. Blaney and coworkers observed that dengue virus adaptation mutations only occur after serial passage into Vero cells but not in C6/36 cells. Therefore, they use C6/36 to propagate the virus to ensure no advent cell adaptation mutations are introduced in the viral genome (Blaney et al., 2003). Based on our findings for IPNV adaptation, RTG-2 would be a better cell line for propagating virulent viruses to prevent the emergence of cell-adapted mutants.

Although absence of Ala at position 221 (Ala 221) in cell-adapted mutants may reduce its virulence *in vivo*, it is not the only determinant of viral virulence, because

Sp103 virus also possesses Ala at that position, but it is avirulent in fish. Therefore, residues other than Ala 221 must play a role in virulence and viral pathogenesis. Sp103 virus grew to higher titer in both CHSE and RTG-2 cells when compared with rNVI15 and rNVI15-15K. Recombinant rNVI15 Δ VP2 and rNVI15-15K Δ VP2 viruses, which have only two residue substitutions at positions 217 (Thr to Pro), and 247 (Thr to Ala) in the polyprotein, exhibited similar grow curve as Sp103 in both cell lines. These results suggest that these two substitutions enhance viral replication *in vitro*, and they may be the involved in viral attenuation.

The possible explanations for the amino acid substitution at 221 only enhances virus grow in CHSE cells, whereas the substitutions at 217 and 247 could improve virus growth in both cell lines is that residues at 217 and 247 may be involved in binding to the common receptors presenting in both CHSE and RTG-2 cells, but the residues at 221 may recognize a specific receptor that is required for virus enter in CHSE cells but not in RTG cells. Imajoh and coworkers have shown that a marine birnavirus, which belongs to the same genus as IPNV, could bind to a common receptor that is present in different fish cell lines, but the virus needs to bind another receptor to penetrate the cells, which is not expressed on the resistant cell line (Imajoh, Yagyu, and Oshima, 2003). Therefore, further characterization of the cellular receptors for IPNV in different cells lines and crystallization of virus particles would shade some light on the mechanism of virus-cell interaction.

By making reassortant viruses between two serotypes, VR299 and Ab, Sano and coworkers demonstrated that virulence of IPNV is associated with genomic segment A (Sano et al., 1992). Using the same approach, they also found that plaque size is dependent on segment A and it is not associated with virulence (Sano, Okamoto, and Sano, 1994). Within the same serotype, there are differences in virulence and pathogenicity among different isolates, although they share the extreme homology sequences (Bruslind and Reno, 2000; Hill, 1982; Silim, Elazhary, and Lagace, 1982). To date, there is no convincing evidence to show that within the same serotype whether VP1 carries the determinants for cell adaptation. In lymphocytic choriomeningitis virus (LCMV), a single amino acid change in the viral polymerase (residue 1079) is a major determinant of macrophage tropism and virulence (Matloubian et al., 1993). Recent studies in our laboratory have shown that VP1 of IBDV affect viral replication kinetics both *in vitro* and *in vivo* (Brandt et al., 2001; Liu, 2003b). Therefore, to exclude the possibility that VP1 of IPNV is involved in virulence and cell adaptation, a reassortant virus was generated in this study. Our results indicate that VP1 is not involved in virus adaptation to cell culture, and it does not carry the determinants for the virus plaque phenotype.

Cell-adapted viruses often confer reduced virulence in natural host animal. It would be of great interest to assess virulence of these cell-adapted and chimeric viruses in fish to identify specific residues important for virulence of IPNV.

Chapter 5

IPNV-induced apoptosis is caspase dependent, and requires NF- κ B activity

ABSTRACT

Infectious pancreatic necrosis virus (IPNV) is an economically important fish pathogen that causes a contagious disease in rainbow trout as well as Atlantic salmon. It has been shown that IPNV infection induces apoptosis in salmon embryonic cells, and apoptosis precedes necrosis (Hong et al., 1998). However, the signal pathways and the nature of the apoptosis have not been well studied. This is the first report of IPNV causing apoptosis in rainbow trout cells (RTG-2). Based on virus single round replication, DNA fragmentation was detected in late stages of the virus replication cycle and caspase-3 was activated during virus infection. A broad-spectrum caspase inhibitor, zVAD-FMK (N-benzylozycarbonyl-Val-Ala-Asp-fluoromethyl ketone) partially inhibits virus-induced DNA fragmentation, contributing evidence that cellular caspases facilitate apoptosis induced by IPNV. Furthermore, after IPNV infection, a transcription factor, NF- κ B is activated. A proteasome inhibitor, MG132, and an antioxidant, PDTC (pyrrolidinedithiocarbamate) inhibit virus-induced NF- κ B activation and apoptosis, but these inhibitors do not affect virus replication. Virus-induced NF- κ B activity is higher in interferon-pretreated cells compared with untreated cells. Our findings suggest that NF- κ B is essential for IPNV induced apoptosis, and potentially plays an important role in interferon-induced antiviral states.

INTRODUCTION

Apoptosis, also called programmed cell death, is a process in which cells activate intracellular death pathways and undergo systematic self-destruction in response to a wide variety of stimuli. It is defined by morphological changes distinct from necrosis, that include: membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and formation of apoptotic bodies (Barber, 2001; Razvi and Welsh, 1995). Cellular cysteine proteases, termed as caspases, are activated during apoptosis and constitute the effective executioners for the selective cleavage of a number of key cellular proteins involved in maintaining the structural and functional integrity of cells (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). Caspases can be functionally divided into initiator caspases, such as caspases 8 and caspase 9, and effector caspases, such as caspase 3, 6, and 7. There are two well-defined pathways of caspase activation cascades that regulate apoptosis: one is initiated from the cell surface death receptor and depends upon caspase 8 activation; the other is triggered by changes in the mitochondria and requires caspase 9 activity (Hengartner, 2000).

The nuclear transcriptional factor kappa B (NF- κ B) is thought to be a central player in apoptosis regulatory pathways. Schlezinger and coworkers first identified NF- κ B in fish, which could bind to the consensus binding sequence of human NF- κ B and be activated by aryl hydrocarbon receptor agonists (Schlezinger et al., 2000). In quiescent cells, NF- κ B is held in the cytoplasm by association with its inhibitor, I κ B (Bauerle and Baltimore, 1988). Upon receipt of various stress signals, such as lipopolysaccharide, tumor necrosis factor, interleukin (IL)-1, and viral dsRNA, I κ B is phosphorylated by an

I κ B kinase. The phosphorylated I κ B becomes ubiquitinated and is subsequently degraded by proteasomes. Once the inhibitory I κ B is destroyed, NF- κ B is free to enter the nucleus, where it activates the transcription of target genes, which regulate inflammation and immune responses, viral replication, nitric oxide production, cell-cell interactions, apoptosis, and proliferation (Janssen-Heininger, Poynter, and Baeuerle, 2000). NF- κ B confers a survival signal to many cells, such as in cells treated with TNF- α (Beg and Baltimore, 1996). NF- κ B is also reported to potentiate apoptosis (Connolly et al., 2000; Grimm et al., 1996; Lin et al., 1995). Some proapoptotic proteins, such as p53, caspase-1, and Fas L, are regulated by NF- κ B, and contain NF- κ B response elements in their promoters (Connolly et al., 2000). Oxidative stresses are common NF- κ B stimuli. Mitochondria are the major source of reactive oxygen species (ROS) and production of ROS is strictly controlled by the mitochondria system (Manna and Aggarwal, 1999; Pahl and Baeuerle, 1995). The unbalanced production of ROS may damage the mitochondria membrane and eventually contributes to cell death by triggering the activation of NF- κ B (Baeuerle and Henkel, 1994). NF- κ B plays an important role in interferon-induced antiviral state. Exposure to dsRNA activates NF- κ B via the interferon-mediated dsRNA-dependent protein kinase R (PKR) (Der et al., 1997; Maran et al., 1994; Yang et al., 1995).

Apoptosis induced by viral infection is mainly a defense mechanism, because it curtails the infection cycle and prevents neighboring cells from being infected. A number of viruses are known to encode anti-apoptotic molecules that suppress or delay apoptosis and consequently keep host cells alive for the production of sufficient quantities of

progeny (Boyed et al., 1994; Hay and Kannourakis, 2002; Henderson et al., 1993; Roulston, Marcellus, and Branton, 1999). On the other hand, certain viruses may depend on apoptosis to facilitate the release and spread of virus progeny or to evade host inflammation and immune responses (Razvi and Welsh, 1995; Roulston, Marcellus, and Branton, 1999). A complete understanding of the mechanisms responsible for the induction and inhibition of apoptosis by viruses would help us develop new strategies to control viral infections.

Infectious pancreatic necrosis virus (IPNV) is the prototype of the family *Birnaviridae*, and its genome consists of two segments of double-stranded (ds) RNA. (Dobos, 1995a). IPNV causes an acute contagious disease of young salmonid fish as well as trout. Pancreatic and intestinal damage had been observed in IPNV-infected fish (Wolf, 1988). Previous studies have shown that IPNV can cause both necrosis and apoptosis in Chinook salmon embryo (CHSE) cells, and apoptosis precedes necrosis (Hong et al., 1998). These investigators also found that IPNV infection up-regulates pro-apoptotic factors but down-regulates anti-apoptotic factors (Hong, Hsu, and Wu, 1999; Hong and Wu, 2002). Apoptosis has been detected in infected trout muscle cells but not in pancreatic cells (Eleouet et al., 2001). However, the role of apoptosis in IPNV infection and pathogenesis has not been well established.

In this study, we investigate IPNV-induced apoptosis in a rainbow trout cell line, which exhibits interferon-like activity after infection with dsRNA or IPNV (Garner, Joshi, and Jagus, 2003; Trobridge, Chiou, and Leong, 1997). We examined the apoptotic

kinetics and possible involvement of caspase-3 and NF- κ B in this process. We also evaluated the effects of interferon treatment on NF- κ B activity during virus infection. The relationship between apoptosis and virus replication was also investigated.

MATERIALS AND METHODS

Virus, cells and reagents. Chinook salmon embryo cells (CHSE-214, ATCC CRL-1681) were maintained at 15°C in minimal essential medium containing Hanks' salts and supplemented with 10% fetal bovine serum (FBS) and used for virus propagation and titration. Rainbow trout gonad cells (RTG-2, ATCC CCL-55) were grown in L-15 medium supplemented with 10% FBS at 15°C. An attenuated IPNV strain, Sp103 was obtained from previously acknowledged sources (Shivappa, Song, and Vakharia, 2003). zVAD-FMK (z-Val-Ala-Asp-fluoromethylketone) or MG-132 (z-Leu-Leu-Leu-CHO) (Biomol Research Labs, Inc.) was dissolved in DMSO (dimethyl sulfoxide) at concentrations of 10 mg/ml or 25 mg/ml, respectively. PDTC (pyrrolidinedithiocarbamate) and Poly (I)·(C) were dissolved in nuclease free water at concentrations of 10mg/ml and 2mg/ml, respectively.

DNA fragmentation assay. The mock or infected cells (1.5×10^6) from 25-cm² tissue culture flasks were harvested at different time points and the low-molecular-weight DNA was extracted as described (Eleouet et al., 1998). At selected times post-infection, the cells were scraped from the plate, collected by centrifugation, washed once with cold phosphate-buffered saline (PBS), resuspended in 400 μ l of ice-cold lysis buffer (10 mM

Tris pH7.5, 10 mM EDTA pH7.5 and 0.2% Triton X-100), and incubated on ice for 30 min. Lysates were clarified by centrifugation at 10,000 ×g at 4°C for 10 min, and the supernatants were subjected to phenol-chloroform extraction. DNA was ethanol-precipitated with 500 mM NaCl. After one wash in 70% ethanol, dried DNA samples were resuspended in 15 µl of sterile water and treated for 15 min at 37°C with RNase A at a final concentration of 1.0 µg/µl. The sample was analyzed by electrophoresis on 2.0% agarose gels. The DNA ladder was visualized by fluorescence using a UV transilluminator.

NF-κB electrophoretic mobility shift assay (EMSA). RTG 2 cells, grown in 25-cm² flasks, were either mock infected or infected with IPNV-Sp103 at an MOI of 10 PFU per cell. After incubation at 37°C for various intervals, total cell extracts were prepared and EMSA was performed as described by Pahl and Baeuerle, (1995). Briefly, cells were trypsinized and washed once with PBS. After centrifugation at 600g for 5min, the collected cell pellet was resuspended in 20µl high-salt buffer, named “Totex buffer” [20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'2'- ethanesulfonic acid)(pH 7.9), 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.1% phenylmethylsulfonyl fluoride (PMSF) and 1% aprotinin], and incubated on ice for 30 min. After centrifugation, the supernatant was collected; the total protein concentration was determined using a BCA protein assay kit (Pierce). An equal amount of protein (15 µg) was added to a reaction mixture that contained 2 µg BSA (Sigma), 2 µg poly (dI) ·(dC) (Amersham Pharmacia Biotech), 2 µl buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF], 4

μl buffer F (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 % PMSF) and 0.2 pmol [γ -³²P]-end-labeled probes in a final volume of 20 μl. For the competition experiments, a 10-fold excess of unlabeled NF-κB consensus oligonucleotide or the double-stranded oligonucleotide of another transcription factor, AP-1 was added to reaction mixtures and incubated for 10 min at room temperature before γ -³²P-end-labeled probes were added. After incubation at room temperature for another 20 min in the presence of γ -³²P-end-labeled probes, all reaction mixtures were separated on 5% nondenaturing polyacrylamide gel at 55 mA at 4°C for 20 min. The gel was then dried, and prepared for autoradiography by exposing to storage phosphor screen (Molecular Dynamics) at room temperature. The [³²P]-labeled bands were visualized and quantified using Phosphorimager storm (Molecular Dynamics).

The double-stranded NF-κB oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3' and the complementary strand, 3'-TCA ACT CCC CTG AAA GGG TCC G-5') (Promega) were labeled using [γ -³²P] ATP (3000 Ci/ mMol; Amersham) and T4 polynucleotide kinase (Promega) according to the instructions provided by the company. The AP-1 oligonucleotide contains the sequence of 5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3' and the complementary strand, 3'-GCG AAC TAC TCA GTC GGC CTT-5'.

Fluorometric assay of caspase-3 activity. Fluorometric assay of caspase-3-like proteolytic activity was performed using BioVision caspase-3/CPP32 fluorometric assay kits (Biovision). RTG-2 cells were cultured in a 6-well plate and mock infected or

infected with IPNV-Sp103 at MOI of 10 and incubated for selected intervals. The cells were trypsinized and collected by centrifugation at 600g for 5min. The cell pellets were resuspended in 50 μ l of chilled cell lysis buffer and incubated on ice for 10 min. The cell lysates were clarified by centrifugation at 14,000 rpm for 10 min. Supernatant (50 μ l) was added to an equal volume of 2 X reaction/DTT buffer supplement with DEVD-AFC (synthetic caspase-3 substrate) and incubated at 37 °C for 90 min. The sample was analyzed using a fluorescent plate reader (Luminescence Spectrometer LS 55, Perkin Elmer Instruments) with a 400-nm excitation filter and 505-nm emission filter. The amount of fluorescence detected is directly proportional to the amount of caspase activity.

Plaque assay. Confluent monolayers of CHSE cells, grown in six-well plates, were infected with serially diluted supernatants from virus stock. After 1hr incubation at 15°C, cells were washed once with PBS and overlaid with 0.6% SeaPlaque Agrose (Difco) in Eagles MEM medium containing 5% FBS and 1% L-glutamine. After 3 days of incubation at 15°C, the overlays were removed. The cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at RT. After rinsing the cells with distilled water, the plaques were counted.

Western blot analysis. To analyze viral protein synthesis during single round replication, RTG-2 cells grown in 6-well plates were infected with IPNV-Sp103 at an MOI of 10 PFU per cell. At selected time intervals, the cells were scraped off the plates and collected by centrifugation. The cells were washed twice with ice-cold PBS,

resuspended in 30µl of PBS and mixed with an equal volume of 2XSDS sample loading buffer (100 mM Tris-Cl pH 6.8, 200 mM DTT, 4% SDS, 0.1% bromophenol blue, 10% glycerol). The samples were then boiled for 5 min, and centrifuged at 13,000g for 3 min. The supernatants were stored at -70°C. For immunoblotting, the cell lysates were fractionated by SDS-PAGE using 5% stacking and 12.5% resolution gels, and proteins were transferred to a nitrocellulose membrane for immunoblot analysis. After electrotransfer, the membranes were blocked in the blocking buffer, 5% nonfat dry milk in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-Cl, pH 7.5), for 90 min at room temperature. The membranes were incubated for 60 min in 10 ml of a 1:400 dilution of a rabbit anti-IPNV polyclonal antibody and then washed for 3 X15min in 1X TBS (0.1% Tween in TBS buffer). The membranes were then incubated in a 1:25000 dilution of a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Amersham) for 60 min and then washed 3X15min as above. The detection of IPNV structural proteins were performed by enhanced chemiluminescence (ECL) Western blot detection system (Amersham).

Production of interferon-like activity. Induction of interferon-like activity was described previously (Garner, Joshi, and Jagus, 2003). Briefly, RTG-2 cells were incubated with 50 µg/ml poly (I)•poly(C) and 200 µg /ml DEAE dextran in complete medium for 24 hrs. After treatment, the cells were washed twice with PBS, and incubated in complete medium for another 24 hr to facilitate accumulation of secreted interferon. The conditioned medium was harvested, and added to fresh cell monolayers for 24 hrs before the virus was inoculated. Aliquots of conditioned medium were routinely tested

for interferon activity by screening for their ability to inhibit IPNV productive replication by plaque assay.

RESULTS

IPNV induces apoptosis in RTG-2 cells in late stages of viral replication. To determine if IPNV induces apoptosis in RTG-2 cells, and study the kinetic of virus-induced apoptosis within viral single round replication, RTG-2 cells were infected with IPNV strain Sp103 at an MOI of 10 PFU per cell. Apoptosis was monitored between 8 hr to 16 hr post-infection (p.i.) by DNA fragmentation assay. As shown in Figure 5-1A, the DNA laddering was first detected at 10 hr p.i., and became prominent at 14-16 hr p.i.. In contrast, DNA laddering did not occur in mock-infected cells. In IPNV-infected cells, the viral protein VP3 was synthesized as early as 4 hr p.i., and reached a peak at 10-12 hr p.i. and decreased thereafter, which may be due to the viral particles releasing from cells (Fig. 5-1 B). Moreover, virus titer increased at 12 hr p.i. (data not shown), by which time the obvious apoptosis was not observed. Therefore, these results indicate that apoptosis occurs during the late stage of viral replication, after the virus has completed protein synthesis and begins to release from the cells.

IPNV-induced apoptosis requires caspase-3 activity. To investigate whether IPNV-induced apoptosis is caspase-dependent, caspase-3 activity was examined at different time points after virus infection at MOI of 10 (Fig. 5-2). The infected RTG-2 cells were collected and the caspase-3 activity was evaluated based on detection of DEVD-AFC fluorometric substrate cleavage. Compared with mock infected cell lysates, the cells infected by IPNV exhibited an increase of caspase-3 activity at 8 hr p.i., and this activity

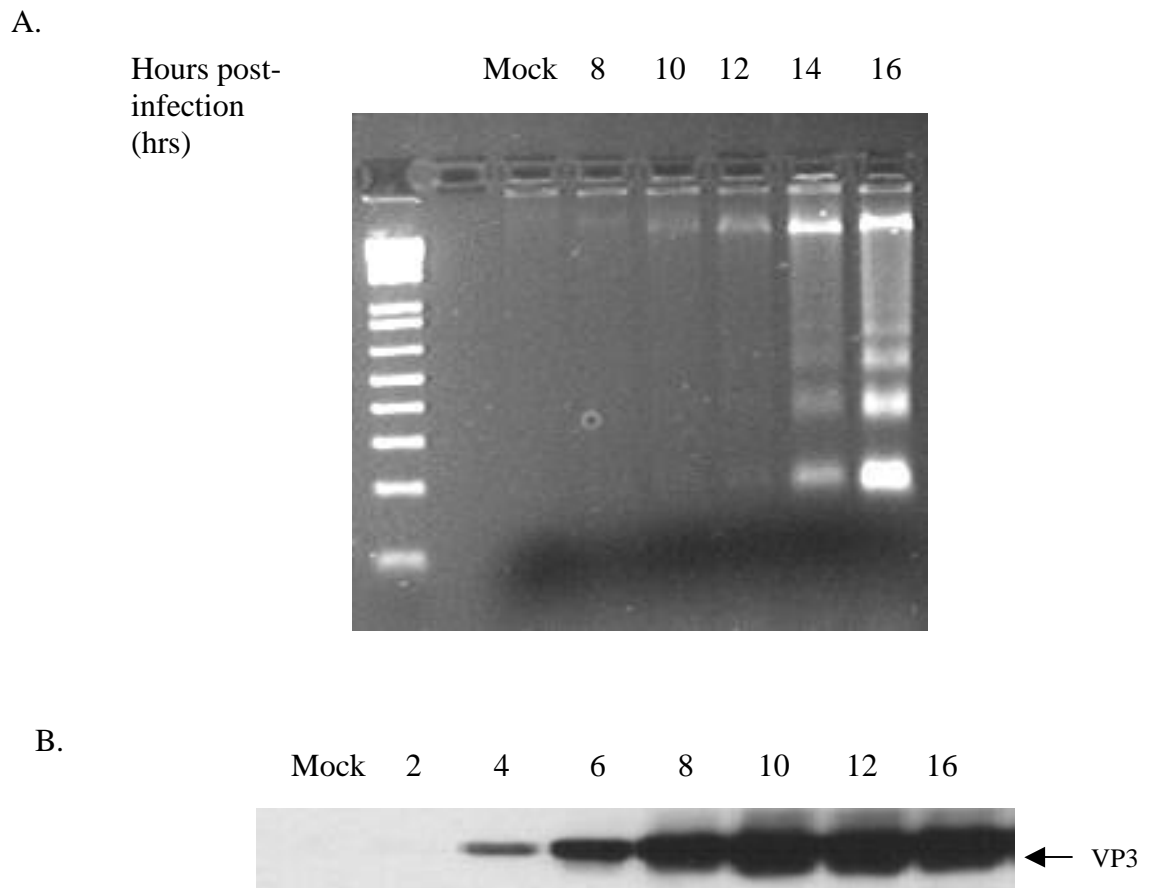


Fig. 5-1. The kinetics of virus protein expression and its induced apoptosis in RTG-2 cells. RTG-2 cells were mock infected or infected with IPNV-Sp103 at an MOI of 10 and the cells were harvested at the indicated time intervals. (A) Time course of IPNV-induced DNA fragmentation in RTG-2 cells. DNA was extracted from IPNV-infected RTG-2 cells and electrophoresed on 2% agarose gel. The 100 bp DNA ladder (Invitrogen) was used as a molecular mass marker. (B) The kinetics of viral protein expression. The cell lysates were subjected to immunoblotting using anti-IPNV polyclonal antibody, the indicated VP3 was used to represent the intensity of viral protein expression.

increased until it reached a peak at 16 hr p.i.. This result correlate to the appearance of DNA laddering, which peaked at 14-16 hr p.i.. To further determine whether IPNV-induced apoptosis requires caspase activation, RTG-2 cells were pretreated with a broad-spectrum caspase inhibitor, zVAD-FMK (zVAD), at a concentration of 25µm and 50µm, respectively. Then the cells were mock infected or infected with IPNV at an MOI of 10. Surprisingly, although zVAD-FMK could completely inhibit caspase-3 activity at concentration of 25 µM (Fig. 5-3B), it can only partially inhibit virus-induced DNA laddering at either concentration of 25 µM or 50 µM (Fig. 5-3 A). Moreover, zVAD-FMK cannot inhibit the cytopathic effects (CPE) caused by viral infection (data not shown). These results suggest that caspase-3 is involved in virus-induced apoptosis, but there are additional factors involved in this process. The presence of zVAD-FMK did not inhibit virus replication or virus titer, when compared to the absence of zVAD-FMK (data not shown), implying that partial inhibition of apoptosis does not affect virus replication.

NF-κB is activated during IPNV infection. NF-κB plays a key role in the regulation of cell survival and death. To determine whether NF-κB is activated in IPNV-infected cells, EMSA was carried out to detect NF-κB gel shift activity during IPNV infection. RTG-2 cells were either mock or infected with IPNV-Sp103 at an MOI of 10, and the cell lysates were prepared at different time intervals after virus absorption. The lysates were incubated with a ³²P-labeled oligonucleotide containing the NF-κB consensus binding sequence and resolved in a nondenaturing polyacrylamide gel (Fig. 5-4A). Following infection with IPNV, due to protein binding, the amount of shifted radiolabeled

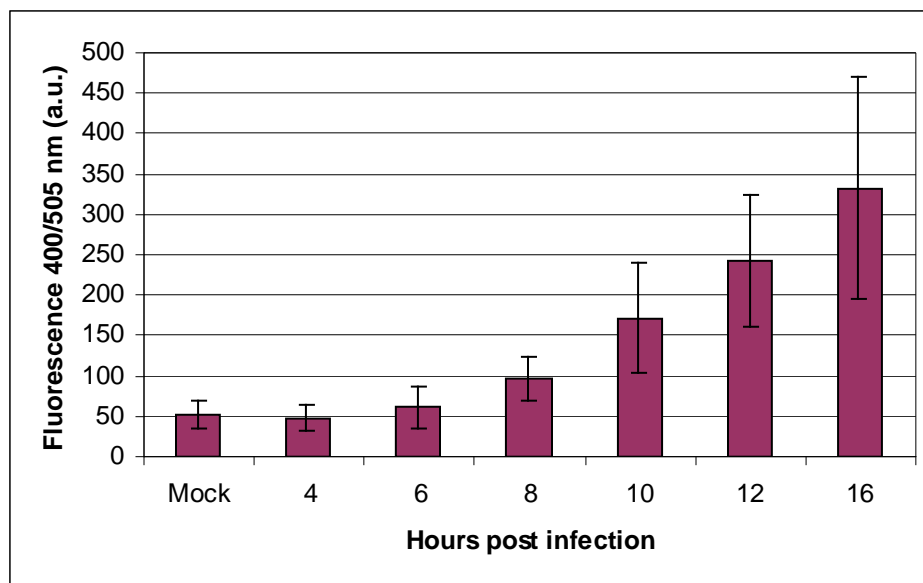
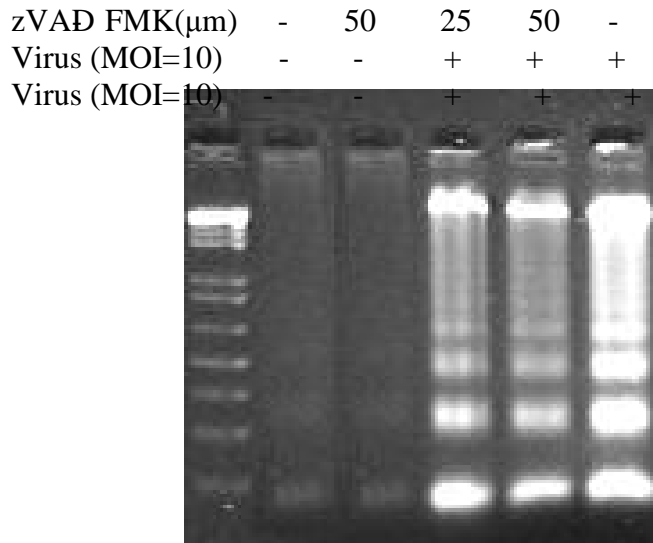


Fig. 5-2. Caspase-3 activities in IPNV-infected RTG 2 cells. RTG-2 cells were mock or infected with Sp103 virus at MOI of 10. The caspase-3 activity was determined by incubation of cell lysates with its specific peptide substrate, DEVD-AFC. The proteolytic activities were quantified by the fluorescent plated reader with a 400-nm excitation filter and 505-nm emission filter.

A.



B.

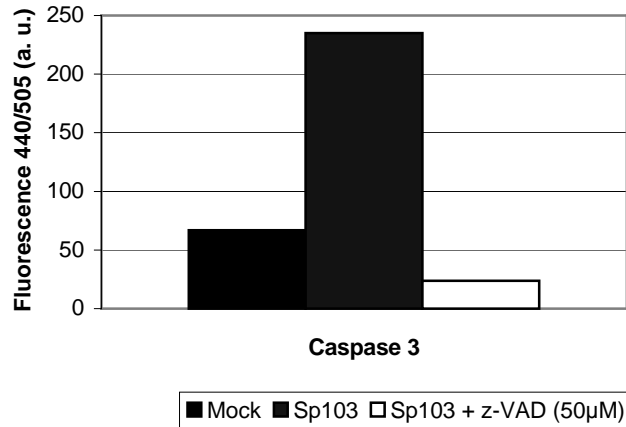


Fig. 5-3. The effect of the broad-spectrum caspase inhibitor zVAD-FMK on caspase-3 activity and IPNV-induced DNA fragmentation. RTG-2 cells were mock infected or infected with IPNV-Sp103 at an MOI of 10 in presence or absence of z-VAD. (A) DNA laddering assay was performed at 14 hr post-infection in presence of 25 μ M or 50 μ M of z-VAD. (B) Caspase-3 activity was determined at 12 hr post-infection in presence of 50 μ M of z-VAD.

oligonucleotides increased, indicating the activation of NF- κ B started at 4 hr p.i., reached the peak at 10-12 hr p.i., and diminished thereafter. Mock-infected cells had only basal level of NF- κ B activity. In order to verify the specificity of the NF- κ B DNA binding in EMSA, competitive EMSA was employed. The binding reaction of cell lysates with a 32 P-labeled NF- κ B consensus oligonucleotide was performed in the presence of a 10-fold excess of either unlabeled NF- κ B consensus oligonucleotide or unlabeled oligonucleotide consisting of the consensus binding sequence for AP-1, another transcription factor. Figure 5-4 B shows that the NF- κ B gel shift activity is completely abolished by 10-fold unlabeled NF- κ B consensus oligonucleotide, but not by unlabeled AP-1 consensus oligonucleotide. These results demonstrated that the gel shift activity after IPNV infection is specific for NF- κ B consensus sequences.

NF- κ B activity is required in IPNV-induced apoptosis. To determine the role of NF- κ B activity in IPNV-induced apoptosis, RTG-2 cells were treated with MG132, a peptide-aldehyde protease inhibitor that blocks NF- κ B activation via its effect on the proteasome (Clifton et al., 1998; Jensen et al., 1995). RTG 2 cells were infected with IPNV-Sp103 at an MOI of 10 in the presence or absence of 25 μ M or 50 μ M of MG132. At 12 hr p.i., cell lysates were prepared and subjected to EMSA (Fig. 5-5A). The treatment of RTG-2 cells with 25 μ M MG132 completely abolished the NF- κ B activity following virus infection. Moreover, DNA laddering, detected at 16 p.i., revealed that the presence of MG132 blocked IPNV-induced apoptosis in RTG-2 cells (Fig. 5-5 B), and CPE caused

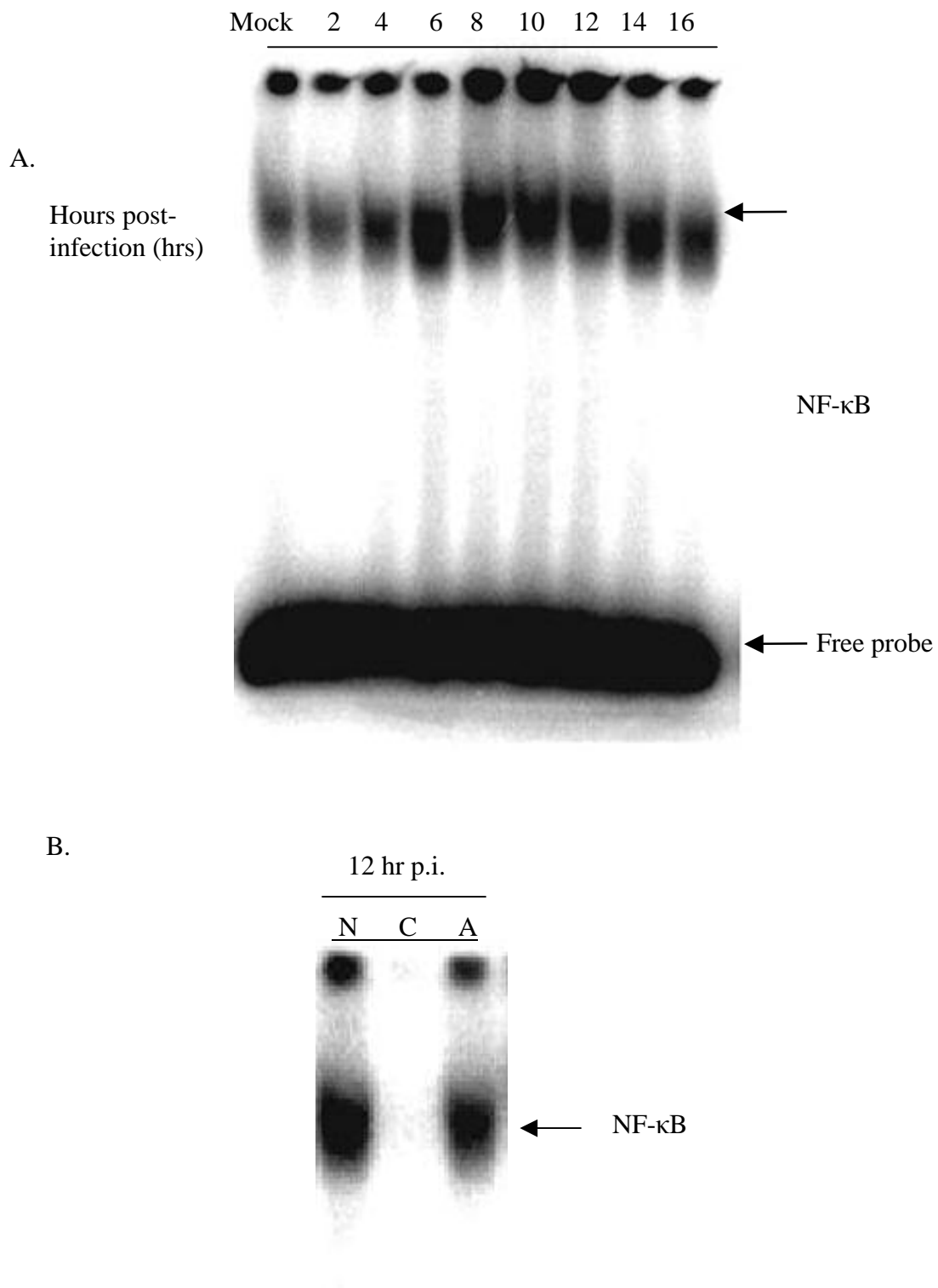


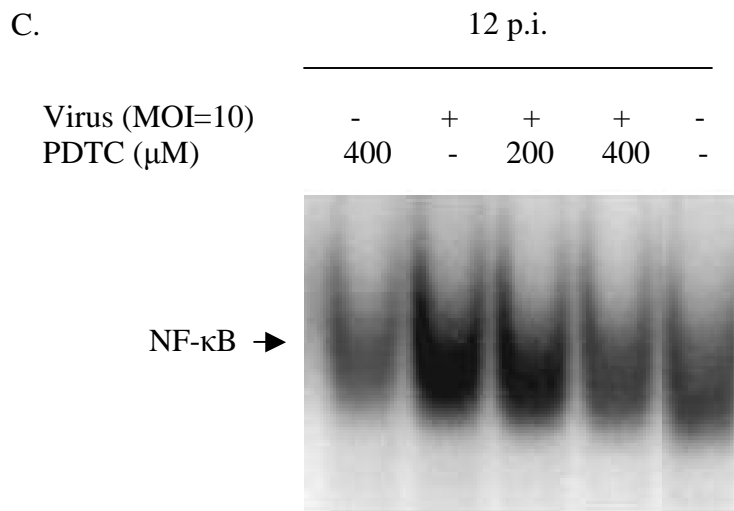
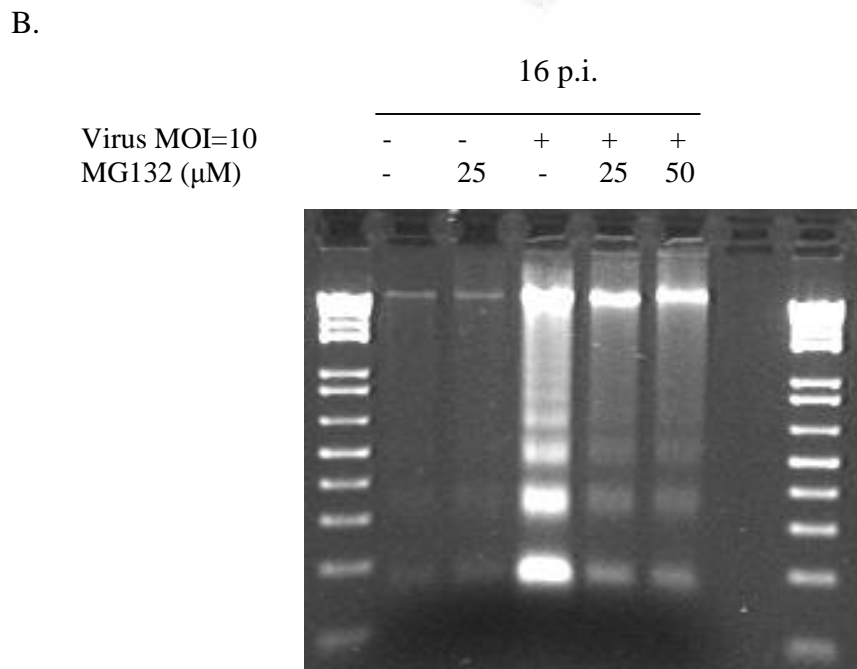
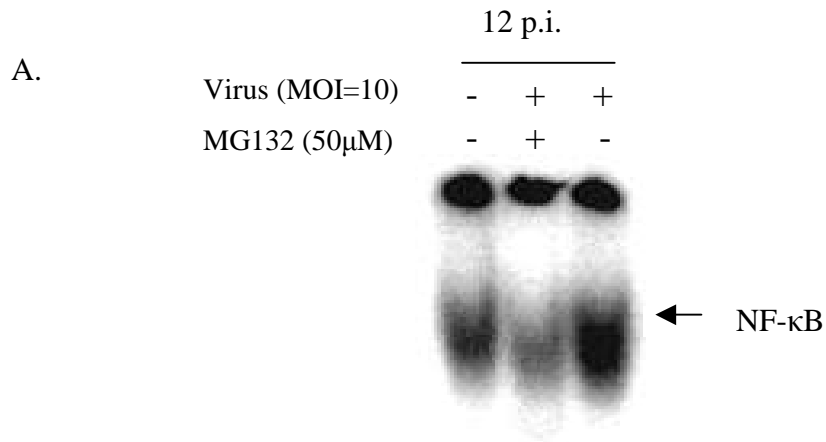
Fig. 5-4. (A) Time course of NF- κ B gel shift activities in the whole cell lysates prepared from IPNV-infected RTG-2 cells. RTG-2 cells were either mock or infected with IPNV-Sp103 at an MOI of 10. Whole cell lysates were prepared at indicated time points and equal amounts of protein (15 μ g) were analysed for NF- κ B activity by EMSA. (B) The

specificity of NF- κ B binding activity in EMSA. Whole cell lysates used in the panel A at 12 hr post-infection were incubated with 32 P-labeled NF- κ B consensus oligonucleotide probe alone (lane N), or in presence of a 10-fold excess of either unlabeled NF- κ B-oligonucleotide (lane C), or unlabeled oligonucleotide probe that binds to transcription factor AP-1 (lane A) . .

by virus infection (data not shown). These results indicate that interference of NF- κ B activation prevents IPNV-induced apoptosis.

Previous studies have shown that elevated ROS enhances NF- κ B activation, and almost all NF- κ B-activating stimuli are inhibited by antioxidants. To determine if ROS accumulation is also upstream of NF- κ B activation during IPNV infection, RTG-2 cells were preincubated in the presence of the antioxidant PDTC (Gong et al., 2001; Pahl and Baeuerle, 1995). The infected cell lysates were harvested for EMSA at 12 hr p.i., and the DNA extracts were harvested at 16 hr p.i. for DNA fragmentation assay (Fig 5-5 C, D). The presence of 200 μ M PDTC could not prevent NF- κ B activity; as a consequence, this concentration also could not effectively block virus-induced apoptosis. However, when RTG-2 cells were pretreated with 400 μ M PDTC, the NF- κ B activity was inhibited, as was virus-induced apoptosis and CPE (data not shown). This suggests that like all other known NF- κ B inducers, IPNV induces the production of reactive oxygen intermediates within cells, which are important upstream messengers for NF- κ B activation. These results also contribute further evidence that virus-induced apoptosis requires NF- κ B activity.

Proteasome inhibitor and antioxidant inhibit caspase-3 activity and have no effect on virus replication. To determine whether inhibition of NF- κ B results in the blockage of caspase-3 activity, RTG-2 cells were pretreated with 50 μ M MG132 or 400 μ M PDTC. At these concentrations, the inhibitors has been shown to effectively inhibit NF- κ B and virus-induced apoptosis. Cell lysates were collected at 12 hr p.i., and their caspase-3



D.

	16PI				
Virus (MOI=10)	-	-	+	+	+
PDTC (μ M)	-	400	-	200	400

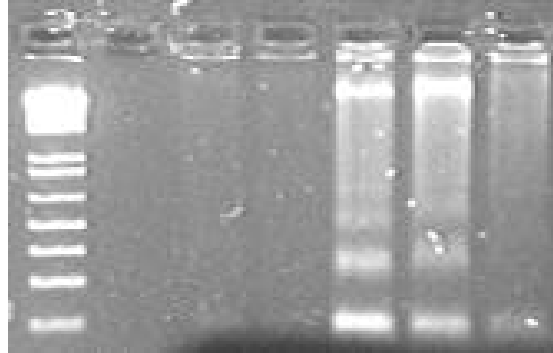
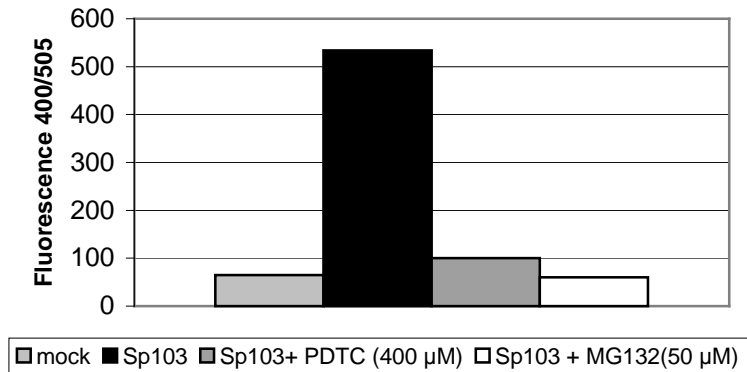


Fig. 5-5. Blocking of NF- κ B activity and DNA fragmentation by proteasome inhibitor MG132 and the antioxidant PDTC. In presence and absence of 25 or 50 μ M of MG132, cells were infected by IPNV-Sp103 at an MOI of 10 and the whole cell lysates were collected at 12 hr p.i. for NF- κ B activity assessment by EMSA (A). At 16 hr p.i., small molecular weight DNAs were also extracted and subjected to DNA laddering assay (B). Antioxidant, PDTC in the different concentrations indicated in panel C and D was added into the cell culture at the same time when the virus was inoculated at an MOI of 10. The cells were harvested and the NF- κ B activity and DNA laddering in the cell lysates were assessed by EMSA at 12 hr p.i.(C) and DNA laddering at 16 p.i. (D).

activity was examined. As shown in Figure 5-6A, caspase-3 activity was inhibited in the presence of either PDTC or MG132, suggesting that the inhibition of NF- κ B might down-regulate some upstream factors that activate caspase-3. Apoptosis caused by virus would facilitate virus release. In addition, the inhibition of NF- κ B may also influence virus replication, since NF- κ B could regulate various genes that may be required for virus productive propagation. Therefore, to evaluate the effect of apoptosis and NF- κ B activity on viral replication, extracellular virus titer was detected in the presence or absence of MG132 or PDTC (Fig. 5-6 B). The presence of either MG132 or PDTC only slightly affected virus titer, which indicates that virus release is not affected by the inhibition of NF- κ B activity and apoptosis.

Interferon treatment inhibits virus replication and induces NF- κ B activity. To address whether NF- κ B activation is involved in an interferon-induced antiviral state, we pretreated RTG-2 cells with IFNs. Since there are no recombinant fish IFNs commercially available, we used a conditioned medium, which is known to have type I interferon-like activity. RTG-2 cells were incubated with normal medium or conditioned medium for 24 hr, and then the cells were infected with IPNV at an MOI of 10. Virus titers were dramatically decreased after IFNs treatments at both 12 hr and 24 hr p.i. (Fig. 5-7A). To detect NF- κ B activation in interferon treated cells, following virus infection, cell lysates from both interferon treated and untreated cells were collected at indicated time intervals, and subjected to EMSA. As shown in Figure 5-7 B, IFNs-treated cells exhibit higher levels of NF- κ B activity compared with untreated cells throughout all

A.



B.

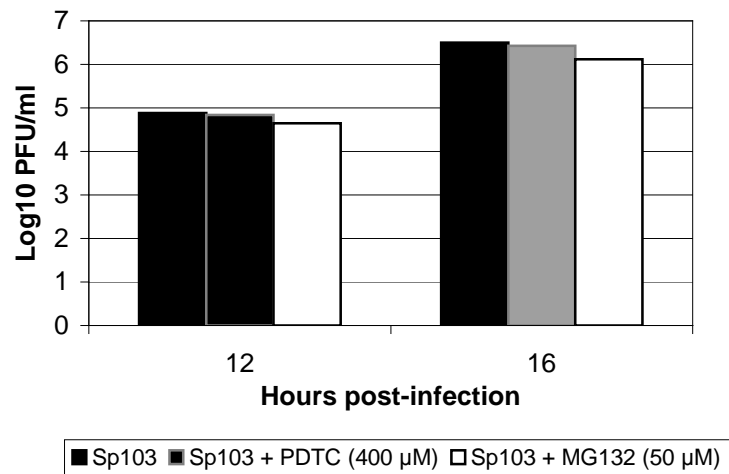


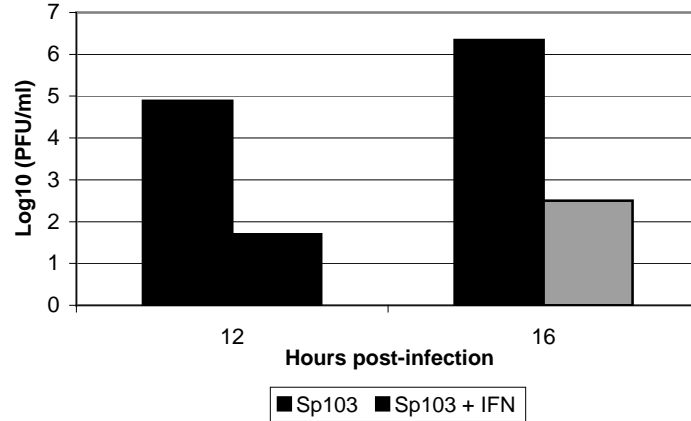
Fig. 5-6. The effect of proteasome inhibitor and antioxidants on virus-induced caspase-3 activity and virus replication. RTG-2 cells were mock or infected with IPNV-Sp103 an MOI of 10 with or without the presence of 50 μM MG132 or 400 μM PDTC. Cell lysates were collected at 12 hours post-inoculation and caspase-3 activity was determined as described in Materials and Methods (A). The cells were freeze-thawed twice at 12 hr or 16 hr p.i., and subjected to plaque assay for the total virus yield (B).

time points post-infection. This result seem to indicate that NF- κ B may be involved in IFN-induced antiviral states.

DISCUSSION

In this study, we demonstrate that IPNV causes apoptosis in RTG-2 cells during the late stage of viral replication. We observed increased DNA fragmentation and caspase-3 activation during virus infection. At an MOI of 10, viral proteins were detected as early as 4 hr p.i., and peaked at 10-12 hr p.i.. Furthermore, virus titer started to increase at 12 hr p.i.. Therefore, arbitrarily, the period between 0 hr to 12-14 hr post-inoculation was considered to be the first round of virus replication. DNA fragmentation were detected at 14-16 hr p.i., suggesting that prior to apoptotic event, the viral proteins were synthesized and packaged into virions for release. Moreover, caspase-3 activation can be only detected at 14-16 hr p.i.. It is known that certain viruses induce apoptosis at the late stages of replication, providing a mechanism for the dissemination of progeny virus (Razvi and Welsh, 1995). One possible explanation for why IPNV induces apoptosis during the late stage of virus replication is that virus may encode an anti-apoptosis protein, which delays apoptosis until sufficient virus progeny have been produced. A number of viruses encode their own anti-apoptosis factors, such as the E1B gene of adenovirus, and the BHRF1 gene of Epstein-Barr Virus (Boyed et al., 1994; Henderson et al., 1993). Recent studies in our laboratory indicated that infectious bursal disease virus (IBDV), another member of Birnavirus family, contains a 17-kDa

A.



B.

Hours post-infection	8	8	4	4	8	8	12	12
Virus (MOI=10)	-	-	+	+	+	+	+	+
IFN	-	+	-	+	-	+	-	+

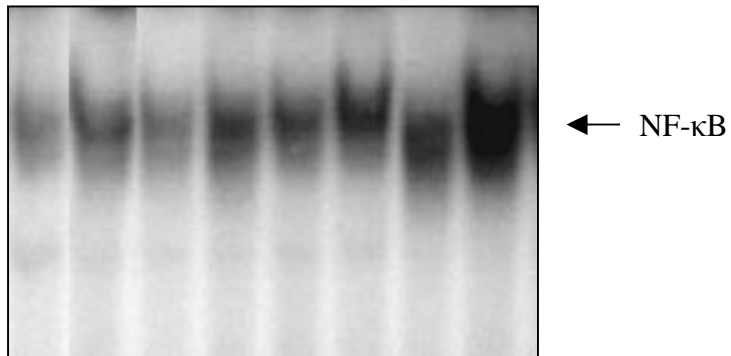


Fig. 5-7. Interferon treatment inhibits virus replication but induces NF-κB activity. RTG 2 cells were either pretreated with IFN conditional medium or normal medium for 24 hours, before the cells were mock or infected by IPNV-Sp103 at MOI of 10. At 12 and 16 hours post-infection, the virus titer was quantified by plaque assay (A). The cell lysates at indicated time points were subjected to EMSA to detect the NF-κB activity(B).

nonstructural (NS) protein that has anti-apoptotic function (Liu, 2003). Similarly to IBDV, IPNV has a 17-kDa NS protein, called VP5 that potentially has anti-apoptosis activity (Hong, Gong, and Wu, 2002). The other possibility is that virus may encode pro-apoptotic proteins, which is only expressed in the late stage of virus infection, helping virus release. For example, E1A of adenovirus and apoptin encoded by chicken anemia virus (Hay and Kannourakis, 2002). Whether a pro-apoptotic factor exists in IPNV has yet to be determined.

zVAD-FMK is a broad-spectrum caspase inhibitor, which inhibits caspase 8 and 9 activity, and leads to total inhibition of the executor caspase, caspase-3 (Sun et al., 1999). Even though zVAD-FMK could totally inhibit caspase-3 activity in this study, it does not block IPNV-induced CPE and only partially inhibits virus-induced DNA fragmentation. This implies that the inhibitor is unable to block all the intracellular caspases or caspase-independent pathways are involved in IPNV-induced apoptosis. By using caspase-3 knockout mice, it has been shown that apoptosis could still proceed in mice, suggesting that some related caspases may exist (Kuida et al., 1996). The partial inhibition of apoptosis by zVAD-FMK has also been reported in Sindbis virus-induced apoptosis (Nava et al., 1998). In some cases, inhibition of virus-induced apoptosis will affect virus growth. For example, inhibition of Vpr-induced apoptosis in human immunodeficiency virus facilitates viral protein synthesis (Stewart et al., 2000). However, in our case, the presence of zVAD-FMK did not affect the titer and replication of IPNV.

It is proposed that NF- κ B may be involved in protecting or potentiating cell death, depending on different stimuli in various cell types (Connolly et al., 2000). Many viruses can activate NF- κ B during their infection cycle. However, to the best of our knowledge, this is the first report that an aquatic virus infection can induce NF- κ B activation. Furthermore we demonstrated that NF- κ B activation is essential for virus-induced apoptosis because the protease inhibitor MG132, which could block NF- κ B activation, also inhibits virus-induced apoptosis. Several viruses, such as reovirus and some flaviviruses require the activation of NF- κ B in virus-caused apoptosis (Connolly et al., 2000; Jan et al., 2000; Liao et al., 2001; Marianneau et al., 1997). Liu and Vakharia showed that NF- κ B activity is also essential for apoptosis induced by IBDV infection (Liu, 2003). Oxidative stress is thought to be the common signal transduction pathway used by various NF- κ B activating stimuli, and NF- κ B is usually activated downstream of ROS (Jan and Griffin, 1999; Jan et al., 2000; Manna and Aggarwal, 1999). In this study, we found that PDTC at a concentration of 400 μ M greatly inhibited IPNV-induced NF- κ B activation and protected cell from apoptosis as well, which confirmed that NF- κ B activity is required in virus-induced apoptosis and is downstream of ROS and oxidative stresses. In addition, both PDTC and MG132 inhibit both virus-induced apoptosis and caspase-3 activity, which suggest that NF- κ B may regulate the transcription of some pro-apoptotic factors that are required in caspase activation. Some pro-apoptotic proteins, which are the upstream messengers for activating caspase cascade, are induced by NF- κ B, such as p53, caspase-1 and Fas L (Connolly et al., 2000).

Some viruses need NF- κ B activity for virus maximal replication. For example, tax protein of human T-cell leukemia virus induces NF- κ B, which in turn enhance the expression of cellular genes that promote virus replication (Mesnard and Devaux, 1999). In other cases, NF- κ B is not essential for virus replication, for example, in flavivirus, specific NF- κ B inhibitor has no effect on virus replication (Liao et al., 2001). In IPNV infected cells, viral protein synthesis and extracellular viral yield were not affected by either PDTC or MG132, suggesting that virus replication is not influence by NF- κ B activation and apoptosis.

Type one interferons are the primary defense mechanism against viral infection in mammal. Upon virus infection, IFNs are induced and subsequently secreted to neighboring cells. Within the neighboring cells, IFN induces the expression of several enzymes, of which 2'-5' A synthetase and PKR are best characterized (Sen, 2001). Activation of these enzymes is dependent on the presence of dsRNA. Activated PKR phosphorylates the α subunit of the eukaryotic translation initiation factor, eIF-2 α , which causes protein synthesis shut off. PKR also can phosphorylate NF- κ B inhibitor, I κ B, leading to NF- κ B activation (Der et al., 1997; Maran et al., 1994; Yang et al., 1995). Interferon-like activity has been elicited in fish cells treated with poly (I)-poly (C) or virus infection (Garner, Joshi, and Jagus, 2003; Nygaard et al., 2000; Trobridge et al., 1997). Recently, the interferon cDNA of zebrafish was cloned (Altmann et al., 2003). The cDNA of fish PKR has not been cloned, but its substrate, eIF-2 α , was cloned from zebrafish and rainbow trout cell lines (Garner, Joshi, and Jagus, 2003). In this study, we found that NF- κ B was upregulated by interferon. This increased activation of NF- κ B is

possibly due to the increased PKR activity in IFN-pretreated cells, because during IPNV infection, the level of e-IF2 α phosphorylation is higher in cells pretreated with IFN than in the untreated cells (Garner, Joshi, and Jagus, 2003).

In this study, we found that IPNV-induced-apoptosis occurs during the late stage of viral replication and this process is caspase-dependent. NF- κ B activity increased in virus-infected cells and its activation is required in virus-induced apoptosis. NF- κ B was upregulated in the presence of IFN, during virus infection. Therefore, our findings indicate that NF- κ B also plays an important role in regulating virus-induced apoptosis and possible interferon mediated antiviral state in lower vertebrates.

Chapter 6

Conclusions and future studies

6.1 Conclusions

Viral structural proteins often affect both virulence and tropism by changing the interaction between the virus and host cell receptors. For example, the capsid proteins of reovirus and poliovirus, and the envelope protein of flavivirus have been shown to carry determinants of virulence and cell tropism (Hrdy, Rubin, and Fields, 1982; Jia et al., 2001; Mandl et al., 2001). Viral polymerases also affect cell tropism and virulence, as reported in LCMV and measles virus (Matloubian et al., 1993; Takeda et al., 1998). Recently, increasing number of RNA viruses has shown new roles of NS proteins in virus replication, inhibition of host-cell functions, and disease process. Examples are the NS proteins of IBDV, influenza virus, reovirus (Gillian and Nibert, 1998; Samuel, 2001; Yao, Goodwin, and Vakharia, 1998). By comparing the deduced amino acid sequence of the virulent and attenuated isolates of Buhl-IPNV, putative residues involved in virulence were identified in VP2 region (Bruslind and Reno, 2000). For IPNV Sp serotype, residues at positions 217, 221 and 247 in VP2 are proposed to be important for virulence (Santi, Vakharia, and Evensen, 2003; Shivappa, Song, and Vakharia, 2003). With the availability of a reverse genetic system for IPNV, more precise functional studies of viral protein in virulence and cell tropisms could be possible.

It is known that IPNV induces apoptosis both *in vitro* and *in vivo*. However, the signaling pathway involved in this process has not been well studied. In most cases,

apoptosis is a defense mechanism, and the infected cells undergo apoptosis before virus complete its life cycle, resulting in reduced viral infectivity. In other cases, apoptosis triggered by virus would facilitate its release. Full understanding of the nature of virus-induced apoptosis and possible signaling pathway would be useful to better understand viral pathogenesis.

The major findings of this research are:

1. 12-kDa VP5 stop codon at nucleotide position 427 in wild-type virus could be read-through, resulting in a lower level of 15-kDa VP5 expression. VP5 is not essential for viral replication *in vitro* and *in vivo*. It is not involved in virulence and does not have anti-interferon activity.
2. 25-kDa ORF encoded by segment A is not essential for virus replication; however, this protein could not be detected *in vitro*.
3. Amino acid substitution at position 221 (Ala to Thr) in VP2 protein occurs rapidly after virus was serially passaged in CHSE cells but not in RTG-2 cells.
4. The residues at positions 217 and 247 of VP2 may be important markers for virus adaptation and attenuation in cell culture.
5. IPNV induces apoptosis in RTG-2 cells at late stage of viral replication. NF- κ B activity is required in this process, and it may be involved in IFN-induced antiviral state.
6. IPNV replication is not affected by NF- κ B inhibitor, MG132 and the antioxidant PDTC.

In this study, we characterized two putative non-structural proteins of IPNV, VP5 and a 25-kDa ORF. Both of these proteins do not affect virus replication kinetics *in vitro*. We could not detect the 25-kDa protein in virus-infected cells, however, it is not certain whether this ORF would express the protein *in vivo*. We demonstrated that the expression of IPNV VP5 is regulated by read-through of a premature stop codon, and the context near the leaky UGA at nt 427 is conserved among other organisms. Unlike the 17-kDa VP5 of IBDV, IPNV VP5 does not play a role in viral virulence (Yao, Goodwin and Vakharia, 1998; Santi, unpublished data). Sequence comparison of these two VP5 proteins reveals more than 30% similarity (Fig. 6-1). However, IBDV VP5 contains a PEST motif and transmembrane helix, which is absent in IPNV VP5 (Liu, 2003a; Lombardo et al., 2000). Usually, the regulatory proteins with short lifetime contain PEST motif and the presence of this PEST region results in the rapid intracellular degradation of the proteins (Rogers, Wells, and Rechsteiner, 1986). It was shown that due to the transmembrane domain, VP5 of IBDV accumulates at the host plasma membrane and induces cell lysis (Lombardo et al., 2000). Therefore, these may be the reasons why these two proteins have different functions in viral pathogenesis.

Virus adaptation to cell culture often leads to loss of virulence in natural host. Study of the molecular determinants of cell adaptation would give us a clue for potential virulence markers. We observed that only one residue substitution at position 221 (Ala to Thr) in VP2 occurred after serial passage in CHSE cells, and this mutation could dramatically enhance virus growth in CHSE cells. The fact that this mutation did not appear even after nine passages in RTG-2 cells suggests that virus may use different

	1	11	21	31	41
IBDvp5	MVSRDQTNDRSDDKPARSNPTDCSVHTEP	SDANMRTGVHSGRHPGEAHSQ			
IPNvp5	-----MQDEHKQGNRNLEIHYASR---	DWTSKHPGRHNGETHLK			
Consensus		d n h		t h grh ge h	
	51	61	71	81	91
IBDvp5	VRDLDLQFDCGGHRVRAN	<u>CLFPWIPWLNCGCSLHTAGQW</u>	EELQ-VRSDAPD		
IPNvp5	TRDLVIQLRGLRIRKWHSC	<u>LFPWGTRLTDRCTLQMECEPDGAGVRPVAGD</u>			
Consensus	r d l q	r	clfpw l	c l	vr a d
	101	111	121	131	141
IBDvp5	<u>CPEPTGQLQLLQASESE</u>	SHSEVKHTSWWR	LCTKRHHKRRDL	PRKPE	
IPNvp5	<u>VAGPEESLQLREVDLKE</u>	IRHPKLHTTGRSLC	SERDAQRCHLRGQSV		
Consensus	p lql	e	ht	lc r r l	

Fig. 6-1. Alignment of VP5 deduced amino acid sequence of IBDV and IPNV. The consensus residues are shaded in yellow color. The PEST domain in IBDV VP5 is underlined. The position of the predicted transmembrane helix in IBDV VP5 is marked in blue rectangle.

receptors for entering these two cell lines. Besides the residue at position 221, the amino acids at positions 217 and 247 also play a role in viral replication kinetics in cell culture. Viruses with only substitutions at these two positions exhibit more rapid growth curve and a higher titer than their parental viruses in both CHSE and RTG-2 cells. These results imply that common receptors present in both cell lines might have a higher affinity for these viruses.

This is the first report indicating that IPNV induces apoptosis in RTG-2 cells, which requires NF- κ B activity. IPNV-induced apoptosis occurs at late stage of virus replication cycle. Caspase-3 is activated during virus infection and its inhibitor (z-VAD-FMK) could partially inhibit virus-induced apoptosis, indicating that other caspases may also be involved in this process. Following IPNV infection, NF- κ B is activated, which is required for IPNV-induced apoptosis, since both NF- κ B inhibitor MG132 and antioxidant could inhibit NF- κ B activity and subsequently apoptosis. Oxidative stress is thought to be the common signal transduction pathway used by various NF- κ B activating stimuli, and NF- κ B is usually activated downstream of reactive oxygen species (ROS) (Jan and Griffin, 1999; Jan et al., 2000; Manna and Aggarwal, 1999). PDTC, an antioxidant, could inhibit NF- κ B activation induced by IPNV infection, therefore, ROS enhances NF- κ B activation possibly as upstream event of IPNV-induced apoptosis cascade. Upon interferon treatment, virus titer was greatly reduced, whereas NF- κ B activity was upregulated, suggesting that NF- κ B is involved in interferon-induced antiviral state. Apoptosis may affect the virus infectivity by limiting viral replication. The transcription factor, NF- κ B, also regulates the transcription of many antiviral genes. However, IPNV

replication was not affected by presence of either NF- κ B inhibitor or antioxidant, which could completely block NF- κ B activity and apoptosis. Therefore, our results suggest that apoptosis is not closely related with viral replication.

6.2 Future studies

The immediate *in vivo* work should to be done to prove our three hypothesis: (1) the adaptation mutation of Ala to Thr at position 221 in VP2 is responsible for loss of viral virulence after several passage in cell culture; (2) the residues at positions 217 and 247 in VP2 are also the virulence markers; (3) VP1 does not play a role in viral virulence. Furthermore, to address IPNV VP5 function in viral pathogenesis, it would be of interest to study whether it is involved in persistent infection, using *in vivo* model.

The mechanism in IPNV-induced apoptosis has been presented in this dissertation. Since VP5 of IBDV has been shown to have anti-apoptosis function, the apoptosis and related signaling pathway induced by wild-type and VP5-deficient virus should be compared in the future (Hong, Gong, and Wu, 2002).

In the present study, we predict that the residues at positions 217 and 247 in VP2 are important for determining cell tropism and possible attenuation in natural host. It would be very interesting to generate mutant viruses in which individual mutation is made in these two residues. This way, we could further narrow down the possible virulence markers.

It has shown in this study that the adaptation mutation acquired in CHSE cells does not affect the virus growth in RTG-2 cells. Sp103 virus replicated faster in cell culture than rNVI15 virus, but the former is attenuated *in vivo*. Therefore, virus may use different receptors to enter different cell lines and their natural host cells. Changing the binding affinity of their receptor would greatly affect the virulence. Hence, efforts should be made to determine the viral receptors for IPNV on the pancreatic cells or CHSE / RTG-2 cells. In the future when IPNV three-dimensional structure becomes available, it would allow for determination of the actual region on VP2 that interacts with the receptor and should lead to increased insights on the amino acid residues responsible for cell tropism and virulence.

With the help of structural information on IPNV, improvements in efficiency of reverse genetic system for IPNV, and functional genomic study in fish cells, further knowledge of the viral disease process, virus-host interaction and host protection will be gained. Based on these future studies, safe and stable live attenuated vaccines would be developed.

Chapter 7

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