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The role of viral proteins in the pathogenesis of infectious hematopoietic necrosis virus (IHNV) was studied at the molecular level. The expression of the viral genes at the protein and RNA level, and their cellular localization, were characterized to further our understanding of viral pathogenesis. The pathogenic effect of individual viral proteins was also investigated and a method for detecting viral RNA in infected fish tissues was developed.

The polarity of transcription was confirmed in terms of the relative amounts of each viral protein. Also, cells treated with glycosylation inhibitors did not exhibit cytopathic effect, demonstrating that a functioning host glycosylation system is necessary for viral replication. These studies also revealed a previously undescribed non-glycosylated protein, S, which appeared to be virus-encoded. The expression of the nonvirion protein (NV), was also detected in infected kidney tissues. The location of M2 and NV in the cell was found to be the nucleus and cytoplasm.

The expression of the NV gene was further analyzed at the level of transcription and the regulation signals for IHNV transcription were investigated. Unique transcriptional initiation and terminational signals for the fish lyssa-like rhabdoviruses were identified. The transcriptional initiation signal, 3'-CGUG-5', was distinctly different from that of the other rhabdoviruses, 3'-UUGU-5'.

The role of the M2 and NV proteins in viral pathogenesis was investigated by transient expression of these proteins individually in cultured fish cells. The M2 protein alone resulted in inhibition of host-directed gene expression at the level of transcription and induction of nuclear fragmentation. The NV protein was not involved in the regulation of the host gene expression, but was involved in another type of cytopathic effect characterized as cell rounding. This is the first biological function attributed to the NV protein.

A PCR method was developed for detecting IHNV N-specific RNA in formalin-fixed, paraffin-embedded fish tissues. The method is sensitive and specific. The technique is capable of detecting viral RNA in samples that have been remained at room temperature in 10% buffered formalin for over 2 years.

A MOLECULAR STUDY OF VIRAL PROTEINS IN THE PATHOGENESIS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

by

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Pinwen Peter Chiou, Author

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CONTRIBUTION OF AUTHORS

Dr. JoAnn Leong was involved in all aspects of this work. Dr. Carol Kim was involved with experiments and interpretation of data for the electron microscopy in Chapter 5, and for the confocal microscopy in Chapter 3 and 5. Dr. Luis Perez was involved in conducting experiments for the glycoprotein labeling in Chapter 3. Dr. Barbara Drolet was responsible for challenging fish and collecting fish samples for the PCR assay in Chapter 6.

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CHAPTER 1
THESIS INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus which causes devastating disease in trout and salmon hatcheries in many countries around the world. The virus can often result in mortality rates of over 90% in young fish and also can cause high mortalities in adult fish of some salmonid species. In order to effectively control the disease, numerous efforts have been devoted to improvement of fish husbandry practices and the development of vaccines and chemotherapeutics. Both improvements must rely on a knowledge of virus replication and pathogenicity.

IHNV infection in fish can result either in death or in the establishment of a persistent virus infection with no apparent disease symptoms or detectable infectious virus (Drolet et al., 1995). In cultured cells, IHNV infection causes shutdown of host protein synthesis (Leong et al., 1983; Hsu et al., 1986), leads to a cytopathic effect characterized as cell rounding, and ultimately, cell death. The pathogenesis of IHNV, including the virus entry, tissue tropism, transmission, and life cycle, has been studied extensively. However, very little is known about the molecular basis of the viral pathogenesis. The work presented in this thesis examines the expression of individual viral genes and their role in pathogenesis. The work provides basic information regarding IHNV replication and should be useful in developing strategies to control the virus at a molecular level.

Two approaches were taken to study the molecular mechanism of IHNV pathogenesis: (1) detection of IHNV protein and RNA synthesis at different periods of the replication cycle; (2) examination of the effect of expression of individual proteins on cell viability. The specific goals were to; (1) further characterize and localize the viral proteins in IHNV infection both *in vitro* and *in vivo* (chapter 3), (2) analyze the expression of the viral mRNA in IHNV infection and identify the transcriptional regulation signals for the virus (chapter 4), (3) investigate the roles of viral proteins in the pathogenesis of IHNV at the molecular level (chapter 5), and (4) develop a PCR technique as a tool to detect the viral pathogen in archived and fixed samples (chapter 6).

These studies have defined roles for the two viral proteins, M2 and NV, in the IHNV pathogenesis. The M2 protein is responsible for the inhibition of host protein synthesis and the induction of the programmed cell death, apoptosis, in IHNV-infected cells. The NV protein was not involved in the regulation of the host gene expression but was able to cause another type of cytopathic effect characterized as cell rounding. Finally, the PCR technique developed here has been used to study IHNV pathogenesis (Drolet et al., 1995).

CHAPTER 2
LITERATURE REVIEW

General Biology of IHNV

Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus that causes an acute, systemic disease which is economically devastating in trout and salmon populations. Because of its economic importance, IHNV has been studied extensively for decades in an attempt to develop appropriate control strategies for the disease.

Historical Perspective

The first reported outbreak of IHNV disease was in 1953 at two Washington state fish hatcheries, Winthrop and Leavenworth (Rucker et al., 1953). The disease was then reported during the 1950's and 1960's, in California (Ross et al., 1960; Parisot and Pelnar, 1962; Yasutake et al., 1965), Oregon (Wingfield et al., 1969), and British Columbia, Canada (Amend et al., 1969).

The viruses isolated in these outbreaks were similar in pathology, cytopathic effect in cell culture, and virion structure as determined by electron microscopy (Amend and Chambers, 1970a & b). It was then proposed that there was a common etiologic agent for the different outbreaks. IHNV acquired its name because necrosis of the hematopoietic tissues of the anterior kidney was the primary internal histological manifestation of the virus (Amend et al., 1969). The other names given to this virus were: British Columbia Virus, the Oregon Sockeye Virus, and the Sacramento River Chinook Disease Virus (Amend and Chambers, 1970 a & b).

Biophysical Properties

Electron micrographic studies of IHNV purified from tissue culture show a bullet-shapped virion of 150 - 190 nm X 65 - 75 nm. The virion is heat, acid, and ether labile. The temperature of replication ranges from 4 to 20 C; with an optimum at 15 C, the virus does not replicate at temperatures higher than 23 - 24 C. Virus added to freshwater at 15 C

survives for 25 days and in sea water for 50 days (Barja et al., 1983; Toranzo and Hetrick, 1982). In cesium sulfate, the buoyant density of the RNA is 1.59 g/ml, with a base composition of 25.4% cytosine, 22.5% adenine, 27.2% uridine, and 24.2% guanine (McCain et al., 1974).

Classification

IHNV is a member of the *Rhabdoviridae* family. Rhabdoviruses are negative sense single-stranded RNA viruses with a host derived envelope. Previously, IHNV and a group of other fish rhabdoviruses including hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV) were classified into the genus *Lyssavirus* with the prototypic *Lyssavirus*, rabies virus. This classification was based on the similarities in virion morphology (Amend and Chambers, 1970a), RNA genome (McCain et al., 1974), and protein profile (McAllister and Wagner, 1975; Hill et al., 1975; Hsu et al., 1986). However, these fish lyssa-like rhabdoviruses differ from the other lyssaviruses by an additional or sixth gene found between the glycoprotein and polymerase genes. These viruses have since been removed from the *Lyssavirus* genus and are currently assigned as unclassified rhabdoviruses (Sixth ICTV Report). Phylogenetic analyses also reveal that these fish lyssa-like viruses form an additional rhabdoviral clade which is separate from those containing members of the *Vesiculovirus* or *Lyssavirus* genera. (Morzunov et al., 1995; Kurath et al., 1996). A new genus, *Piscivirus*, has thus been proposed for these fish lyssa-like rhabdoviruses since all its known representatives have fish hosts (Morzunov et al., 1995; Kurath et al, 1996).

Five types of IHNV have been classified on the basis of the molecular weight differences of the viral glycoprotein (G) and nucleocapsid protein (N) (Table 2.1). The IHNV types showed that a particular virus is geographically specific and that the disease in progeny fish arises from the same type of isolate found in the spawning adults (Hsu et al., 1986). Host species does not determine the IHNV biochemical type. Recently, different methods, including T1 ribonuclease fingerprinting (Oshima et al., 1995), RNase protection assay (Kurath et al., 1995), and phylogenetic analysis (Nichol et al., 1995), have been employed to further characterize the different types of IHNV isolates. These methods all demonstrate that variation in IHNV isolates is geographically related.

Table 2.1 Biochemical types of IHNV (Hsu et al., 1986)

IHNV Type	N protein	G protein
I	40.5 *	67.0
II	42.8	67.0
III	43.25	67.0
IV	40.5-41.0	70.0
V	41.0-44.0	67.0

* Molecular weight x 10^3 dalton

Geographic Distribution

Since the first reports of IHNV, this virus has been identified in many states in the U.S.A. and several countries throughout the world. In the U.S.A. and Canada, the virus is considered to be enzootic in the Pacific Northwest rim. IHNV has also been identified in Minnesota (Plumb, 1972), Montana (Holoway and Smith, 1973), South Dakota (unpublished, cited in Wolf et al., 1973), and West Virginia (Wolf et al., 1973). In Europe, IHNV has been reported in France (Laurencin, 1987), Italy (Bovo et al., 1987), and Belgium (Hill, 1992). In Asia, IHNV has been isolated in Japan (Sano et al., 1977), Taiwan (Chen et al., 1985), and recently Korea (Park et al., 1993). The spread of IHNV is believed to be the consequence of shipment of IHNV-contaminated eggs and fry from the Pacific Northwest of the U.S.A. and Canada (Plumb, 1972; Holway and Smith, 1973; Wolf et al., 1973; Sano et al., 1977; Chen et al., 1985).

Host Range and Susceptibility

The host range of IHNV is relatively broad. IHNV is known to naturally infect rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), coho salmon (*O. kisutch*), amago salmon (*O. rhodurus*), yamame salmon (*O. masou*), Atlantic salmon (*Salmo salar*), and cutthroat trout (*S. trutta*) (for review, see LaPatra, 1987). Bull trout (*Salvelinus*

confluentus), mountain whitefish (*Prosopium williamsoni*) and the marine fish, seabream (*Archosargus rhomboidalis*) and turbot (*Scopthalmus maximus*) have been experimentally infected with IHNV (Castric and Jeffroy, 1991; Bootland et al., 1994). Other hosts may include mayflies (*Callibaetis sp.*) and leeches (*Piscicola salmositica*), (Shors and Winston, 1989; Mulcahy et al., 1990; see transmission below).

The severity of IHNV disease is highest in very young fry (Pilcher and Fryer, 1980); however age is not the only deciding factor in susceptibility since IHNV epizootics have been also found in fish older than one year (Yasutake, 1978; Burke and Grischowsky, 1984; Traxler, 1986; Banner et al., 1991). Other factors might include the virulence of the virus isolates as well as the genetic makeup of the host. It has been demonstrated that there are IHNV strains which are more virulent for particular species of salmonid fish (Chen, 1983). Also, various salmonid hybrids generated by genetic manipulation are less susceptible to IHNV (LaPatra et al., 1992; LaPatra et al., 1993).

Control of IHNV

Currently, the most practical and practiced means for containing the spread of IHNV is avoidance of contaminated stocks and elimination of infected fish. Other methods for controlling the IHNV infection include chemical control, water treatment and vaccination.

(1) Chemical treatment: Addition of a low concentration of iodine to water can produce a 99.9% reduction in infectious IHNV titers after a 7.5 second exposure (Amend and Pietsch, 1972; Batts et al., 1991). In hatcheries, newly fertilized eggs are routinely treated with diluted iodine to reduce the risk of vertically transmission of IHNV.

(2) Water treatment: Ozone and U.V. treatment of water has been shown to be efficient to reduced the mortality in IHNV-infected fish (Wedemeyer et al., 1978; Yoshimizu et al., 1991). However, irradiation of water is not an economically viable method for treating water for hatchery production of fish.

(3) Vaccination: Five types of vaccines have been developed to protect from IHNV infection. These vaccines include killed virus (Amend, 1976; Nishimura et al., 1985), attenuated live virus (Fryer et al., 1976; Tebbit, 1976; Rohovec et al., 1981), purified IHNV glycoprotein (Engelking and Leong, 1989 a & b), subunit vaccines produced *in E coli* (Gilmore et al., 1988; Oberg et al., 1991, Xu et al., 1991), and genetic immunization (Anderson et al., 1996b). These vaccines have been partially successful in conferring a

protective immune response in fish against the IHNV infection. However, to date there are no commercially available vaccines.

Molecular Biology of IHNV

IHNV Genome

IHNV is a member of the *Rhabdoviridae* family whose genomes are typically single-stranded negative sense RNA encapsidated with the viral nucleocapsid protein (N) and phosphoprotein (variously named P, NS, or M1). The typical genome order is 3' - leader (*l*) - N - P - matrix protein (M or M2) - glycoprotein (G) - polymerase (L) - trailer (*l'*) -5' (Banerjee, 1987; Banerjee and Barik, 1992). The IHNV genome has a sedimentation value of 38 to 40 S (Hill, 1975; McCain et al., 1974). The molecular weight of IHNV is 3.7 x 10^6 daltons as measured by migration of the denatured genome RNA in both glyoxal and methyl mercury agarose gels (Kurath and Leong, 1985). IHNV has a sixth gene encoding a nonvirion protein (NV; Kurath and Leong, 1985) and its gene order determined by cDNA cloning, R-loop mapping (Kurath et al., 1985), and nucleotide sequence analysis (Morzunov et al., 1995; Schutze et al., 1995) is 3' - *l* - N - M1 - M2 - G - NV - L - *l'* -5'.

The 3' and 5' ends of IHNV contain sequences thought to be involved in replication. Sequence analysis has revealed the presence of a 60 nucleotide putative leader sequence at the 3' end and a 101 nucleotide trailer sequence at the 5' end of the genome (Morzunov et al., 1995; Schutze et al., 1995). For vesicular stomatitis virus (VSV), the prototype rhabdovirus, the leader sequence was found in a non-polyadenylated form. For IHNV, the leader sequence is part of the N mRNA as revealed by sequence analysis of the IHNV N mRNA (Gilmore and Leong, 1988). The 3' and 5' ends of IHNV genomic RNA are complementary to each other, which is a common feature of the negative-stranded RNA viruses (Morzunov et al., 1995; Schutze et al., 1995). It has been suggested that the complementary termini of these viruses may form panhandle structures which may prime the initiation of RNA synthesis (Banerjee and Barik, 1992).

The internal gene junctions of IHNV, and the other fish lyssa-like rhabdoviruses are different from that of other rhabdoviruses. In IHNV, HIRRV and VHSV, a uniquely conserved sequence, 3'UCURUC(U)7RCCGUG(N)4CACR -5', exists between internal genes on the genome (Morzunov et al., 1995; Schutze et al., 1995; Kurath et al., 1996).

The sequence, 3' -CGUG-5', has been suggested as a putative transcriptional initiation signal, and the sequence, 3' - UCURUC(U)7, is a putative transcriptional termination/polyadenylation (TTP) signal. A shorter intergenic sequence, 3' -C(U)7NNUUGU -5' (vRNA-sense), is conserved in the internal gene junctions of all other rhabdoviruses characterized to date (Banerjee, 1987; Banerjee and Barik, 1992).

The leader-N junction and L-trailer junction sequences have a lower degree of similarity relative to the internal gene junctions, reflecting differences in the function of these control regions. The IHNV leader-N junction contains sequences which are highly similar to the putative initiation signal seen at regular gene junction, but lacks the (U)7 sequence considered to be essential for polyadenylation. The lack of the (U)7 signal in the leader-N junction is consistent with the presumed lack of polyadenylation of the leader (Gilmore and Leong, 1988). Also, the L-trailer junction possesses the TTP signal but lacks the putative transcriptional initiation signal, consistent with the lack of a downstream transcript (Morzunov et al., 1995).

The NV Genes and the G-L Intergenic Regions of Rhabdoviruses

The G-L junction of the *Rhabdoviridae* is genetically plastic. The typical genome structure of rhabdoviruses is 3'- N-P(NS, M1)-M(M2)-G-L -5'. However, IHNV has a sixth gene, NV, and was the first rhabdovirus found to vary from that organization (Kurath and Leong, 1985; Kurath et al., 1985). Additional genetic information has since been identified in the G-L junction of several rhabdoviruses. Recently, the NV genes have also been found in two other fish lyssa-like viruses. An NV gene is found in a close relative of IHNV, hirame virus (HIRRV) (Kurath et al., 1996), and a distant relative, viral hemorrhagic septicemia virus (VHSV) (Basurco and Benmansour, 1995). As such, the NV gene is considered to be a specific feature of these fish lyssa-like rhabdoviruses. No significant similarity has been found between the NV genes and the additional nucleotide sequences of other rhabdoviruses at the amino acid level.

Sequence analyses have shown that VSV has only a dinucleotide spacer, GG, at the intergenic region between G and L (Tordo et al., 1992). However, numerous base insertions and deletions have been found in the 5' noncoding region (v-sense) of the G genes of VSV isolates (Indiana serotype) from Central America. These changes resulted in significant variation of the G gene sizes, with gene lengths ranging from 1,652 to 1,868

nucleotides. The ORF of the G gene is constant, but the 3' noncoding regions of the G mRNAs vary (Bilssel and Nichol, 1990).

The G-L junction of the rabies virus genome was found to have 423 nt of sequence flanked by two TTP-like signals (Tordo et al., 1986). This sequence, named ψ is similar in length to the NV genes of IHNV, HIRRV and VHSV. It was suggested that in RV, y was a remnant or pseudogene which was no longer required by the adaptation of the virus to replication in mammalian cells. Further analyses revealed that only a few attenuated strains of RV derived from the original Pasteur strain had the ψ gene (Ravkov et al., 1995). The wild-type rabies viruses were missing the upstream TTP signal immediately following the G coding region that marked the beginning of the presumptive rabies pseudogene (Ravkov et al., 1995). The exact function of these noncoding sequences is still unknown. It has been suggested that they might influence mRNA interaction with host proteins and RNA stability (Ravkov et al., 1995). In the attenuated rabies, two types of mRNA were transcribed from the G gene, a smaller G mRNA and a larger G-ψ mRNA. There were distinct differences in the ratio of the smaller and larger mRNA species in the different cell types (unpublished data cited in Tordo et al., 1992). Phylogenetic analysis of sequence differences provided no evidence that the attenuated strains containing two TTP-like signals were ancestral to any of the wild-type viruses possessing only the downstream TTP sequence motif (Ravkov et al., 1995).

The ephemeroviruses also have additional genes in the G-L junctions. The bovine ephemeral fever virus, BEFV, has 8 to 9 genes including 4 to 5 genes located at the G-L junction. There was a nonvirion glycoprotein (Gns) gene following the virion glycoprotein (G), and 4 additional open reading frames (α1, α2, β, and γ) located between the Gns and the L gene (Walker et al., 1992). The α1, α2, β, and γ ORFs were transcribed as polycistronic (α1-α2), (α1-α2-β-γ) and (β-γ) mRNAs and monocistronic, γ, mRNA. The Adelaide River virus, another member of this genus, was shown to have α1, α2, and β ORFs but not the γ ORF. These ORFs were also located between the Gns and the L gene (Wang et al., 1994). The function of these additional genes is still unknown.

Among some rhabdoviruses, additional genes have been identified in genome regions other than the G-L junction. Sonchus yellow net virus (SYNV), the best characterized plant rhabdovirus, has a sixth gene, sc4, encoding a membrane-associated protein of unknown function located between the P and M genes (Heaton et al., 1989; Scholthof et al., 1994). The sigma virus, a pathogen of *Drosophila* species, has four genes mapped upstream of the G gene (Teninges et al., 1993). The gene 2 has been shown to encode the phosphoprotein and the gene 3 encodes a protein related to the reverse

transcriptase of retroelements (Devauchelle et al., 1995). Flanders virus, a rhabdovirus of birds, also encodes at least seven virus-associated proteins but the gene order has not been determined (Boyd and Whitaker-Dowling, 1988). In addition to the N, P, G and L proteins, the virus has a 43 kDa protein which is cleaved to 23 kDa, 27 kDa and 19 kDa proteins.

Transcription of IHNV mRNAs

mRNA Species

Six IHNV mRNA species were identified in infected cells as determined by denaturing gel electrophoretic separation of [^{3}H]-uridine-labeled polyadenylated RNA (Kurath and Leong, 1985). No readthrough mRNA species has been reported for IHNV although readthrough transcription products have been identified in some rhabdoviruses (Masters and Samuel, 1984; Wunner et al., 1993; Wang et al., 1994). The six mRNA species are: L mRNA, 2.26 x 10^6 Da; G mRNA, 5.63 x 10^5 Da; N mRNA, 4.48 x 10^5 Da; M1 mRNA, 3.00 x 10^5 Da; M2 mRNA, 3.00 x 10^5 Da; and NV mRNA, 1.95 x 10^5 Da. The M1 and M2 mRNAs comigrate in both glyoxal and methylmercury gels. The N mRNA is thought to be the first mRNA species produced intracellularly in infected CHSE-214 cells. The molar ratio of these mRNA species was determined as follows: N mRNA, 1.00; M1 and M2 mRNAs, 2.52 ±0.40; G mRNA, 0.49 ± 0.03; NV mRNA, 0.41 ± 0.14; and L mRNA, 0.02 ± 0.01 (Kurath and Leong, 1985). The combined ratio of 2.52 for the M1 and M2 mRNA species indicates that one or both of these mRNA species are present in molar quantities greater than the N mRNA.

The mRNAs of rhabdoviruses are generally considered monocistronic because they each encode a single translation product. However, it has been shown among numerous paramyxoviruses and rhabdoviruses, that the phosphoprotein genes may encode more than one protein (Lamb et al., 1976; Curran et al., 1992; Spiropoulou and Nichol, 1992). These proteins are encoded by the same ORF or by overlapping ORFs recognized through leaky ribosomal scanning (Chenik et al, 1995) or through scanning-independent internal ribosomal initiation (Currant and Kolakofsky, 1989). So far, there is no evidence that the M1 or other genes of IHNV encodes a polycistronic mRNA.

Transcriptional Signals

The sequence 3'-UUGU-5', which is located in the conserved intergenic region, has been shown to function as a transcriptional initiation signal for the rhabdoviruses (Banerjee and Barik, 1992). However, in fish lyssa-like rhabdoviruses, a uniquely conserved sequence, 3'- UCURUC(U)7RCCGUG(N)4CACR -5', exists between the genes on the genome. Some researchers have suggested the sequence, 3'-CGUG -5', based on the conservation of its composition, and locations on the viral genome as a putative transcriptional signal unique to the fish lyssa-like rhabdoviruses (Morzunov et al., 1995; Bjorklund et al., 1996). Other researchers have shown that the M2 mRNA of VHSV initiates at or near the sequence, 3'-UUGU-5' (Benmansour et al., 1994). However, unlike other rhabdoviruses, for the fish lyssa-like rhabdovirus, the tetranucleotides, 3' -UUGU -5', is not well conserved and is not located at the corresponding position on the genome as in other rhabdoviruses. Therefore, further studies were needed to verify these hypotheses. In chapter 4, we have demonstrated that the sequence, 3'-CGUG-5', instead of the sequence, 3'-UUGU-5', serves as the transcriptional initiation signal of IHNV. We have also demonstrated that the sequence, 3' -UCURUC(U)7, serves as the transcriptional termination/polyadenylation signal for the IHNV RNA polymerase.

In vitro Transcription/Translation System

An *in vitro* transcription system for IHNV was first reported by McAllister and Wagner (1977). The transcripts produced *in vitro* ranged from 9 S to 17 S in size, with no discrete species. Hybridization to the viral genome showed that these RNA transcripts were IHNV-specific. In later studies, the *in vitro* synthesized transcripts were identified by translation of hybrid selected mRNA (Kurath and Leong, 1987). Optimal polymerase activity was obtained when purified virion was incubated in HEPES buffer supplemented with S-adenosyl-L-methionine. The RNA transcripts produced in this system contained polyadenylated species which comigrated with IHNV N, M1, M2, G, and NV mRNAs from IHNV-infected cells. The transcripts were shown to be functional mRNAs by an *in vitro* translation reaction in which the N, M1, and M2 proteins were identified. This system has demonstrated that the transcription and translation of IHNV can be accomplished in a cell-free environment.

Transcription and Replication

The transcription of negative single-stranded RNA viruses is thought to be initiated at a single site at the 3' end of the viral genome (Banerjee, 1987). All negative-stranded RNA viruses have complementary termini which may form panhandle structures of 10 to 20 bp in length, and may be part of the RNA synthesis initiation signal. Two important features of VSV transcription are the polarity of mRNA accumulation in relation to the gene order and sequential genes transcription. The quantities of the transcripts follow the order of the genes from the 3' to the 5' end of the genome (Iverson and Rose, 1981; Villareal et al., 1976). IHNV transcription is also attenuated sequentially as suggested by the molar ratios of the IHNV mRNA in infected cells (Kurath and Leong, 1985).

The mechanism of IHNV genome replication is unknown. In VSV, continued viral protein synthesis is essential for replication. It is postulated (Blumberg et al., 1981) that the N protein, available in large quantities in the late phase of viral growth, binds to the nascent RNA transcript and prevents the viral polymerase from recognizing termination signals on the genome. This results in synthesis and encapsidation of full length antigenome (+ strand RNA), which serves as a template for synthesis of the genome (- strand RNA). In a similar manner, N protein may also act as an antiterminator at each intergenic junction and suppress accessory events such capping and polyadenylation. It is believed that IHNV replication should follow a similar mechanism.

IHNV Proteins

The virion of IHNV consists of five virion-associated proteins, including N, M1, M2, G, and L (McAllister and Wagner, 1975; Hill, 1975; Kurath et al., 1985). In addition, a non-virion protein, NV, with a molecular weight of 12 kDa was synthesized in IHNV-infected cells (Kurath and Leong, 1985; Kurath et al., 1985). All of the genes encoding these six proteins have been cloned and sequenced. Overall, as for most other fish virus proteins, only the structural similarities are conserved when the IHN viral proteins are compared with other viruses from the same family (Bernard and Bremont, 1995). However, putative functional domains conserved in all reported L proteins of rhabdoviruses are also present in the IHNV L protein.

Nucleocapsid protein (N)

The complete nucleotide sequences of cDNA clones that encode the N proteins from three IHNV isolates have been reported (Gilmore and Leong, 1988; Morzunov et al., 1995; Schutze et al., 1995). The N protein is 413 amino acids in length (Gilmore and Leong, 1988) and has been shown to be a phosphorylated protein as evidenced by incorporation of ^{32}P-labeled orthophosphate (McAllister and Wagner, 1975; Hsu et al, 1984). Rhabdovirus genomes are tightly associated with the viral N protein. The carboxyl terminal of the IHNV N protein may be involved in binding of the N protein to the viral RNA (Gilmore and Leong, 1988). The intracellular concentration of the N protein of VSV plays a crucial role in regulating the balance between viral transcription and replication in the cell as described above. It is believed that the IHNV N protein performs the same function in the IHNV replication cycle in the infected cell.

N protein is the most abundant protein in the viral virion as well as in infected cells. Because of this, the N protein has been used as a diagnostic probe for IHNV infection and for the study of IHNV pathogenesis (reviewed later).

Phosphoprotein (M1)

The M1 protein has been shown to be a phosphorylated protein by incorporation of ^{32}P-labeled orthophosphate (McAllister and Wagner, 1975; Hsu et al, 1984). The phosphorylation of the phosphoprotein of VSV has been shown to be essential for the transcriptional activity (Banerjee and Barik, 1992). The deduced M1 amino acid sequence of IHNV RB1 and K strains contain 5 potential phosphorylation sites, SXXD/E, which are concentrated mostly within the first half of the amino acid sequence (Ormonde, 1995). However, the reported consensus sequence, TXXD/E, for the VHSV M1 protein has not been found in the M1 protein of IHNV (Benmansour, 1994). The M1 protein of IHNV is a basic protein with an estimated pI of 8.4, similar to that for the M1 protein of VHSV, which has a pI of 9.46 to 9.89 (Benmansour et al., 1994; Ormonde, 1995). This property is drastically different from the M1 proteins of RV and VSV whose pI is 4.36 and 4.84 respectively.

The phosphoproteins among VSV strains are extremely divergent with only 33% similarity at the amino acid level as reported for the P proteins of the major serotypes, although the overall structure of the protein is conserved (Gill and Banerjee, 1985). In

contrast to the high degree of divergence of the phosphoprotein in VSV strains, the phosphoproteins of RV strains shows a high degree of similarity (92 to 98%; Larson and Wunner, 1992). The IHNV M1 genes also share a high degree of similarity (94%) between the RB1 strain and a European K strain (Ormonde, 1995). The IHNV M1 gene also shares 63% homology with HIRRV and 38% with VHSV (Ormonde, 1995). Sequence analysis revealed that the M1 genes of different IHNV strains potentially encode a second overlapping ORF. This putative ORF is highly basic with an estimated pI of 10.1 to 12.8 and is rich in arginine (Morzunov et al., 1995; Ormonde, 1995). The existence of this peptide has not yet been proven, but the characteristics of its deduced amino acid sequence are similar to the proteins encoded within the P genes of VSV (Spiropoulou and Nichol, 1993) and RV (Chenik et al., 1995). These proteins are found in infected cells and are not virion-associated.

The VSV phosphoprotein, P, is associated with the viral polymerase and is essential for viral transcription and replication (for review, see Banerjee and Barik, 1992). Although the IHNV M1 has been cloned and sequenced, the analysis of its biological function(s) in the IHNV life cycle has not been analyzed.

Matrix Protein (M2)

The IHNV M2 protein is highly basic, with an estimated pI of 10.08, similar to that of VHSV (10.07 for 07-71 strain and 10.23 for Makah strain) (Benmansour et al., 1994; Ormonde, 1995). VSV M protein is known to be phosphorylated in VSV-infected cells (Clinton and Huang, 1981); however, the IHNV M2 is not phosphorylated as indicated by the lack of incorporation of ^{32}P-labeled orthophosphate (McAllister and Wagner, 1975; Hsu et al, 1984). The deduced IHNV M2 amino acid sequence shares 74% identity (84% similarity) with HIRRV M2 and 39% identity (60% similarity) with VHSV M2. However it shares no significant homology with either VSV, RV or the fish vesiculovirus, SVCV (Benmansour et al., 1994; Ormonde, 1995). Despite low similarity in the amino acid sequence, the IHNV M2 is similar to RV and VSV in being a highly basic protein that contains a high content of charged amino acids clustered at the amino terminus (Rose and Gallione, 1981; Bourhy et al., 1993; Ormonde, 1995). This region has been shown in VSV to be essential for stable interaction with the plasma membrane (Chong and Rose, 1994) and for viral assembly (Black et al., 1993).

Rhabdoviral matrix proteins (M or M2) are multifunctional proteins (Coulon et al., 1990). M protein is a major structural component of the virion and plays a central role in the initiation of virion assembly by forming a bridge between the ribonucleocapsid core and the host plasma membrane (Bergmann and Fusco, 1988). The M protein of VSV alone is responsible for the cytopathic effect as evidenced by inducing the rounding of polygonal cells, which is typical of VSV infection (Blondel et al., 1990). In addition, VSV M protein has been shown to inhibit host-directed transcription of a plasmid-encoded target gene in transfected cells (Black and Lyles, 1992). Interestingly, VSV M protein can stimulate host-directed translation under the same conditions in which it potently inhibits transcription (Black et al., 1994). Under these conditions, the combined effect of M protein expression results in a 20-fold decrease in host gene expression (Black et al., 1994). Furthermore, cotransfection with an infectious clone of human immunodeficiency virus type 1 (HIV-1) demonstrates that VSV M protein alone can inhibit expression from chromosomally integrated DNA. This finding strongly supports the hypothesis that the VSV M protein is involved in the shutoff of host cell transcription (Paik et al., 1995). It remains unknown whether the matrix proteins of other negative-stranded RNA virus have similar effects.

Glycoprotein (G)

The IHNV glycoprotein is a membrane-associated protein which forms spikelike projections on the surface of the mature virion (McAllister and Wagner, 1975). The glycosylation of IHNV G protein has been demonstrated by incorporation of ^{3}H-labeled glucosamine (McAllister and Wagner, 1975) and by enzymatic digestion (Engelking, 1987). Differences in the forms of oligosaccharides appear to account for the differences of the molecular weight of G proteins among several isolates (Engelking, 1987). Like the other rhabdoviruses, the IHNV G protein has a domain of 20 amino acids at the N terminus which might act as a signal peptide during transport of the G protein to the plasma membrane (Koener et al., 1987).

For VSV and RV, the G proteins are responsible for eliciting both neutralizing antibody and a protective immune response (Kelly et al., 1972; Cox et al., 1977; Wiktor et al., 1984). The IHNV G has also been demonstrated to be the only IHN viral protein capable of evoking neutralizing antibody in rabbits, and inducing protective immunity in fish vaccinated by immersion or injection with the purified G protein (Engelking and Leong, 1989b). Also, trout vaccinated with IHNV in Freund's complete adjuvant elicited a

response to G by immunoblotting (Mourich and Leong, 1991). Cross-protective immunity to different biochemically defined types of IHNV has been demonstrated in fish immunized with the purified G protein from a type 1 isolate. This result indicates the existence of at least one common neutralization epitope on the G protein (Engelking and Leong, 1989a). The altered carbohydrates of the G protein may modify the host immunological response by hiding epitopes or directing antibodies to other epitopes (Engelking, 1987). However, the viral G protein in its nonglycosylated form is sufficient for developing a subunit vaccine for IHNV (Xu et al., 1991) as suggested by the finding that some G protein epitopes are not carbohydrate in nature, or dependent on carbohydrates for the immunogenic conformation (Engelking and Leong, 1989b). Genetic immunization of rainbow trout against IHNV infection has been recently evaluated with plasmids encoding the IHNV G or N protein (Anderson et al., 1996). Fish injected with the plasmid DNA encoding the G protein alone or in combination with plasmid DNA encoding the N protein elicited a G protein-specific antibody response and were protected from subsequent IHNV challenge.

A relation between mutation of the G protein sequence and the virulence of the virus has been observed. Kim et al. (1994) analyzed the virulence and pathogenecity of four IHNV neutralization-resistant variants, which were selected with a G-specific monoclonal antibody (Roberti et al., 1991). This study demonstrated a strong correlation between the secondary structure of the G protein with altered tissue tropism and virulence. Thus, IHNV G , like that of RV and VSV, plays an integral part in the pathogenesis of viral infection.

Nonvirion Protein (NV)

The nonvirion protein, NV, was first found in IHNV RB1-infected cells but was not present in the purified virion (Kurath and Leong, 1985). The molecular weight of the NV protein was determined by SDS-PAGE analysis to be approximately 12 kDa (Kurath and Leong, 1985). The nucleotide sequence of the NV gene of the IHNV RB1 isolate has been characterized and shown to contain one major and one potential secondary open reading frame (ORF), capable of encoding 12 and 6.5 kDa proteins respectively (Chiou and Leong, chapter 4). Recently, NV gene was also identified in the G-L junction of a closely related virus, HIRRV (Kurath et al., 1996), and a distantly related virus, VHSV (Schutze et al., 1996).

The NV gene is highly conserved among IHNV isolates. The NV gene sequence has now been determined for 14 strains including 13 isolates from North America and one from Europe (Nichol et al., 1995; Schutze et al., 1995; Kurath et al., 1996; Chiou and Leong, chapter 4). These genes have greater than 97% identity at the deduced amino acid level and are 111 amino acids in length, with a high content of charged amino acids. However, the overall pI is approximately 7. The potential secondary ORF is also maintained in some of these isolates, but the protein's existence has not been confirmed. The NV amino acid identity values between VHSV and IHNV or HIRRV are not conclusively higher than background levels of identity to clearly unrelated proteins. However, if the amino acid similarity values are compared between these proteins the similarity is significant (amino acid identity/similarity of 23.3%/47.6% and 16.5%/40.4% for VHSV vs. IHNV and HIRRV respectively), indicating a distant but discernible relationship between these genes (Kurath et al., 1996). Putative phosphorylation sites have been identified in the NV proteins of IHNV, VHSV, and HIRRV (Nichol et al., 1995; Schuetze et al., 1995; Kurath et al., 1996; Chiou and Leong, chapter 4). However, it is unknown whether the NV proteins are indeed phosphorylated proteins.

The expression of the NV protein in infected cells has been problematic. The NV protein was found to be expressed in high quantities in IHNV-infected cells in an original study by using [^{35}S]-methionine labeling (Kurath and Leong, 1985). However, subsequent reports have shown that the expression of the NV protein is either extremely low or below detection limit by [^{35}S]-methionine labeling in IHNV-, VHSV- and HIRRV-infected cells (Nishizawa et al., 1991 a & b; Basurco and Benmansour, 1995). In the case of VHSV, a protein with the expected NV gene product size (14 kDa) was precipitated from radioactively-labeled, virus infected cells only by immunoprecipitation (Basurco and Benmansour, 1995). Nonetheless, a recent report by Schutze and colleagues (1996) documented both VHSV and IHNV NV protein expression in cell culture at levels detectable by immunofluorescence and Western immunoblot. This discrepancy of different investigator's ability to detect the NV protein has not yet been resolved.

The function of the NV protein is also unknown. No significant functional motifs were identified between the deduced NV protein sequences of IHNV and VHSV or any proteins encoded by other rhabdoviruses. Efforts to find homology of NV to other characterized protein sequences in the GenBank database failed to detect any highly significant regions of amino acid identity (Nichol et al., 1995; Basurco and Benmansour, 1995). Apparently the NV protein is not essential for viral transcription as suggested by the fact that the transcription of IHNV mRNAs can be accomplished in *in vitro* reactions

containing lysed purified virion (Kurath and Leong, 1987). However, the high degree of conservation of the NV gene among IHNV isolates implies that the NV gene should have an important role in the life cycle of the virus (Nichol et al., 1995). Thus the NV gene may be involved with other steps in viral replication or may influence host cell functions. The limited sequence similarity between NV proteins of VHSV and IHNV suggests that the structure, rather than the sequence, is important for the NV protein function (Basurco and Benmansour, 1995). The fact that the NV genes have been found only in viruses infecting fish living in water of low temperature has lead to the hypothesis that the NV genes may be specific to virus replication at colder temperatures (Kurath and Leong, 1985; Kurath et al., 1996). In addition, the extremely low expression of the NV proteins in both IHNV and VHSV indicates that the NV proteins are probably required in catalytic amounts or, alternatively, may be nonfunctional (Basurco and Benmansour, 1995).

Polymerase (L)

In rhabdoviruses, the L protein, by interacting with phosphoprotein and nucleocapsid protein, is responsible for viral RNA transcription and genome replication (Banerjee and Barik, 1992). Genetic and biochemical reconstitution experiments have suggested that the VSV L protein itself may encode all transcriptional activities. These activities include; RNA-dependent RNA polymerase (De and Banerjee, 1985), cap methylase (Hammond and Lesnow, 1987; Hercky et al., 1988), and poly(A) polymerase (Hunt et al., 1984). The L protein is also a protein kinase involved in the phosphorylation of the phosphoprotein, which is essential for viral transcription and replication (Sanchez et al., 1985; Masters and Banerjee, 1986).

The partial or full length cDNA sequence has been determined for the L gene of three IHNV isolates (Bjorklund et al., 1995; Morzunov et al., 1995; Schutze et al., 1995). However, a cDNA clone encoding a functional L protein has not been reported so far. The L gene is more conserved than the other viral genes at the amino acid level when compared to RV and VSV (Morzunov et al., 1995). All of the six conserved amino acid motifs in the reported L gene amino acid sequences are present in the IHNV L protein with some variation in the proposed catalytic QGDNQ motif (QGDNV in IHNV) (Morzunov et al., 1995; Schutze et al., 1995). It is believed that the IHNV L protein performs the same functions in viral transcription and replication as the VSV L protein. As an example, the

RNA species transcribed in an *in vitro* transcription reaction were polyadenylated (Kurath and Leong, 1987).

All five IHN virion-associated proteins exhibit the structural characteristics of their rhabdoviral counterparts. So far, the genes encoding N, G, M1, M2 and NV proteins have been cloned and sequenced, but, except for the G protein, the role of the other genes in IHNV pathogenesis has not been determined.

Pathology of IHNV

IHNV infection causes severe mortality in young fish, fry or fingerlings. Typically, in an experimental IHNV epizootic, young fish infected with virus will have external signs of infection 3 to 5 days post exposure (dpe). Mortalities usually begin 4 to 5 dpe, reach their peak approximately 10 dpe, and then decline. By 40 to 50 dpe no further mortalties are observed.

Clinical Signs

Fish infected with IHNV are typically found to exhibit several clinical signs; anorexia, lethargy, with brief episodes of frenzied swimming, avoidance of currents, rolling, swimming vertically or circling and flashing are all common. Externally, typical signs caused by IHNV infection include; abdominal swelling, petechial hemorrhages on the ventral surface and at base of pectoral and pelvic fins, exophthalmia, a pale ventral surface, a dark dorsal surface and occasionally fecal casts (Amend et., 1969). Internally, the kidney and liver are anemic, and hemorrhages may be found in the muscle, fat and liver.

Internal pathology includes severe necrosis of the hematopoietic tissues in the anterior kidney, and, to a lesser degree, in spleen, liver, pancreas, and the alimentary tract (Yasutake et al., 1970; Yasutake and Amend, 1972)..

Serological changes in infected fish were first reported by Amend and Smith (1975). The changes include reduced corpuscular counts, hemoglobin, and packed cell volume. But, the mean corpuscular volume, the mean corpuscular hemoglobulin, and the mean corpuscular hemoglobin concentration remain normal. The percentage of immature

erythrocytes increases, but the percentage of leukocytes do not changed. There is a significant decrease in neutrophils and an increase in lymphocytes. Plasma electrolytes and osmolality are also significantly reduced. The pH of plasma increases.

Diagnosis

In the laboratory, IHNV typically is detected by isolating the virus in tissue culture and then confirming its identity by serum neutralization. These methods are sensitive for infectious virus but are labor intensive and often do not provide an answer until long after the epizootic. For example, the recommended incubation time for tissue culture plaque assay is 10 to 14 days, although this can be shortened if cells are pretreated with polyethylene glycol (Batts and Winton, 1989), or the polycation, polybrene (Leong et al., 1982).

Recently, new methods for detecting both infectious and noninfectious forms of IHNV have been developed using immunological and molecular biological techniques. Immunoassays have been developed for IHNV detection and to characterize the progression of IHNV infection. A staphylococcal coagglutination test has been developed to detect virus in cell cultures and infected fish in 15 minutes, but a titer of 10^6 pfu/ml is required to obtained a positive reaction (Bootland and Leong, 1992). Immunodot (McAllister and Schill, 1986; Eaton et al., 1991; Ristow et al., 1991), immunoblot (Schultz et al., 1989), and immunofluorescence (LaPatra et al., 1989; Arnzen et al., 1991; Jfrgensen et al., 1991) assays are used to rapidly detect IHNV using monoclonal and polyclonal antisera. Immunohistochemistry techniques have been used to detected IHNV proteins in fixed tissue culture cells (Drolet et al., 1993) and to reveal the route of entry and pathogenic progression of IHNV in fish (Drolet et al., 1994).

Molecular techniques such as PCR and in situ hybridization have also been employed to study IHNV and for rapid detection. Viral mRNA can be detected in situ by hybridization with a probe to the N gene, and specific infected cells in the major target organs have been identified (Anderson et al., 1991). A polymerase chain reaction (PCR) has been developed to amplify a portion of the N gene of IHNV directly from viral genomic RNA in freshly sampled fish tissues (Arakawa et al., 1990). This method was shown to be rapid, specific and very sensitive. Additionally, as described in chapter 6, a PCR method has been developed to detect IHNV RNA in formalin-fixed, paraffin-embedded tissues

(Chiou et al., 1995). This technique is capable of detecting viral RNA in samples that have remained at room temperature in 10% buffered formalin for over 2 years.

Pathogenesis

Entry and Progression

Several studies have suggested the gills as the initial site of IHNV entry and infection (Mulcahy et al., 1983; Burke and Grischkowsky, 1984; Yamamoto et al., 1989; Yamamoto and Clermont, 1990; Drolet et al., 1994). It was also reported that the skin was shown to be the major site of IHNV replication and the gill played a lesser role in the initial stages of infection (Yamamoto et al., 1990). Some studies also suggest an alternate or additional origin of IHNV infection. Mulcahy et al. (1983) reported that infectious virus was detected in the viscera of naturally infected sockeye in the absence of virus in the gills. Moreover, virus has been detected in the visceral organs of rainbow trout before it appeared in the gills (Yamamoto et al., 1990; Yamamoto and Clermont, 1990).

Recently, a detailed study of the route of entry and progression of IHNV in rainbow trout has been reported (Drolet et al., 1994). This study revealed that IHNV infection progressed from two major sites: the gills and the gastrointestinal tract. It appeared that both routes of entry eventually resulted in systemic viremia through the circulatory system and that the connective tissue is the major site of virus replication. The study also showed that the skin was more indicative of a transient infection than a productive infection.

Tropism

The main target of IHNV is the hematopoietic tissue in the kidney of the fish. IHNV is also found in epithelial cells of numerous organs. Among them, the brain is the most restrictive area (Drolet et al., 1994). Drolet et al. (1994) showed that IHNV exhibits a specific tissue tropism for connective tissue, while the virus readily infects epithelial cells. It has been shown that the infection of epithelial cells appears to be transient and not highly productive, and that the majority of cells undergoing productive viral infection are of connective tissue origin. Recently, Kim et al. (1994) has demonstrated a connection

between the altered tissue tropism and mutations in the G protein of IHNV. The virulence and tissue tropism of IHNV is strongly correlated to a change of threonine to an isoleucine at amino acid 78 and glutamic acid to glycine at amino acid 218 of the G protein.

Transmission

Two routes of transmission of IHNV, horizontal and vertical, has been documented. Virus can be transmitted horizontally through feces, urine, and ovarian or seminal fluid (Pilcher and Fryer, 1980; Mulcahy et al., 1983; Mulcahy and Batts, 1987; Nishimura et al., 1988). Also, it has been demonstrated that IHNV can remain infective for several months in water near spawning fish and can infect yearling fish held down stream (Mulcahy et al., 1983).

Evidence for vertical transmission is, however, circumstantial. It has been shown that infectious IHNV can be isolated from dead egg (Mulcahy and Pascho, 1985), ovarian fluid (Mulcahy and Batts, 1987; Lapatra et al., 1990), and sperm (Mulcahy and Pascho, 1984). The strongest evidence for vertical transmission comes from the association made between the appearance of the disease and the shipment of infected eggs into new geographic areas (Holway and Smith, 1973; Plumb, 1972; Wolf et al., 1973). In the laboratory, eggs or sperm incubated with IHNV, or eggs injected with IHNV, did not result in mortalities in the progeny (Yoshimizu et al., 1989). However, virus injected into eyed eggs did cause infection in 90% of the embryos. Recently, an extensive fertilization study demonstrated no vertical transmission in steelhead populations (LaPatra et al., 1991). Therefore, there is still no conclusive evidence for vertical transmission of IHNV.

Efforts have been undertaken to identify a reservoir for IHNV. IHNV has been isolated from a freshwater leech and a copepod (*Salmincola* sp.) obtained from the gills of infected, spawning sockeye salmon (Mulcahy et al., 1990). Also, virus has also been isolated from mayflies found near IHNV epizootic sites (Shors and Winston, 1989). These organisms may serve as a reservoir of infection; however, it is still unclear whether the virus replicates in the invertebrate host or whether there is virus transmission from the host to fish.

Molecular Pathogenesis

Although pathogenesis of IHNV has been studied extensively, the molecular basis of IHNV pathogenesis is still unknown. It has been shown, however, that IHNV infection can shut down the synthesis of host proteins in infected cells (Leong et al., 1983; Hsu et al., 1985). Inhibition of cellular macromolecules synthesis is a common effect caused by rhabdoviruses in susceptible cells. The VSV wild type leader RNA and M protein have been shown to inhibit cellular transcription presumably at different stages in infection (Wagner et al., 1984; Black and Lyles, 1992; Pike et al., 1995). The leader RNA was also shown to inhibit cellular DNA synthesis (Wagner et al., 1984; Remenick et al., 1988). Translation-inhibition by VSV appears to occur at the initiation stage, but it is not clear whether the target is eIF-3/eIF-4B or eIF-2 (Wagner,1984), and which viral component may be involved in the regulation.

Persistence of IHNV

Persistence of IHNV has been shown to occur in infected CHSE-214 cells (Engelking and Leong, 1981). High levels of virus were found to be released from the persistently infected cells, which continued to grow well in culture. Persistence is mediated by several factors including the development of temperature-sensitive mutants, small plaque mutants, and defective interfering (DI) virus particles. Endogenous interferon production is probably not involved in the maintenance of virus persistence, since persistently infected cells were susceptible to infection with a heterologous virus.

Persistence of IHNV in fish has been controversial for the last three decades. In general, infectious virus can be detected by plaque assay from fish surviving an epizootic for approximately 40 to 50 days postexposure, after which infectious virus can no longer be isolated. However, infectious virus can be detected again when these fish reach sexual maturity and return from the ocean to spawn. It is unknown whether infectious virus isolated from spawning adults is the result of reinfection from secondary reservoirs during their migration upstream or whether reactivation of a latent form of IHNV occurs in the survivor. Two studies so far have provided evidence for the persistence of IHNV. Amend (1975) reported that IHNV was recovered from the reproductive fluids from 33% of the survivors at their maturity. A similar result reported by Busch (1984) showed that virus was isolated from 2.56% of male and 2.84% from female IHNV survivors.

Despite these findings, many other trials have not been able to confirm these findings and thus doubt has arisen about the existence of a life long carrier state. Recently, IHNV has been identified in mature rainbow trout survivors 1 year after the epizootic. The presence of virus in the survivors was shown by the presence of viral protein detected by immunohistochemistry, viral RNA detected by PCR amplification, and IHNV-truncated particles detected by immunogold electron microscopy. These last results provide evidence for a life long carrier state in IHNV survivors.

Necrosis and Apoptosis

The interaction between viruses and their hosts often result in the death of the infected cells. There are two major morphologically and biochemically distinct modes of death in eukaryotic cells, necrosis and apoptosis. Necrosis is considered a pathological reaction that occurs in response to major disturbances in the cellular environment, such as complement attack, severe hypoxia, hyperthermia, and lytic viral infection. These stimuli increase the permeability of the plasma membrane resulting in irreversible swelling of the cells (Wyllie et al., 1980). Apoptosis, also called programmed cell death, is considered to be a physiological process that is part of homeostatic regulation during normal tissue turnover. Apoptosis also occurs during embryogenesis, aging and tumor regression (Wyllie et al., 1980). However, recent evidence suggests that apoptosis contributes to the pathogenesis of a number of diseases, including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and human acquired immunodeficiency syndrome (AIDS) (reviewed by Thompson, 1995).

There is mounting evidence that the induction of apoptosis contributes directly to the pathogenesis of a number of viruses. On the other hand, some viruses have developed different strategies to inhibit the process, through which they can themselves benefit by delaying the death of the host cells, or by establishment of a persistent infection. A list of viruses involved in induction or suppression of apoptosis is given in table 2.2. These viruses include representatives from a wide spectrum of viral families indicating that apoptosis plays an important role in viral pathogenesis.

Table 2.2 Viruses that induce or suppress apoptosis

Virus	Protein	Apoptosis induction	Reference	Apoptosis suppression	Reference
DNA viruses					
Adenovirus	E1A	+	Rao et al., 1992		
	E1B			+	Rao et al., 1992
EBV	LMP-1			+	Henderson et al., 1991
HPV	E7	+	White et al., 1994		
	E6			+	Scheffner et al., 1990
SV40	Large T			+	Levine et al., 1990
HSV-1	g1 34.5	+	Chou and Roizman, 1992		
CAV	VP3	+	Noteborn et al, 1994		
RNA viruses					
HTLV-1	Tax	+	Chlichlia et al., 1995	+	Laherty et al., 1993
PRRS	p25	+	Suarez et al., 1996		
Poliovirus	?	+	Tolskaya et al., 1995	+	Tolskaya et al., 1995
Alphavirus	?	+	Levine et al., 1993		
Influenza virus	?	+	Takizawa et al., 1993		
Measles	?	+	Esolen et al., 1995		
VSV	?	+	Koyama, 1995		
LCMV	?	+	Razvi and Welsh, 1993		

EBV: Epstein Barr virus, HPV: Human papilloma virus, SV40: Simian virus 40, HSV-1: Herpes simplex virus-1, CAV: Chicken anemia virus, HTLV-1: Human T cell leukaemia virus, PRRS: Porcine reproductive and respiratory syndrome virus, VSV: Vesicular stomatitis virus, LCMV: Lymphocytic choriomeningitis virus

Morphological Comparison of Necrosis vs. Apoptosis

The majority of morphological changes of necrosis are in the cytoplasm. The earliest changes include a mild degree of cytoplasmic edema, dilation of the endoplasmic reticulum, slight mitochondrial swelling, and the appearance of multiple small aggregates of condensed chromatin around the nuclear membrane. These changes are probably all reversible. The second stage includes "high amplitude" swelling of mitochondria with rupture of internal cristae. Later lethal changes include extensive cytoplasmic swelling, dissolution of cytoplasmic organelles and rupture of plasma membranes. The nuclear changes, in contrast to the cytoplasmic changes, are relatively unremarkable. The nucleus may rupture or it may escape when the plasma membrane bursts (Lockshin, 1981).

Apoptotic cells undergo several dramatic morphological changes, mainly in the nucleus. The changes include extensive chromatin condensation, development of fragmented nuclei, plasma membrane blebbing, and loss of cell volume. Ultimately, the cell breaks up into several membrane-bounded smooth-surfaced "apoptotic bodies" containing a variety of cytoplasmic organelles, and some including nuclear fragments. Even at this stage, mitochondrial structure is frequently intact (Lockshin, 1981; Hale et al., 1996).

Mechanism of Apoptosis

Apoptosis occurs through the activation of a cell-intrinsic suicide program after cells receive a "death signal". The basic machinery to carry out apoptosis appears to be present in essentially all eukaryotic cells, but the activation of the program is regulated by many different signals that originate either from within, or outside the cell (Hermann, 1995). There is increasing evidence that apoptosis occurs by a mechanism that has been conserved throughout animal evolution. At least, some components of the apoptosis program have been shown to be conserved among worms, insects, and vertebrates.

Signal Transduction

The vulnerability to different death signals and the ability to be saved from death by different inhibitors of apoptosis varies from between cell types. Particular signaling routes to death give each cell type its own unique pattern of susceptibility to some death signals,

and immunity to others. Even among one general cell type such as lymphocytes, some subtypes succumb to Fas activation while others are oblivious, and some are killed by tumor necrosis factor (TNF) while others are spurred to produce more inflammation (Hale et al., 1996).

Currently, two cell-surface receptor-mediated signaling pathways have been best studied: the Fas receptor-mediated (reviewed by Nagata and Golstein, 1995; Barinaga, 1996) and the TNF receptor mediated pathway (reviewed by Barinaga, 1996; Hale et al., 1996). The Fas pathway is a short and straight route. The Fas receptor is activated by a protein called Fas ligand (FasL), which is released by the killer cells of the immune system. The signaling continues through two molecules, MORT1/FADD and FLICE/MATCH, which are believed then to trigger the activities of a group of proteases belong to the human interleukin-1b converting enzyme (ICE) family. It is believed that activation of one ICE could start a chain reaction in which that enzyme activates other family members, which in turn go on to cleave other substrates, eventually leading to cell death through extensive cleavage of cellular proteins.

The TNF pathways are more convoluted. Cell activated by the TNF may lead to two different pathways: apoptosis or activation of a NF-kB pathway, which activates genes that ultimately produce inflammation. A protein called TRADD is a bridge between these two routes. There are two different TNF receptors, TNFR1 and TNFR2. The path leading from TNFR1 goes through TRADD, which can either 1) "cross-talk" to the MORT1/FADD protein, which eventually leads to apoptosis as described above, or 2) bind to a protein called TRAF2, which will initiate the NF-kB route. Unlike activation through the TNFR1, the TNFR 2 directly binds to the proteins TRAF1 and TRAF2. Therefore the TNFR2 path can also lead to the NF-kB activities or to apoptosis through the binding of the TRAF2 and TRADD.

Protease Activities

Proteolytic activity plays an important role and has been reported in a number of different apoptotic systems. Proteins shown to be cleaved during apoptosis include poly-(ADP-ribose) polymerase (Lazebnik et al., 1994), lamin B (Neamati et al., 1995), topoisomerase I and II, histone H1 (Kaufmann, 1989) etc. Two groups of proteases have been studied extensively. The first group is the ICE family. As described above, ICE or an ICE-like cysteine protease, appears to play a role in Fas-mediated and TNF-induced

apoptosis. Overexpression of the ICE-like proteins results in apoptosis in culture cells (Kumar et al., 1994; Casciola-Rosen et al., 1994). However, whether or not these proteins will induce apoptosis at their physical levels has yet to be determined.

A protease, CPP32 (also called Yama), which can cleave poly(ADP-ribose) polymerase (PARP), has been found to play an important role in apoptosis (Fernandes-Alnemri et al., 1994; Tewari et al., 1995). PARP is involved in DNA repair and in the supervision of genome structure and integrity in stressed cells (Nicholson et al., 1995). The cleavage of PARP to an 85-kDa fragment is an early event in apoptosis and is observed in virtually every form of apoptosis examined (Hale et al., 1996). Loss of PARP function could lead to the activation of the Ca^{2+}/Mg^{2+}-dependent endonuclease which is implicated in the internucleosomal DNA cleavage occurring during apoptosis. CPP32 is cleaved to its active form, composed of two subunits, 12-kDa and 17-kDa. It is assumed that apoptotic signals lead to the activation of a protease which cleaves CPP32 to its active form, which then cleaves PARP and probably other substrates whose proteolysis culminates in apoptosis (Hale et al., 1996).

Regulators

A family of mammalian ced-9-related genes encode both inhibitors and promoters of apoptosis (Hale, 1996). These genes and their encoded proteins are given in table 2.3.

Table 2.3 Intracellular regulators of apoptosis

Gene	Protein	Role
bcl-2	Bcl-2	inhibitor
bcl-x	Bcl-xL	inhibitor
	Bcl-xS	promoter
mcl-1	Mcl-1	inhibitor
bax	Baxa	promoter
bak	Bak	promoter
bad	Bad	promoter

Among these regulators, Bcl-2 has been studied most extensively. Bcl-2 is a potent inhibitor and has been used in many experiments as an inhibitor to the apoptosis process. The mechanism of Bcl-2 action is unclear. Bcl-2 is able to inhibit apoptosis caused by dysregulated expression of the ICE proteases (Miura et al., 1993). But there is no published evidence that it acts directly on these enzymes. Bcl-2 may block apoptosis through regulating cytosolic free $Ca2^+$ concentration, by modulating the transport of protein through the pores of the nuclear envelope (Borner et al., 1994), or by preventing Bax from inducing apoptosis. It is possible that Bcl-2 acts in more than one way either in order to prevent the induction of apoptosis by different stimuli or in order to control different aspects of the apoptotic effector pathway.

Viral Infection and Apoptosis

As mentioned and listed in the table 2.2, the induction or inhibition of apoptosis contribute directly to the pathogenesis of a number of viruses. Recent research has revealed several different mechanisms involved in virus-induced or virus-suppressed apoptosis. Influenza virus infection can trigger apoptosis by inducing the Fas antigen mRNA in the early infectious stage, followed by the expression of the Fas antigen on the cell surface (Takizawa et al., 1995). Some viruses, like adenovirus, HPV, EBV, and SV40, can suppress apoptosis by binding to specific cellular proteins, such as pRB and p53. The tumor repressor p53, implicated in induction of both growth arrest and apoptosis following DNA damage, is a positive regulator of bax gene expression and a negative regulator of bcl-2 gene expression. Some viruses inhibit apoptosis by inducing expression of genes involved in the regulation of apoptosis. The viral protein LMP-1 of EBV can protect cells from apoptosis by inducing expression of the bcl-2 gene in B cells (Henderson et al., 1991). It also induces expression of an apoptosis suppressor gene A20 (Tewari, et al., 1995). Interestingly, the Tax protein of HTLV-1 has both apoptosis-suppressing and inducing capabilities. Expression of Tax leads to activation of NF-kB-like factors, can induce expression of myc, and upregulates the A20 gene to suppress apoptosis (Laherty et al, 1993). However, it has been also shown that expression of Tax leads to apoptosis (Chlichlia et al., 1995). The level of the apoptotic response is shown to be dependent on the duration of the Tax stimulation.

CHAPTER 3
FURTHER CHARACTERIZATION AND CELLULAR LOCALIZATION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRAL PROTEINS

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This paper reports a portion of the work described in a thesis to be submitted to Oregon State University Department of Microbiology in partial fulfillment of the requirements for a PhD degree.

Abstract

The kinetics of viral protein synthesis for infectious hematopoietic necrosis (IHN) virus in cells was examined by pulse labeling and Western blot analysis. Sequential expression of the viral genes correlated with their relative location on the viral genome. The strict dependence of viral replication on host glycosylation was also confirmed. The study also resulted in the observation of a previously undescribed, small, non-glycosylated protein, (S) that was not found in purified virions. The S protein was expressed as early as the viral N, M1, and M2 proteins. Pulse-chase studies demonstrated no product-precursor relationship between S and the other viral proteins. Although the gene encoding the S protein is unknown, it is probable that the S protein is virus-encoded since S proteins with slightly different electrophoretic mobilities were found with different IHNV isolates.
An extensive survey of NV protein expression was undertaken. The presence of the NV protein in IHNV-infected tissues was detected by immunofluorescence; however, under several different growth conditions, NV protein was either expressed in such low quantities as to be undetectable by pulse labeling or was extremely labile. The location of each viral protein in infected cells in culture and in the kidney cells of infected fish was analyzed by confocal microscopy. While N, M1 and G proteins were found to be confined in the cytoplasm, M2 and NV proteins were identified in the nucleus and cytoplasm of infected cells.

Introduction

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that produces an acute disease in salmon and trout. When the virus infects young fish, destruction of the hematopoietic tissue in the kidneys ensues, and the fish die of extensive hemorrhaging and tissue damage (Drolet et al., 1994). The disease at a fish hatchery may lead to the loss of its entire production for that season (Pilcher and Fryer, 1980). IHNV is a member of the *Rhabdoviridae* family whose genome is typically organized in the order 3' - nucleocapsid protein (N), phoshoprotein (P or M1), matrix protein (M or M2), glycoprotein (G), and polymerase (L) - 5'. IHNV has also been shown to contain an additional gene, the nonvirion (NV) protein, which is located between the G and L gene (Kurath and Leong, 1985; Kurath et al., 1985).

Although IHNV is now classified as belonging to a group of fish rhabdoviruses genetically more related to the lyssavirus (rabies) genus (ICTV, 1995), there is a petition before the International Committee on the Taxonomy of Viruses to create a separate genus for those fish rhabdoviruses that contain an intervening gene between the glycoprotein and polymerase genes. The NV gene is considered a unique feature of fish lyssa-like viruses and for that reason, an understanding of its role in virus replication is important. We have found that the expression of this protein is uncertain. In the original report, NV protein was easily detected in infected CHSE-214 cells (Kurath and Leong, 1985); however, subsequent reports have indicated that the detection of NV can be problematic (Nishizawa et al., 1991a, 1991b; Schutze et al., 1995). In our most recent efforts to detect NV by standard pulse labeling or immunoprecipitation, we have been unable to detect the protein. In fact, NV was not detectable under many different experimental conditions and suggested that NV was expressed in amounts below the detection limits of pulse labeling or was extremely labile. What we found, instead, was a previously undescribed protein of 5.8-6.5 kDa that appeared to be virus-encoded. No antigenic or product-precursor relationship to the other viral proteins was established.

Immunofluorescent staining of NV in infected cells was possible and subsequent confocal microscopy of the stained cells indicated that NV was located in both the cytoplasm and nucleus. Unlike the viral proteins, G, M1, and N which are found exclusively in the cytoplasm, NV and M2 are found in both the nucleus and cytoplasm. Thus, interaction of viral proteins with the host cell nucleus may be important in IHNV replication.

Materials and Methods

Cell and Virus

Virus stocks were propagated in chinook salmon embryo cells (CHSE-214, Fryer et al. 1965; Lannan et al., 1984) or *epithelioma papillosum cyprini* (EPC, a carp cell line; Fijan et al., 1983). The RB1, a type 1 isolate, was taken from an adult steelhead trout at the Round Butte Hatchery in the central Oregon in 1975 and found to be virulent for rainbow trout (*Oncorhynchus mykiss*) and sockeye salmon (*O. nerka*) in laboratory trials. A type 2 isolate, RA, taken in 1983 from dead rainbow trout fry at the International Aquaculture Research Center (Rangen Research), Hagerman, Idaho, was also included in the protein

expression assays. The isolate, RB4 (type 1), was derived from a moribund yearling steelhead trout at Round Butte Hatchery in 1983 and the Elk River isolate (type 3) was obtained from chinook salmon (*O. tshawytscha*) at the Elk River Hatchery, Oregon, in 1986.

Radioactive Labeling of Viral Proteins

At different intervals after infection with IHNV at a multiplicity of infection (M.O.I.) of 10, the cells were starved in methionine-free medium for 1 h and then exposed to [^{35}S] methionine (30 μCi/ml) for 1 h or longer. In the pulse-chase experiment, cells were exposed to [^{35}S] methionine for 5 min followed with excess unlabeled methionine for 0, 30, 60 and 120 min. For glycoprotein labeling, the cells were starved in sucrose-free medium and exposed to [2,6-^{3}H] mannose (30 μCi/ml) for 1 h or longer. The cells were lysed with a buffer containing 0.5 M urea, 2% Nonidet P-40 and 5% b-mercaptoethanol, and the lysate was applied to a 12% polyacrylamide gel.

For the glycosylation inhibition assay, infected cells were incubated with medium containing tunicamycin at 0, 0.5, 1.0 5.0 and 10.0 mg/ml at 1 h postinfection (hpi). The same concentrations of tunicamycin were added to methionine-free medium for pulse labeling.

Cloning

Two cDNA clones containing the NV open reading frame (ORF) were constructed. The RB1 mRNA-derived cDNA clone, pNV 137, constructed by Kurath et al. (1985) was employed as a template for the subsequent subcloning. A fragment containing the NV ORF starting from the first ATG was generated by PCR amplification. PCR was carried out with two primers: the first primer was designed in the sense orientation (5'-AGA GAC AAT GGA TCC CCG TGA CAC AAA C-3') with two point mutations (C to T and A to C) to create a BamHI site; the second primer (5'-CGC AAG CTT CTA TCT GGG ATA AGC AAG AAA-3'), was designed in the antisense orientation and carried an additional Hind III site. Although these two primers introduced new restriction sites to the NV fragment, they did not change the encoded amino acid. The PCR fragment was digested with appropriate

restriction enzymes and subsequently cloned into the trpE expression vector pATH2 (Koerner et al., 1991) to generate the clone, pNV-1.

In another preparation, the NV fragment was cloned into an expression vector, pQE60 (Qiagen), which resulted in the attachment of a 6X-Histidine affinity tag to the C-terminus of the NV protein. Because the NV ORF contains two in-frame ATG codons within 24 nt of each other (Chiou and Leong, chapter 4), the NV fragment starting with both the first and the second ATG were generated by PCR priming and then cloned into the vector to create the plasmids, pQE-NV1 at the first ATG and pQE-NV2 at the second ATG. The insertion of the NV gene into the correct reading frame and the nucleotide sequence were verified by DNA sequence analysis.

Antisera and Western Blot Analysis

Anti-IHNV serum was prepared by injection of a New Zealand White female rabbit with a preparation of purified IHNV RB1 virions mixed in Freund's adjuvant. The RB1 virion was purified by ultracentrifugation through a sucrose-gradient as described previously (Hsu et al., 1985). The mouse anti-N (14D) and anti-G (136J) monoclonal antisera were kindly provided by Dr. Sandra Ristow at Washington State University (Arnzen et al., 1991). The rabbit anti-M1 and anti-M2 polyclonal antisera were provided by Dr. H. Mark Engelking at Oregon State University (Engelking and Leong, 1986). Two different antisera against an NV mRNA derived protein were prepared. Antisera were generated by immunizing rabbits with purified trpE-NV fusion protein or with purified NV(ATG2)-His fusion protein in Freund's adjuvant. The trpE-NV fusion protein was induced with b-indoleacrylic acid (Sigma) in *E. Coli* strain DH5a transformed with pNV-1. The fusion protein was first isolated in purified inclusion bodies by the method of Lin and Cheng (1991) and then run on a 10% SDS polyacrylamide gel. The trpE-NV fusion protein band was excised and the gel was crushed with a plastic pestle in a microfuge tube. The fusion protein was eluted in PBS and stored at -20 C. The NV(ATG2)-His fusion protein was induced with IPTG in *E. Coli* strain, M15 (Qiagen), transformed with pQE-NV2, and was then purified by Ni-NTA resin according to the manufacturer's protocols.

Western blot analyses were carried out as previously described (Hsu et al., 1986). The protein samples were subjected to electrophoresis and blotted onto nitrocellulose membranes. The proteins were detected with antisera and developed with goat anti-rabbit IgG conjugated to alkaline phosphatase or biotin.

Immunofluorescence Staining and Confocal Microscopy Examination

Rainbow trout from a group infected with the RA isolate of IHNV and from a group of age-matched control fish were collected at 9 days postexposure to the virus. The kidney tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned as described by Kim et al. (1994). The sections were deparaffinized and blocked with 1% BSA and 0.1% Tween-20 in PBS for one hour at room temperature. At this point, the blocking solution was used as the diluent for the primary and secondary antibody preparations. The immunofluorescence staining was carried out as described by Trobridge et al. (1996) with modification. Briefly, the sections were incubated with primary antibody for 1 h as follows: monoclonal antibody 14D directed against the N protein of IHNV (1:2000), polyclonal anti-M1(1:200), polyclonal anti-M2 (1:200), polyclonal anti-NV (1:200), and monoclonal antibody 136J directed against the G of IHNV (1:50). The sections were washed several times with 0.1% Tween-20 in PBS and subsequently incubated with a Texas Red conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Molecular Probes , Inc., Eugene, OR) at a concentration of 10 mg/ml for 30 minutes. The tissues were washed and then counterstained with 500nM DAPI for 10 minutes. The tissues sections were permanently mounted in Crystal Mount (Biomeda, CA). Confocal images were captured using a Leica TCS4 confocal microscope and compiled using Adobe Photoshop software (Mountainview, CA).

Results

Kinetics of Intracellular IHN Viral Protein Synthesis

A study on the kinetics of intracellular IHN viral protein synthesis during virus replication was carried out by pulse labeling the infected cells with [^{35}S] methionine for one hour. The N and M2 proteins appeared as early as 8-9 hpi, the G and M1 proteins appeared later at 12-13 hpi and the L protein was not detected until 24 hpi (Fig. 3.1A). This result is similar to that of a previous report on the kinetics of the IHN virally structural proteins (Leong et al., 1983). However, the NV protein was not detected at any time in the infection cycle (Fig. 3.1A). A previously undescribed protein was observed at the position

Figure 3.1 Time course study on IHN viral proteins synthesis in cells by pulse-labeling (A) and Western analysis (B). CHSE-214 cells were infected with IHNV RB1 isolate at M.O.I. of 10. In panel A, infected cells were exposed to [^{35}S]methionine (30μCi/ml) for one hour at the times indicated after infection. Note the appearance of a previously unidentified protein (S) at the position of 6.5 kDa. In panel B, same samples were analyzed by Western blotting with an anti-IHNV serum. The position where an NV protein might migrate is shown by the arrow.

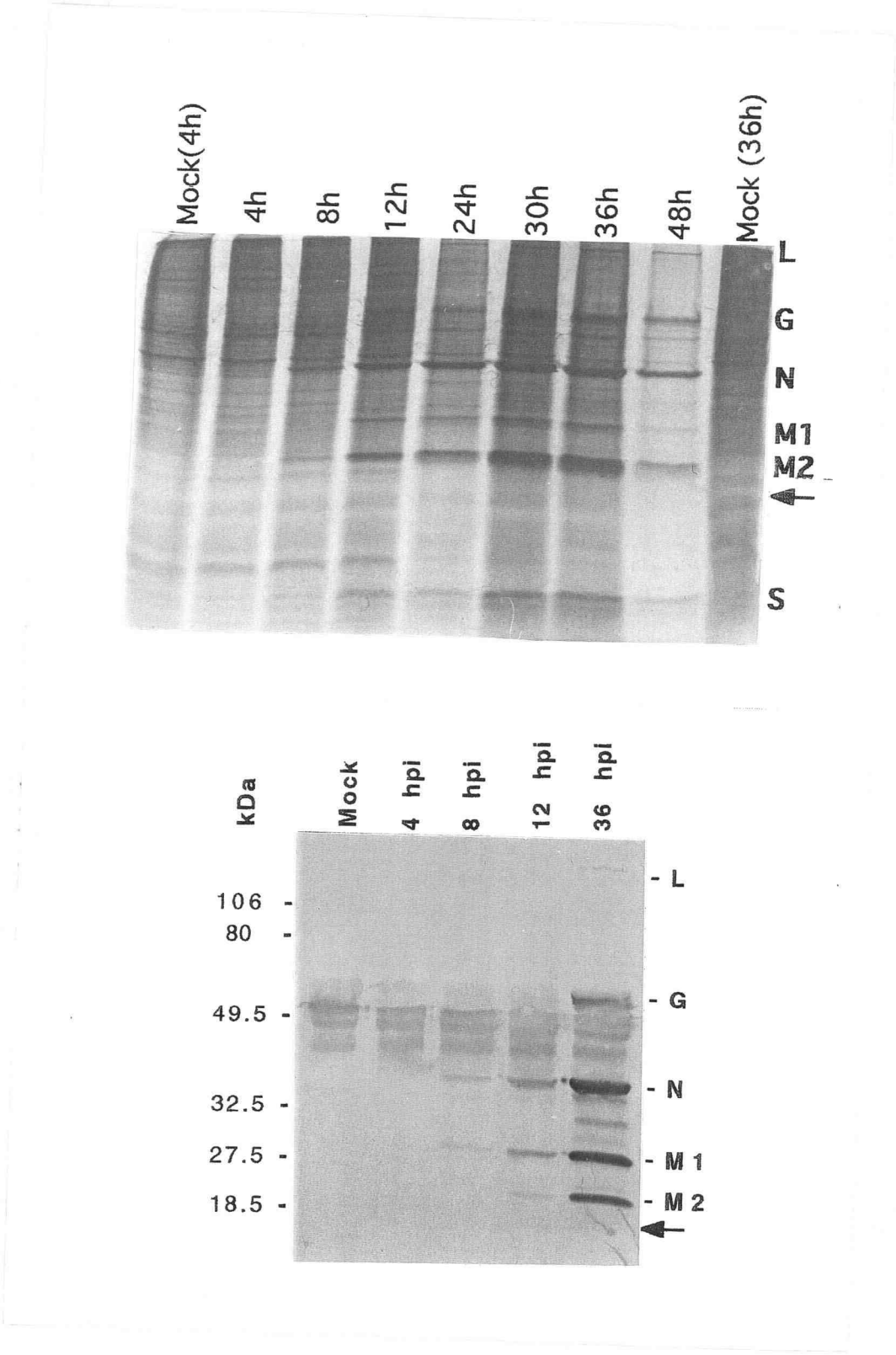
Mock(4h)
4h
8h
12h
24h
30h
36h
48h
Mock (36h)
L
G
N
M1
M2
S
kDa
Mock
4 hpi
8 hpi
12 hpi
36 hpi
106
80
49.5
32.5
27.5
18.5
L
G
N
M 1
M 2

Figure 3.1

of 6.5 kDa on the SDS-acrylamide gel during the infection cycle (Fig. 3.1A and see next section). The kinetics of intracellular IHN viral protein was also assayed by the Western blot analysis with an anti-IHNV serum. Interestingly, in this assay the M1 protein was recognized with the anti-IHNV sera earlier than the M2 protein was (Fig. 3.1B). The same samples were further analyzed using both the anti trpE-NV and anti NV-Histidine antisera. Both antisera did not react with a 12 kDa protein on the gel (data not shown) while all the other five virion proteins were identified by the anti-IHNV antiserum.

Identification of a 6.5 kDa Protein in IHNV-Infected Cells

A previously undescribed small (S) protein was detected in the IHNV-infected cells (Fig. 3.1A). This 6.5 kDa protein appeared as early as the N, and M2 proteins at 8 hpi and did not appear in the mock-infected cells. To determine whether this S protein was a viral protein or a virally-induced cellular protein, different cell lines infected with IHNV were examined for production of the S protein. In addition, the CHSE-214 cell line was infected with several IHNV isolates of different types and examined by pulse-labeling. In Figure 3.2A, the S proteins of same size appeared in three different cell lines infected with RB1 isolate suggesting that the induction of the S protein is not specific to only one cell line. The expression of the S protein was also detected in cells infected with different IHNV isolates (Fig. 3.2B). In this case, the S protein was induced by all four IHNV isolates. Interestingly, the S protein produced by IHNV type-2 isolate, RA, migrated differently at the position of 5.8 kDa on the gel (Fig. 3.2B). This finding suggests that the S proteins produced by different IHNV isolates are slightly different in electrophoretic mobility and therefore, provides direct evidence that the S protein is encoded by the IHN virus. Also, no protein band was found migrating at the position of 5.8 - 6.5 kDa in the lane of an IPNV-infected cell lysate on the gel (data not shown) supporting the deduction that the S protein is not a virally-induced cellular protein.

To determine whether the S protein is associated with the mature IHN virion, virions were purified by ultracentrifugation through a sucrose gradient and then analyzed by SDS-PAGE followed by Coomassie blue staining. Only five structural proteins (N, M1, M2, G and L) were identified in the gel (data not shown). The purified virions were further analyzed by Western blot with an anti-IHNV serum, which is capable of recognizing the viral proteins associated with the virion. No protein migrating at the position of 5.8-6.5 kDa was recognized by the antiserum (data not shown). These results demonstrate that the

Figure 3.2 Detection of S protein. (A) [^{35}S]methionine labeling of the viral proteins in different cell lines infected with IHNV. Three cell lines, two different passage levels of CHSE-214 and an EPC cell line were infected with IHNV RB1 at a M.O.I. of 10 and labeled with [^{35}S]methionine for 1 h at 12, 24, and 36 hpi. CHSE-214 #1 has been passaged over 350 times routinely and CHSE-214 #2 was recovered from a frozen stock which has been passaged 270 times. (B) [^{35}S]methionine labeling of the viral proteins in the cells infected with different IHNV isolates. CHSE-214 cells were infected with four different IHNV isolates at a M.O.I. of 10: RB1(type 1), RB4 (type 1), RA (type 2) and Elk (type 3). The position where an NV protein might migrate is shown by the arrow.

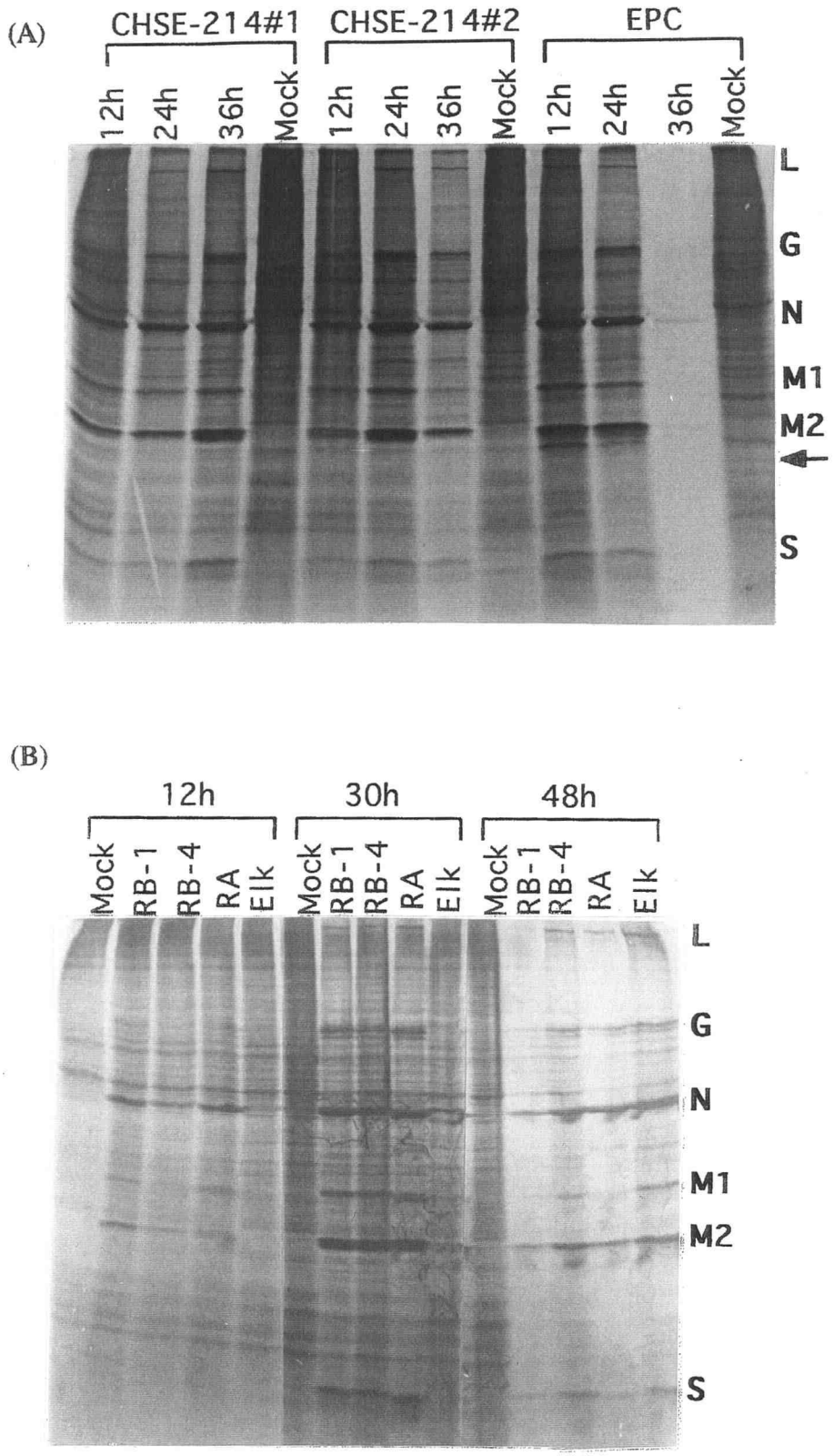
(A)
CHSE-214#1
CHSE-214#2
EPC
12h
24h
36h
Mock
L
G
N
M1
M2
S
(B)
12h
30h
48h
Mock
RB-1
RB-4
RA
Elk
L
G
N
M1
M2
S

Figure 3.2

Figure 3.3 Detection of S protein (cont.) (A) Glycosylation inhibition assay. IHNV-infected cells were treated with different concentrations of tunicamycin at 1 hpi and pulse labeled with [^{35}S]methionine for 1 h at 24 and 36 hpi. At both time points, the inhibition of G protein glycosylation was observed. The position of the S protein remained unchanged like other non-glycosylated viral proteins. (B) Pulse-chase assay. At 30 and 36 hpi, IHNV-infected cells were exposed to [^{35}S] methionine for 5 min followed with excess unlabeled methionine for 0, 30, 60 and 120 min. After a 5 min exposure to the radioactive methionine resulted in a predominant G_2 band and the predominantly labeled band switched to G_1after a further chase with excess unlabeled methionine. G1 was suggested to be a fully glycosylated G (Leong et al., 1983). The signal intensity of N, M1, M2 and S during the whole cycle maintained unchanged.

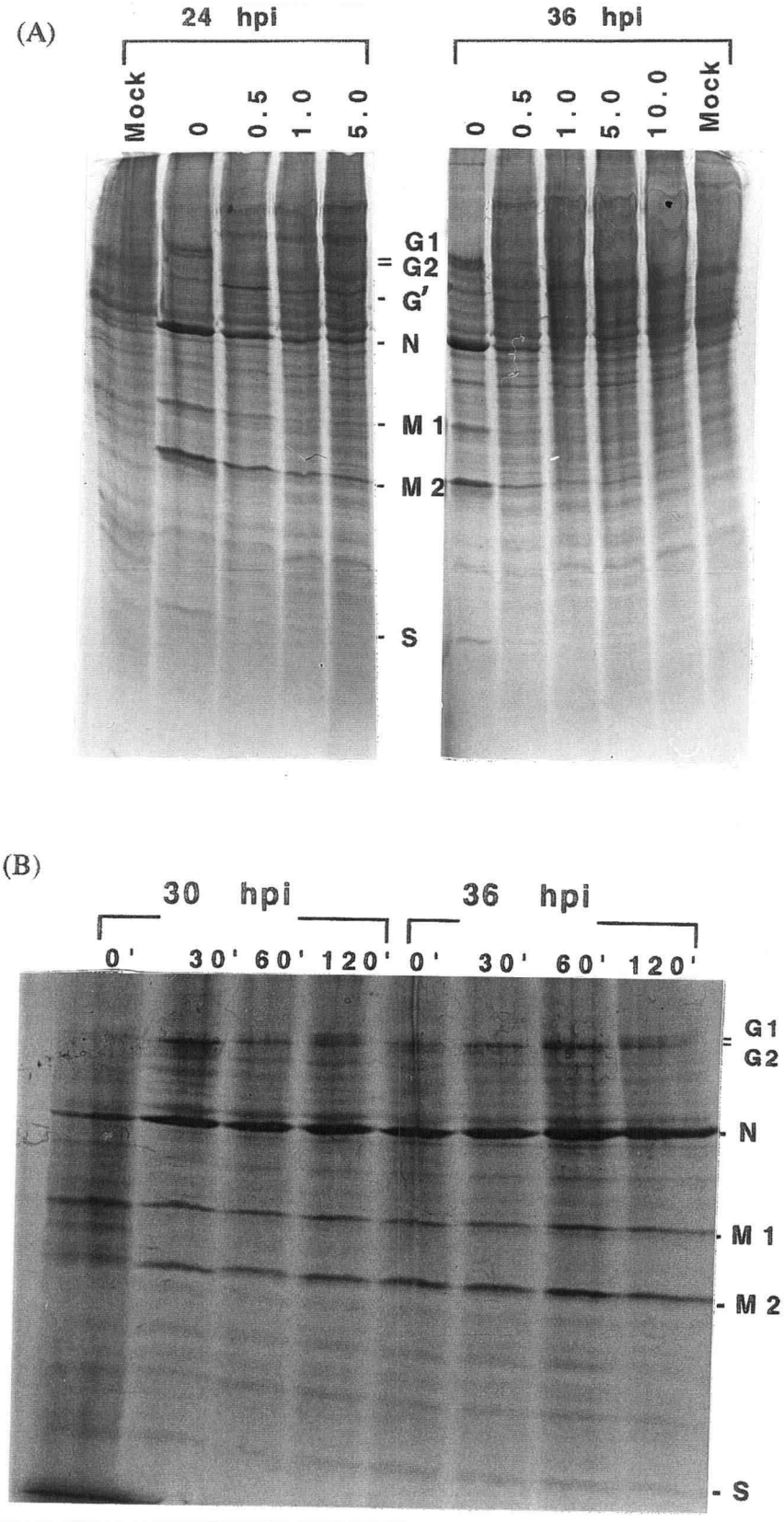
(A)
24 hpi
36 hpi
Mock
0
0.5
1.0
5.0
0
0.5
1.0
5.0
10.0
Mock
G1
G2
G'
N
M 1
M 2
S
(B)
30 hpi
36 hpi
0'
30'
60'
120'
0'
30'
60'
120'
G1
G2
N
M 1
M 2
S

Figure 3.3

S protein is not a virion-associated protein, which indicates that its function is not required for virus attachment or penetration, and is probably not required for assembly.

The differences in the electrophoretic mobility of the S proteins of different isolates may be due to the differences in the size of the peptides or due to posttanslational modifications. Two experiments were performed to further investigate whether the S protein is a glycosylated protein and whether the difference in the observed MW is due to the extent of glycosylation. By direct labeling of the viral proteins with [2,6-^{3}H]mannose, no band migrating at the position of 5.8 - 6.5 kDa was observed (data not shown). G protein was the only glycosylated protein identified in this assay. Another investigation was conducted by employing a glycosylation inhibitor, tunicamycin. Tunicamycin is an antibiotic which is capable of inhibiting N-linked glycosylation in eukaryotic cells. IHNV-infected cells were treated with different concentrations of tunicamycin at 1 hpi and pulse labeled with [^{35}S] methionine for 1 h at 24 and 36 hpi. At both time points, inhibition of glycosylation of the G protein was observed as the concentration of tunicamycin increased. Nonetheless, the position of the S protein remained unchanged as did the other non-glycosylated viral proteins (Fig. 3.3A). These results support the conclusion that the S protein is not glycosylated, and that the difference in the observed MWs of the S protein of the different IHNV isolates is not due to glycosylation. This assay also demonstrated that viral protein synthesis, as well as the CPE of the IHNV-infected cells, were drastically reduced as the concentration of tunicamycin increased. Hence, unlike IPNV (Perez et al., 1996), a fish birnavirus, the replication of IHNV is strictly dependent on the host glycosylation machinery.

A pulse-chase experiment was conducted to verify the relationship of the intracellular synthesis and accumulation between the S protein and other viral proteins (Fig. 3.3B). At 30 and 36 hpi, IHNV-infected cells were exposed to [^{35}S]methionine for 5 min followed by treatment with excess unlabeled methionine for 0, 30, 60 and 120 min. As reported previously by Leong et al. (1983), for the G protein, a 5 min exposure to the radioactive methionine resulted in a predominant G_2 band. After a further chase with excess unlabeled methionine, the predominantly labeled band switched to G_1. There are two forms of glycosylated G protein, G_1 and G_2, which migrate at 67 and 65 kDa respectively. G_1 is considered to be a fully glycosylated G, while the lower MW of G_2 is considered to be less glycosylated, rather than a breakdown product (Leong et al., 1983). The signal intensity of the S protein during the whole cycle remained unchanged, suggesting no product-precursor relationship between the S protein and the other IHN viral proteins. This result suggests that the translation of the S protein is independent of the other viral proteins.

Expression of NV Protein

Upon recognition that the NV protein levels observed in previous work (Kurath et al, 1985) were no longer detectable, a series of experiments (summarized in Table 3.1) were designed to investigate potential explanations for this discrepancy. Efforts to confirm the original finding that NV was detectable in IHNV-infected cells by [^{35}S] methionine pulse-labeling were carried out in different cell lines with different IHN virus isolates and with different virus growth conditions (media components, temperature of growth, and different M.O.I.s). In all cases, there was no NV protein detectable by pulse-labeling with [^{35}S] methionine (data not shown). When infected cells were double labeled with [^{35}S] methionine and [^{3}H] leucine, no immunoprecipitable protein at 12 - 13 kDa was recognized by anti-NV sera prepared to an NV fusion protein synthesized in bacteria. No NV was detected despite the fact that there were 15 leucine residues available for [^{3}H] leucine labeling in the NV ORF (Chiou and Leong, unpublished data). The addition of protease inhibitors to the immunoprecipitation medium did not result in NV detection. In addition, lysis of cells with different extraction buffers designed to inactivate proteases, break non-covalent protein-protein interactions, and reduce disulfide bonds, also did not result in NV detection. In addition, the NV protein was not detected in the liver, kidney and blood samples from IHNV-infected fish by Western blot analysis with anti-NV sera (data not shown) even though the other five virion proteins were clearly identified in these samples.

Two different antisera against the NV ORF were employed in these assays. One antiserum was generated to a trpE-NV fusion protein and the other was to a NV-Histidine tagged fusion protein. Both antisera were able to recognize trpE-NV and NV-Histidine tagged protein by Western blot analysis. However, neither antisera recognized any protein produced in the IHNV-infected cell (data not shown). The anti-NV sera, by immunohistochemistry, did specifically stain cells transfected with plasmid encoding the NV gene (Chiou and Leong, unpublished data). Thus, the antisera were capable of detecting NV produced in transiently transfected eukaryotic cells. Further, the anti-NV-Histidine serum has been successfully used to identify the NV protein in the IHNV-infected tissues by confocal microscopy employing an immunofluorescence technique (Fig. 3.4). These assays demonstrate that the NV protein, under most circumstances, is expressed in a very low amount and hence contradicts the original report by Kurath et al. (1985).

Table 3.1 Parameters of virus growth and gel analyses tested for effect on NV protein expression

Parameter	Description
Cell	Different cell line: EPC, RTG-2, and CHSE-214 Cells of different age: CHSE-214 cells at 1, 2, and 3 days post plating
Virus	Different isolates: RB1, RB4, RA, ELK, Carson Different M.O.I.: 0.01, 0.1, 1, 10 PFU/cell
Medium	Depletion of antibiotics Depletion of fetal bovine serum Addition of Actinomycin D Double labeling medium: methionine- & leucine-free
Temperature	10C, 17C and 20C; Temperature shifting: 10 to 20 C, 20 to 10C
Lysis Buffer	Buffer I: 0.5 M urea, 2% Nonidet P-40, 5% b-mercaptoethanol Buffer II: 50 mM tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 Buffer III: 50 mM tirs, pH 8.0, 1% Nonidet P-40, 0.1% SDS. Addition of protease inhibitors: PMSF, Aprotinin^{R}

PMSF, phenyl methyl sulfonyl fluoride

Cellular Localization of IHN Viral Proteins

The virus positive staining of the N protein by alkaline phosphatase immunohistochemistry (APIH) was persistent and strongest in the kidney of fish during an IHNV epizootic (Drolet et al., 1994). Therefore, we chose the kidney cells from infected fish during an IHNV epizootic to determine the cellular localization of the IHN viral proteins. Figure 3.4 illustrates the localization of the N, M1, M2, G and NV proteins in an infected posterior kidney. Confocal microscopy was conducted on the kidney tissue samples, capturing single optical sections through the nucleus of the cells. The N protein was intensely stained in the cytoplasm of the tubular epithelium particularly in the basal portion of the epithelium (Fig. 3.4F). The N protein was also found in the cytoplasm of the surrounding hematopoietic cells which have been found to be subsequently infected after the virus buds out of the basement membrane of the tubules (Drolet et al., 1994). The lumen of the kidney tubule was also stained indicating the existence of virus particles as reported by Drolet et al. (1994). The M1 protein was expressed in the cytoplasm of the epithelial cells as well as the surrounding hematopoietic cells (Fig. 3.4G). Unlike the N protein, the M1 protein was detected evenly in the cytoplasm. The lumen of the tubule also stained positively for M1 supporting the existence of virus in this area.

The M2 protein was expressed in the cytoplasm and additionally, the nucleus of the epithelial cells as demonstrated in the Figure 3.4H. The same staining pattern was also found in the neighboring hematopoietic cells. Since the viral proteins are synthesized in the cytoplasm, M2 must translocate from the cytoplasm to the nucleus. As with M1, the lumen of the tubule was stained positively with the anti-M2 sera. As illustrated in Figure 3.4I, the G protein was expressed evenly in the cytoplasm of the epithelial cells and the hematopoietic cells. Also, the lumen of the tubule was stained positively as expected.

While the N, M1, M2 and G proteins were extensively expressed in the kidney cells, only a few cells were stained positively with the anti-NV serum (Fig. 3.4J). This result supports the finding that NV protein is expressed in an extremely low amount in the IHNV-infected cells as demonstrated. The NV protein was expressed in the cytoplasm of the epithelial cells in the tubule, and in some positive cells the NV protein was also found in the nucleus (arrow). This result indicates, that like the M2 protein, the NV protein translocates from the cytoplasm to the nucleus. There was no positive staining in the lumen of the tubule with the anti-NV serum. This result is expected as the NV protein is not found in the IHN virion, and the lumen is likely staining positive with antisera to other viral proteins because of the accumulation of virions in this area.

Figure 3.4 Confocal micrograph of IHN viral proteins in fish tissue. Rainbow trout infected with IHNV RA isolate (panels F, G, H, I, and J) and age matched control fish (panels A, B, C, D, and E) were collected at day 9 postexposure. The kidney tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. The section were deparaffinized, blocked, and incubated with primary antibody for 1 h as follows: anti-N (panels A and F), anti-M1 (B and G), anti-M2 (C and H), anti-G (D and I), and anti-NV (E and J). The sections were subsequently incubated with a Texas Red conjugated goat anti-mouse or goat anti-rabbit secondary antibody, and counterstained with DAPI. Confocal images were captured using a Leica TCS4 confocal microscope and compiled using Adobe Photoshop software.

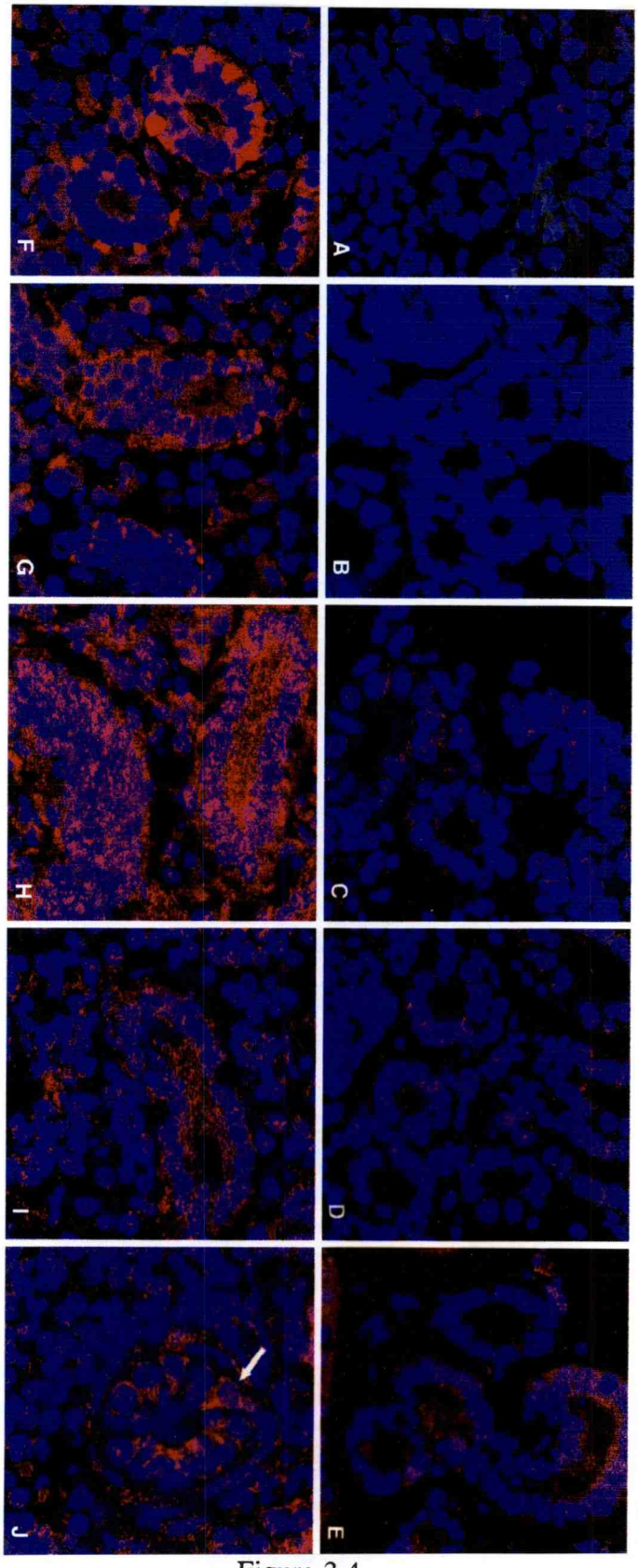

Figure 3.4

Discussion

The transcription of vesicular stomatitis virus, a prototype of rhabdoviruses, proceeds sequentially from the 3' end of the viral genome and there is a progressive decrease in the amount of RNA transcribed for each gene that is directly related to its distance from 3' end (Banerjee, 1987; Banerjee and Barik, 1992). The differences in mRNA concentration is reflected in the amount of viral protein synthesized for each gene. This polarity was also observed for IHNV where the most abundant intracellular viral protein was N, and the least abundant, L (Fig. 3.1). However, there is a discrepancy in the concentrations of M1 and M2 proteins since M1, which precedes M2 on the viral genome, is produced in apparently lower amounts than M2. This discrepancy may be an artifact because of the pulse-labeling method used to detect the viral proteins; for the IHNV isolate RB1, there are 3 methionine residues in the M1 gene, but there are 6 methionine residue in M2 (Ormonde, 1995) available for labeling with the [^{35}S] methionine. This explanation is also supported by Western blot analysis that showed the M1 protein was recognized with anti-IHNV sera earlier than the M2 protein (Fig. 3.1B).

We have identified a novel small intracellular protein, S, associated with IHNV-infection. The SDS-PAGE gels used in this study (12% polyacrylamide) contained a higher concentration of polyacrylamide and had a higher resolution for small proteins than the gels used by previous investigators. These 12% gels may have allowed us to identify the S protein because of this increased resolution. Although the gene encoding the S protein is unknown, it is likely that the S protein is indeed virus-encoded because S proteins with slightly different electrophoretic mobilities were found when different virus isolates were used to infect the same cell (Fig. 3.2B). For many single stranded negative RNA viruses (paramyxoviruses and rhabdoviruses), the M1(P) gene has shown to encode multiple proteins. These proteins may be encoded by the same ORF or by overlapping ORFs recognized through leaky ribosomal scanning (Chenik et al, 1995) or through scanning-independent internal ribosomal initiation (Currant and Kolakofsky, 1989). The S protein did appear as early as N, M1 and M2 (Fig. 3.1A) suggesting the S protein may be a product of one of these three genes. Sequence analysis of the M1 gene of IHNV isolate RB1 revealed a potential second ORF encoding a protein with calculated MW of 4.8 kDa (Ormonde, 1995). In future studies, antisera to this ORF or to other potential ORFs within other viral genes should be generated to determine the gene encoding the S protein.

The original report by Kurath et al. (1985) demonstrated the NV protein was expressed in a dominant amount in the IHNV-infected cells. We present a contradictory result in this report. We examined NV expression using different; cell lines, growth conditions, virus isolates, times of infection, labeling methods, SDS-gel running conditions, media, and lysis methods. In all cases, we have been unable to detect the NV protein in IHNV-infected cells by the same pulse-labeling method reported by Kurath et al. (1985). However, by an immunofluorescence technique, the NV protein was identified in the cytoplasm and nucleus of the IHNV-infected kidney cells. Other reports (Nishizawa et al., 1991 a and b; Schutze et al., 1995) also suggest that the NV protein is expressed in an extremely low amount. The discrepancy between the original finding by Kurath et al. that NV is dominantly expressed and our finding of weak expression of NV is still unclear. It is possible that the NV gene has been mutated after passage in the cultured cells. We have recently found that in our hands the NV mRNA is not degraded and is available for translation during the IHNV infection cycle (see chapter 4).

We determinedthe cellular localization of the IHN viral proteins. Confocal microscopy was conducted to distinguish nuclear and cytoplasmic localization. The N, M1 and G proteins were all confined to the cytoplasm of the infected cells, whereas the M2 and NV protein were detected in the nucleus as well as the cytoplasm. This finding indicates translocation of these two proteins from the cytoplasm to the nucleus in infected cells. The intranuclear localization of VSV M protein has also been demonstrated (Lyles et al.,1988). These findings suggest that intranuclear localization is a common feature of the matrix proteins of rhabdoviruses. However, the mechanism of translocation is unknown.

A targeting signal is usually found in nuclear proteins. For example, the T antigen of simian virus 40 contains a short amino sequence which is able to specify nuclear location (Kalderon et al., 1984). The IHNV M2 protein, similar to all reported rhabdovirus matrix proteins, is highly basic and contains a high content of charged amino acids at the amino terminus (Ormonde, 1995). Future studies should be carried out to determine if this region contains a targeting signal resulting in nuclear localization.

VSV is able to replicate in enucleated cells, demonstrating that interaction of viral proteins with the host nucleus is not essential for replication for this virus *in vitro*. This experiment has not been performed for IHNV. However, for IHNV both transcription and translation of IHNV genes can be accomplished in a cell-free environment (Kurath and Leong, 1987), suggesting nuclear interaction is not necessary for these steps of viral replication . Despite the fact that nuclear interaction is not necessary for replication *in vitro* it is known that the VSV M protein is able to inhibit host-directed transcription in the nucleus

of infected cells (Black and Lyle, 1992). The intranuclear localization of M2 and NV proteins suggests that interaction of these viral proteins and host nuclear components may be important for efficient replication *in vivo*.

The localization of L and S proteins are not included in this assay because the immunological reagents were not available. S protein was not associated with the mature IHN virion. For both RV and VSV, additional proteins encoded within the phosphoprotein gene are found in the cytoplasm of infected cells and also are not associated with the mature virion (Spiropoulou and Nichol, 1993; Chenik et al., 1995) . Interestingly, some of these additional proteins also localize in the nucleus of infected cells (Chenik et al., 1995) suggesting these proteins perform a role in the nucleus. It will be interesting to determine the location of the S protein in infected cells.

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CHAPTER 4
TRANSCRIPTION OF THE NV GENE OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS: IDENTIFICATION OF A UNIQUE TRANSCRIPTIONAL INITIATION SIGNAL

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This paper reports a portion of the work described in a thesis to be submitted to Oregon State University Department of Microbiology in partial fulfillment of the requirements for a PhD degree.

Abstract

The sixth gene of infectious hematopoietic necrosis virus (IHNV) encodes a nonvirion protein, NV, which was first identified from the Round Butte 1 (RB1) strain of IHNV. We characterize here the expression of the NV gene at the transcriptional level. The nucleotide sequences derived from the NV mRNA and the viral genome were compared and only one nucleotide difference resulting in a single amino acid change was detected between cDNA clones of the original isolate and progeny virus cloned after passage three times in tissue culture. The NV mRNA appeared in the infected cells as early as 12 hpi and was maintained stably throughout the infection. The presence of the NV mRNA was estimated to be approximately 3×10^{-7} pmole (1.8×10^5 molecules) per infected cell at 18 hpi, when 100% of the cells expressed cytopathic effect (CPE). These results suggest an important role of the NV gene in the viral infection despite the fact that the NV protein product was extremely difficult to detect. The transcriptional initiation and termination signals for IHNV mRNA synthesis were examined. Nucleotide sequence examination of the NV mRNA confirmed the sequence, (3'-UCURUC$(U)_7$-5', vRNA-sense), serves as transcriptional termination signal. In addition, these studies identified a unique transcriptional initiation signal for fish lyssa-like rhabdoviruses. The signal, (3'-CGUG----CA-5'), located by primer extension analysis of the NV, M1, and M2 mRNAs, is distinctly different from that of the other lyssaviruses and vesiculoviruses, (3'-UUGU----GA-5').

Results and Discussion

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus of salmonid fish that causes severe losses at hatcheries in western North America (Pilcher and Fryer, 1978), Europe, and Asia. IHNV infection is acute in juvenile fish and can result in up to 100% mortality in affected populations. The virus, along with such fish rhabdoviruses as viral hemorrhagic septicemia virus (VHSV) and hirame rhabdovirus (HIRRV), has an electrophoretic protein pattern similar to the lyssaviruses and are considered to be lyssa-like rhabdoviruses (Nishizawa et al., 1991a & b). The IHNV particle is typically bullet-shaped and consists of a distinctive nucleocapsid core containing a viral genome of minus sense ssRNA that is approximately 11,500 nucleotides (nt). The prototype rhabdoviruses have five genes organized in the order 3'- nucleocapsid protein (N) - phosphoprotein (M1)- matrix protein (M2) - glycoprotein (G) - polymerase (L) - 5' (Peters, 1991; Tordo et al.,

1992). IHNV has an additional gene, NV, which is located between the G and L genes (Kurath and Leong, 1985; Kurath et al., 1985). Two other fish lyssa-like rhabdoviruses, VHSV (Basurco and Benmansour, 1995; Schutze et al., 1996) and HIRRV (G. Kurath, personal communication), have also been found to contain an additional gene between the G and L genes. This extra gene may be a signature for the fish lyssa-like rhabdoviruses. The fact that the NV protein was originally found only in the infected cells but not in the purified virion suggested that it is a nonvirion protein (Kurath and Leong, 1985; Kurath et al., 1985).

There are several reasons for studying the expression of the NV gene at the transcriptional level. First, the expression of the NV protein has been uncertain. The NV protein was found to be in high quantity in IHNV-infected cells in the original study using [35S]-methionine labeling (Kurath and Leong, 1985). However, subsequent reports have shown that the expression of the NV protein is either extremely low or below detection level by [^{35}S]-methionine labeling in IHNV-, VHSV- and HIRRV-infected cells (Nishizawa et al., 1991a & b; Basurco and Benmansour, 1995). In the case of VHSV, a protein with the expected NV gene product size (14 kDa) was precipitated from radioactively-labeled, virus infected cells only by immunoprecipitation (Basurco and Benmansour, 1995). However, a recent report by Schutze et al. (1996) documented both VHSV and IHNV NV expression in cell culture at levels detectable by immunofluorescence and Western immunoblot. Recently, we have conducted an extensive survey on the NV expression
under a series of different conditions. The expression of NV protein was confirmed by immunofluorescence in IHNV-infected tissues; however, under several growth conditions in the study the NV protein was expressed in an extremely low amount which is below the detection limit by the pulse labeling or double labeling techniques (Chiou et al., Chapter 3). One hypothesis to explain these inconsistencies is that the NV gene we are currently working with may have mutated after several passages. A comparison of the nucleotide sequence of the current NV transcript with that of the original isolate would address this possibility. Second, although the NV gene is well maintained in different isolates of IHNV (Nichol et al., 1995) and VHSV, it is possible that the NV gene product may not be essential for virus replication. The NV transcript may be turned off or may decay rapidly in the infected cells. Differences in degradation rates among mRNAs do contribute significantly to the control of gene expression in eukaryotes (Beelman and Parker, 1995). Thus, the expression of the NV mRNA over time was assessed to verify the presence and the stability of the NV RNA transcript in the cell.

Another source of uncertainty is that the regulatory signals for mRNA transcription have not been defined for these viruses. The internal gene junctions of fish lyssa-like rhabdoviruses have been shown to be different from that of other rhabdoviruses. In other rhabdoviruses the intergenic sequence, 3'- $C(U)_7NNUUGU$ -5' (vRNA-sense), is conserved in the gene junctions of the internal genes on the genome. The sequence 3'-UUGU-5' has been shown to function as a transcriptional initiation signal for these rhabdoviruses (Banerjee, 1987; Banerjee and Barik, 1992). In fish lyssa-like rhabdoviruses, a uniquely conserved longer sequence, 3'- $UCURUC(U)_7RCCGUG$ (N)4 CACR -5' , exists between internal genes on the genome. Some researchers have suggested the sequence, 3'-CGUG-5', based on the conservation of its composition and locations on the viral genome as a putative transcriptional initiation signal unique to the fish lyssa-like rhabdoviruses (Morzunov et al., 1995; Bjorklund et al., 1996). Other researchers have shown that the M2 mRNA of the Makah strain of VHSV was initiated at or near the sequence, 3'-UUGU-5', which is 46 nt downstream of the intergenic region (Benmansour et al., 1994). However, unlike mammalian rhabdoviruses, for the fish lyssa-like rhabdoviruses, the tetranucleotide, 3'-UUGU-5', is not well conserved and is not located at the corresponding positions on the genomes as other rhabdoviruses. Analyses of the 3' ends of IHNV RNA transcripts have not been done to date, and would test the hypothesis of the putative transcriptional initiation signal which is specific to the fish lyssa-like rhabdoviruses.

In this report, we characterize the expression of the NV gene at the transcriptional level. We analyzed the nucleotide sequences derived from the NV mRNA and the viral genome of the IHNV RB-1 strain in which the NV gene was first found. We also compare the nucleotide sequences between the original isolate and the progeny virus after the virus was passaged an additional three times in a fish cell line. The stability and the quantity of the NV mRNA in the IHNV-infected cells were also examined. Another interesting result of this study is the confirmation of the putative transcriptional initiation signal, 3'-CCGUG-5', which exists uniquely in the fish lyssa-like rhabdoviruses. This signal was identified by the primer extension analysis of the NV mRNA and further confirmed with the analysis of the M1 and M2 mRNAs of IHNV.

The virus stock used in this study was derived from the same RB1 stock that was used by Kurath et al. (1985) to generate mRNA-derived cDNA clones. The RB1 stock, which had originally been isolated from an adult steelhead trout, had been passaged an additional three times in CHSE-214 cells before use in the current study. In order to detect any changes in the sequence information, three clones were synthesized from $polyA^+$ RNA

Figure 4.1 NV gene of the IHNV RB1 and intergenic region between G-NV and NV-L. Sequence is presented as a cDNA positive strand. The consensus sequences of the intergenic region are underlined with a solid line and the intergenic $(A)_7$ motif is shown in bold. The start sites of the ORF and the potential second ORF are indicated in bold at nt 29 and 60. One nucleotide change at nt 299 (in bold) from A to T changing the encoded amino acid from N to Y was observed in the sequences obtained from three cDNA clones to viral mRNA and three cDNA clones to viral genomic RNA prepared after the virus stock had been passaged three more times in the tissue culture. The sequence of the original pNV137 clone is shown.

3' G gene

```
1556                                                                1625
GCAGAGGATC CCCATGTATC ACCTGGCAAA CCGGTCCTAA AGGACTCAAT CTTCACTTCC TCCCCACCAG
 Q  R  I   P  M  Y  H   L  A  N   R  S  *
1626       1636
ACAGAAAAAA A
```

5' NV gene

```
1                                                                     70
CGGCACATTT GTCGTGTAAA AAGAGACAAT GGACCACCGT GACACAAACA CGAACATGGA GGCACTCAGA
                              M   D  H  R   D  T  N  T   N  M  E   A  L  R   14
                                                                    140
GAAGTTCTGC GATACAAGAA CGAGGTGGCC GGACACGGCT TCCTCTTTGA CGACGGAGAC CTGGTATGGC
E  V  L  R   Y  K  N   E  V  A   G  H  G  F   L  F  D   D  G  D   L  V  W  R  38
141                                                                   210
GTGAAGAGGA CGACGCAACA TGGAGGCGGC TTTGCGATGT CGTCAACGCA CTGATCTCCT CCAAGAGGAT
  E  E  D   D  A  T   W  R  R  L   C  D  V   V  N  A   L  I  S  S   K  R  M   61
                M   E  A  A   L  R  C   R  Q  R  T   D  L  L   Q  E  D    17
211                                                                   280
GCAGCGAGTA TTGTACATGG ACCTCAGCAT CACCAAGGGC GAGGGGCATC TACTTTTTGT GGATCTCCAG
 Q  R  V   L  Y  M  D   L  S  I   T  K  G   E  G  H  L   L  F  V   D  L  Q    84
A  A  S  I   V  H  G   P  Q  H   H  Q  G  R   G  A  S   T  F  C   G  S  P  G  41

281                                                                   350
GGGACCAAGA ACCGCCTGTA CAAAGAACCC CGATTCAGGA GACATCTGAT CCTGATTGAA GACTTTCTTG
G  T  K  N   R  L  Y   K  E  P   R  F  R  R   H  L  I   L  I  E   D  F  L  A 108
  D  Q  E   P  P  V   Q  R  T  P   I  Q  E   T  S  D   P  D  *                59

351                     371
CTTATCCCAG ATAGAAAAAA A
  Y  P  R   *
```

5' L gene

```
1                                                                     70
TGGCACTTTT GTGCAAAAAA ACTCAAGCGC GACTCACAGA GAACATAACC AGCAACGACA GCACCCCATC
```

Figure 4.1

from IHNV-infected cells by RACE-PCR and three clones from viral genomic RNA by RT-PCR. The sequences of these clones showed that the complete NV gene of the IHNV RB-1 (Fig. 4.1) spans 364 nucleotides and is separated from the G and L genes by the sequence 3'-UCUAUC$(U)_7$RCCGUG - 5', which fits the sequence, 3'-UCURUC$(U)_7$RCCGUG$(N)_4$CACR - 5', conserved at all internal gene junctions of IHNV (Nichol et al., 1995), VHSV (Schutze, GeneBank x73873), and HIRRV (Bjorklund et al., 1996). The NV gene of the RB-1 isolate was highly similar, ranging from 96.4% to 99.7%, to the NV gene of the European IHNV K-strain (Schutze et al., 1995) and the other 12 IHNV isolates reported by Nichol et al. (1995). The NV ORF of all known sequences encodes a protein of 111 amino acids. We also noted a second smaller ORF extending from nucleotide 160 to 336 in most isolates. The significance of the second ORF is unknown. With the RACE-PCR technique, the 3' end of the cDNA clones derived from NV mRNA was found to be terminated at the conserved AGATAG$(A)_7$ (mRNA-sense) followed by a long stretch of poly A residues. This result strongly supports the role of the conserved sequence, UCURUC$(U)_7$ (vRNA-sense), as a transcriptional termination/polyadenylation signal for the viral RNA polymerase. The termination codon (UAG) of the RB-1 NV transcript is immediately adjacent to the poly A tail confirming the lack of any downstream untranslated region of the NV transcript, as has been suggested by other researchers (Nichol et al., 1995). This unique feature is conserved in all of the IHNV NV genes reported so far; its significance to the regulation of gene expression is unknown.

A comparison of the sequences from all six clones indicated that there was no difference in the sequence of the encoded NV protein between the mRNA and genome-derived cDNA clones suggesting that there was no RNA-editing involved in the expression of the NV gene product. When the sequence was compared with that of pNV137, the original mRNA cDNA clone prepared from RB-1 virus infected cell polyA$^+$ RNA, only one nucleotide change was found at position 299 (A to T), which changed the encoded amino acid from N to Y (Fig. 4.1). Thus, after three passages in tissue culture, the RB-1 stock virus appeared to have sustained a single base mutation in NV.

A study of the time course of NV mRNA expression during the virus replication cycle was conducted to examine the stability of the NV mRNA expression and to determine the quantity of NV mRNA produced in the infected cells. Northern dot blot analyses were use to determine the quantity of the NV mRNA produced. At different intervals after infection at a multiplicity of infection (M.O.I.) of 10, total RNA was extracted from IHNV-infected CHSE-214 cells with RNAzol™ (Tel-Test, Inc., Friendswood, Texas). Separation of polyA$^+$ RNA from total RNA was carried out with Oligotex-dTTM (Qiagen, Chatsworth,

CA). For Northern blot analysis, the RNA samples were electrophoresed on 1% agarose gels after denaturation with glyoxal and dimethyl sulfoxide and then transferred to Maximum Strength Nytran Plus (Schleicher & Schuell, Keene, New Hampshire). An internal oligonucleotide antisense probe, ME139 (5'-GCC ATA CCA GGT CTC CGT CG-3') complementary to nt 140 to 121 of the NV gene was end-labeled with digoxigenin (Boehringer Mannheim, Indianapolis, Indiana) by terminal deoxynucleotidyl transferase (Promega) and used to hybridize to NV mRNA at 37 C. Chemiluminescent detection of the hybridized digoxigenin-labeled probed with anti-digoxigenin was carried out as described by Holtke et al . (1992). The appearance of NV mRNA in IHNV-infected cells, shown as a 475 nt transcript (Fig. 4.2A), was first detected at 12 hpi when the CPE was observed in 30-40% of the cells. This is much later than the N gene transcripts which appear at 1-2 hpi (data not shown). NV transcript levels increased as infection continued and remained high through 24, 28, 32 and 36 hpi. At 18 hpi, 100% of the infected cells showed CPE, and at 36 hpi, 60-70% of cells were no longer attached to the plate surface; however, the NV mRNA was still detectable.

The detection of a single 475 nt transcript in IHNV infected cells indicated that there was no significant readthrough transcription of the G or L gene. The presence of only one NV transcript made it possible to develop an RNA dot blot assay to determine the quantity of NV mRNA in the infected cell. As a concentration marker, NV RNA transcripts were generated by in vitro transcription from an NV cDNA clone constructed with a T7 promoter and were quantitated by spectrophotometry. At 12 hpi, 30% of the cells showed CPE and the total NV mRNA was estimated at 20 ng per 2 x 10^6 cells by laser scanning densitometry (Fig.4.2B). At 18 hpi, 100 % of the cells expressed CPE and the cell number remained at 2 x 10^6. At this time point, the total NV mRNA was estimated to be 100 ng per 2 x 10^6 cells. Since the intracellular NV mRNA molecule is 475 nt, the total NV mRNA at 18 hpi was calculated to be 0.6 pmole or 3 x 10^{-7} pmole/cell (i.e. 8 x 10^5 molecules/cell). PolyA$^+$ RNA selected from mock-infected cells was included in the assay as a negative control. As shown in the Fig.4.2B, there was no background in the control. These results has shown that NV mRNA was synthesized in the infected cells as early as 12 hpi and increased in concentration after that time. The size of the NV specific RNA remained at 475 nt during the entire replication cycle suggesting that there was no significant degradation of the NV transcript in the infected cells.

The precise site of transcriptional initiation of NV mRNA was determined by primer extension of polyA$^+$-selected RNA with a ^{32}P end-labeled DNA primer, 5'- GCC ATA CCA GGT CTC CGT CG -3', complementary to a portion of the 5' end of the NV mRNA

Figure 4.2. (A). Time of appearance of the NV mRNA in IHNV-infected cells. Poly A^+ RNA extracted from mock-infected and IHNV-infected CHSE-214 cells at 3, 6, 12, 18, 24, 28, 32 and 36 hpi, were analyzed by Northern blot with an anti-sense NV probe. (B) Quantitation of the NV mRNA in IHNV-infected cells by dot blot hybridization. Poly$^+$ RNA samples were extracted from mock-infected and IHNV-infected CHSE-214 cells at 12 and 18 hpi. As a concentration marker, NV transcript was generated in vitro. A longer exposure of the blot demonstrated the detection limit of the probe was 160 pg.

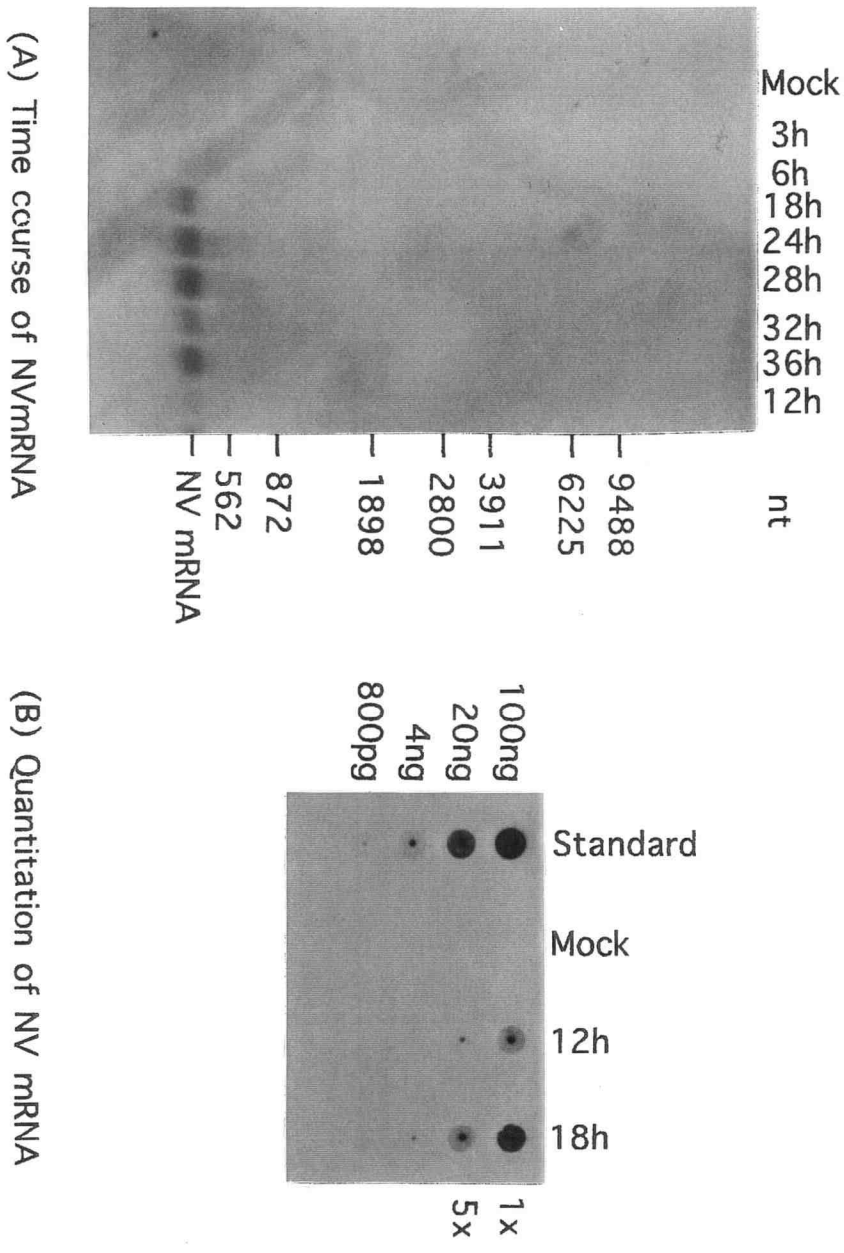
(A) Time course of NVmRNA
Mock
3h
6h
18h
24h
28h
32h
36h
12h
9488
6225
3911
2800
1898
872
562
NV mRNA
nt
(B) Quantitation of NV mRNA
100ng
20ng
4ng
800pg
Standard
Mock
12h
18h
1x
5x

Figure 4.2

120 bases downsteam of the end of the $(U)_7$ putative termination signal of the G gene. Two reactions were carried out with RNA extracted at 48 h and at 66 h postinfection from CHSE-214 cells infected with IHNV at a M.O.I. of 0.01. Hybridization of the primer was carried out overnight at 30 C, after which the primer was extended with AMV RT followed by digestion with pancreatic ribonuclease A. The primer extension product was analyzed on a 8% sequencing gel with a DNA sequencing reaction of a genomic G-NV cDNA clone with the same primer. Primer extension (Fig.4.3) revealed doublet bands which is similar to the result of the primer extension analysis for SYNV mRNAs (Heaton et al.., 1989; Scholthof et al.., 1994). The more rapidly migrating band coincided with a band initiated at the third residue (G) from the intergenic $(U)_7$ motif, and may represent the first nucleotide of the NV transcript. The more slowly migrating band, which comigrated at the position of the second residue (G) from the $(T)_7$ motif, is presumed to represent the 7-methyl-guanylate (cap) linkage to the first nucleotide of the NV transcript. This result suggests that the NV mRNA consists of 362 nt and the 5' end of the molecule consists of a cap structure followed by a tetranucleotide (5'-GCUC-3'); furthermore, since the NV mRNA is about 475 nt in length (Fig. 4.2A), the polyA tail was estimated to be 110 residues. The result provides evidence that the genomic consensus sequence, 3'-CGUG-5', serves as the transcriptional initiation site for the viral RNA polymerase.

This transcriptional initiation site was further confirmed by the primer extension analysis of the M1 and M2 mRNAs of IHNV with 32P end-labeled DNA primers, 5'- CGG GAT TCT AGC CTC AGT ATG TCT TCG CCT-3' and 5'- TTT GCT CTC TTG AAA ATA GAC ATG CTC TCG-3', complementary to a portion of the 3' end of the M1 and M2 genes (in the genomic sense) respectively (sequence data from Patty Ormonde, Master thesis, Oregon State University). Similar to the NV mRNA, the primer extension product of the M1 mRNA (Fig. 4.4A) revealed doublet bands migrating at positions corresponding to the second residue (G) and the third residue (G) from the $(T)_7$ motif, demonstrating the existence of a cap structure and the initiating of the M1 transcript at the same signal as the NV mRNA. Interestingly, an additional third band migrating at the fourth residue (C) was presented in the primer extension product of the M2 mRNA (Fig. 4.4B). This extra band might represent a premature product of the reverse transcriptase or another M2 transcript initiated at a second start site, the fourth residue. These results have confidently demonstrated the tetranucleotide, 3'-CGUG-5', as the transcriptional initiation site of IHNV. Since this tetranucleotide, instead of the 3'-UUGU-5', is also located at the same position in the gene junctions of the internal genes on the genomes of VHSV and HIRRV, it is very likely that this sequence serves as a transcriptional initiation signal common to these

Figure 4.3 Determination of the initiation site of transcription for the IHNV NV gene. A synthetic oligonucleotide end-labeled with ^{32}P was annealed to PolyA$^+$ RNA samples extracted from IHNV-infected CHSE-214 cells at 48 and 66 hpi separately. The reverse transcription products were analyzed on 8% urea-polyacrylamide gel. DNA sequencing reactions prime with the same primer are shown adjacent to the primer extension products. The arrow at the third nt, G, after the $(A)_7$ motif is considered the actual initiation site for the transcription.

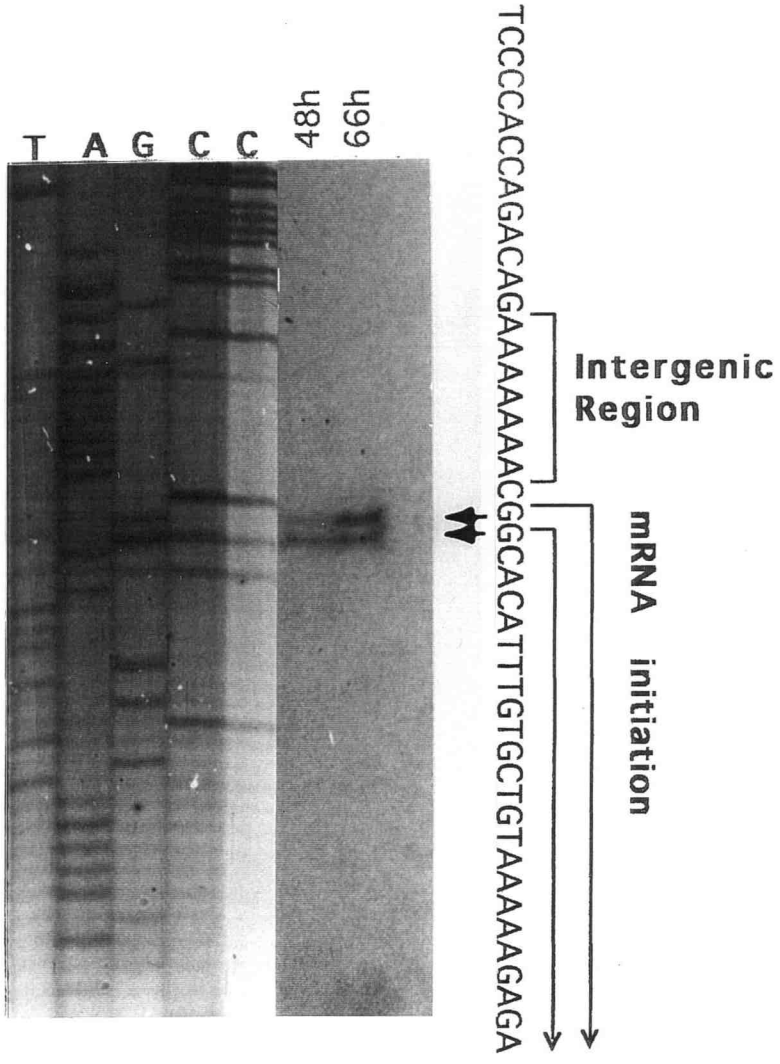
T A G C C
48h
66h
TCCCCACCAGACAGAAAAAAACGGCACATTTGTGCTGTAAAAAGAGA
Intergenic Region
mRNA initiation

Figure 4.3

Figure 4.4 Confirmation of the transcriptional initiation signal of IHNV M1 and M2 genes. (A) Determination of the initiation site of transcription for the M1 gene. The reverse transcription product of M1 mRNA reveals doublet bands, similar to the NV mRNA, indicating a cap structure and the tetranucleotide 3'-CGUG-5' as a transcriptional initiation signal. (B) Determination of the initiation site of transcription for the M2 gene. In addition to the doublet bands, an extra band exists at the at the fourth residue from the $(T)_7$ motif. This extra band may represent a premature product of the reverse transcriptase or a second initiation site starting at the second nt of the tetranucleotide 3'-CGUG-5'. DNA sequencing reactions were carried out with a cDNA clone containing the N, M1 and M2 genes of IHNV. The cDNA clone was kindly provided by Dr. G. Kurath.

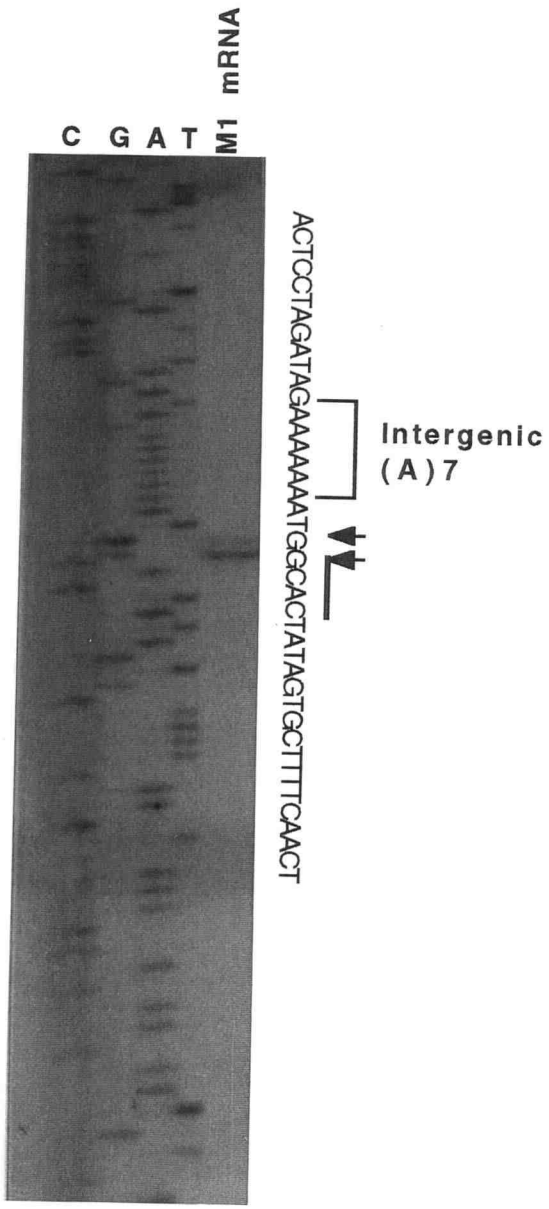
C G A T M1 mRNA
ACTCCTAGATAGAAAAAAATGGCACTATAGTGCTTTTCAACT
Intergenic
(A)7

Figure 4.4 (A)

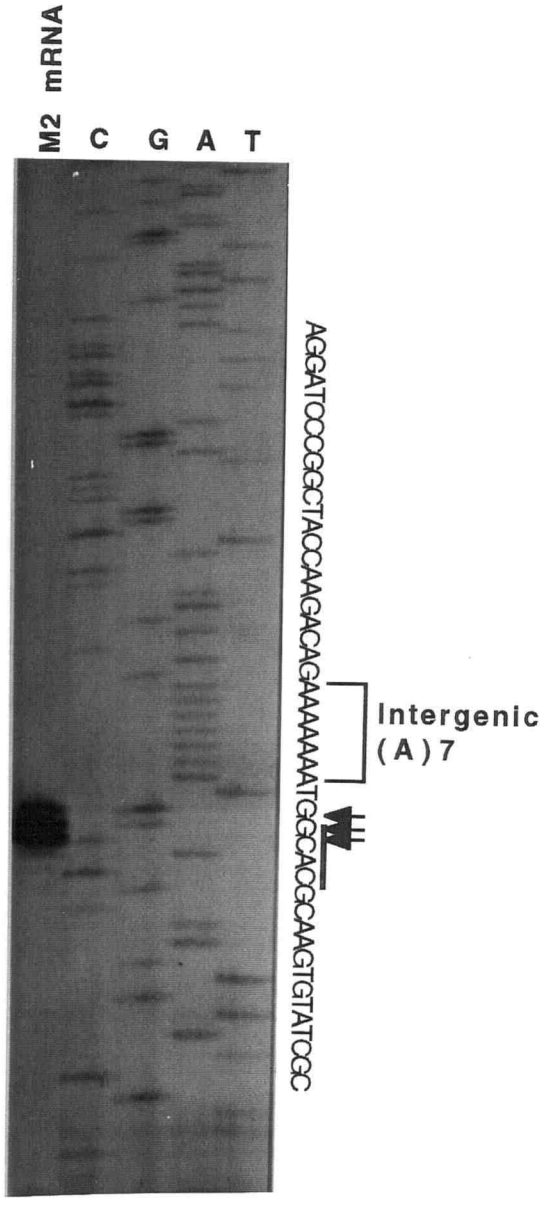
M2 mRNA
C G A T
AGGATCCCGGCTACCAAGACAGAAAAAATGGCACGCAAGTGTATCGC
Intergenic
(A)7

Figure 4.4 (B)

fish lyssa-like rhabdoviruses. However, it has been reported previously that the M2 mRNA of the Makah strain of VHSV was initiated two nucleotides after the sequence, 3'-UUGU-5', which is 46 nt downstream of the intergenic $(T)_7$ motif (Benmansour et al, 1994). In addition, the authors also found two smaller products of primer extension, one initiating at position 75 and the other at position 94. It is possible that these three products may represent the premature products of the reverse transcriptase. It is thus suggested that a reexamination on the 5' of the RNA transcripts of the VHSV may be necessary to determine if the transcriptional initiation sites are the same or if they differ for these fish lyssa-type rhabdoviruses.

In summary, we report here the analyses of the IHNV NV gene expression at the transcriptional level in vitro. We also provide the first experimental evidence confirming the function of the novel transcriptional initiation signal which is unique to fish lyssa-like rhabdoviruses. Because of the difficulty of detecting the NV protein both in vitro and in vivo as described previously, it has been suggested that the NV protein may not be essential to the viral infection (Basurco et al., 1995). We have clearly observed that the viral replication cycle in cell culture takes place in the absence of detectable amounts of the NV protein by pulse labeling (Chiou et al., chapter 3). However, the sequence stability in tissue culture, and among different isolates, and the conservation of the gene in different viruses strongly indicate the biological importance of the NV gene (Nichol et al., 1995; Schutze et al, 1995). In this report, the NV transcript has been shown to be stably expressed in the IHNV-infected cells throughout the infectious cycle, suggesting an important role of the NV gene in viral replication. It has been suggested that the NV protein is required in catalytic amount (Basurco and Benmansour, 1995). The turn-over of this protein may be very rapid in the infected cells and, as a result, the protein can be detected in the NV-overexpressed cells by an immunohistochemistry assay. Supporting this hypothesis, a transient transfection assay showed that less than 1% of the cells, transfected with the NV gene driven by a CMV4 promoter, were identified as positive by immunohistochemistry with the transfection efficiency of 20% (data not shown). With these characteristics, it is possible that the NV protein may serve its function by triggering, by itself or as a cofactor, a cascade of an important pathway in the cells.

Previously, IHNV, HIRRV, and VHSV were classified into the genus *Lyssavirus* with the prototypic *Lyssavirus*, rabies virus. This classification was based on the similarities of the virion morphology and the protein profile (McAllister and Wagner, 1975; Hill et al., 1975; Hsu et al., 1986). However, these fish lyssa-like rhabdoviruses differ from the other lyssaviruses by the presence of the NV gene. These viruses have since been

removed from the *Lyssavirus* genus and are currently assigned as unclassified rhabdoviruses (Sixth ICTV Report). Phylogenetic analyses also reveal that these fish lyssa-like viruses form an additional rhabdoviral clade which is separate from those containing members of the *Vesiculovirus* or *Lyssavirus* genera. (Morzunov et al., 1995; Kurath et al., 1996). A new genus, *Piscivirus*, has been proposed for these fish lyssa-like rhabdoviruses since all its known representatives have fish hosts (Morzunov et al., 1995; Kurath et al, 1996). In this work, we have identified a unique transcriptional signal whose nucleotide sequences are also specifically conserved in the intergenic gene junctions of all fish lyssa-like rhabdoviruses. Thus, our finding provides further evidence to support such proposal of a new genus for the fish lyssa-like rhabdoviruses.

Acknowledgments

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CHAPTER 5
TRANSIENT EXPRESSION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS MATRIX PROTEIN (M2) INHIBITS HOST-DIRECTED GENE EXPRESSION AND INDUCES NUCLEAR FRAGMENTATION IN CELL CULTURES

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This paper reports a portion of the work described in a thesis to be submitted to Oregon State University Department of Microbiology in partial fulfillment of the requirements for a PhD degree.

Abstract

The role(s) of the phosphoprotein (M1), matrix protein (M2) and nonvirion protein (NV) of infectious hematopoietic necrosis virus (IHNV) in viral pathogenesis were examined by transgenic expression of these proteins in fish cells. Expression of the M2 protein alone inhibited host-directed gene expression in a dosage-dependent manner as evidenced by the reduced activity of the reporter gene, luciferase, both *in vitro* and *in situ*. The M1 and NV proteins did not produce a similar effect. The M2 inhibition of host-directed gene expression was observed at 12 h posttransfection and continued up to 100 h. M2-mediated inhibition is selective. Northern blot analysis demonstrated that the M2-induced inhibition in luciferase activity was due to the reduction of luciferase mRNA; however, M2 protein did not inhibit the expression of its own mRNA. The effect of IHNV M2 protein on host-directed gene expression was also analyzed with cells cotransfected with the M2 gene and either the human ß-galactosidase gene under control of a same viral promoter or the luciferase gene under control of an IFN-inducible promoter. The data demonstrated that IHNV M2 protein-induced inhibition was not a gene-, viral promoter-, nor host-specific event. Fragmented nuclei were found in some cells transfected with the M2 gene, but not with cells transfected with either M1, NV, or the ß-galactosidase gene. IHNV infection also caused nuclear fragmentation, as evidenced by the presence of DNA laddering in infected cells. These data indicate that expression of the IHNV M2 protein may be responsible for triggering apoptosis in infected cells. Additionally, we found that transient expression of the NV gene was associated with cytopathic effect, as evidenced by cell rounding. The implications of these results on IHNV pathogenesis is discussed.

Introduction

Infectious hematopoietic necrosis virus (IHNV) causes an acute disease with high mortality among young trout and salmon (Amend et al., 1969). IHNV infection in fish can result in death, or in virus persistence in carrier fish with no apparent disease symptoms or detectable infectious virus (Drolet et al., 1995). In tissue culture cells, IHNV infection causes shutdown of host protein synthesis (Leong et al., 1983; Hsu et al., 1986), and leads to a cytopathic effect characterized as cell rounding, and ultimately cell death. Persistent infection has also been established in fish cells infected with IHNV (Engelking and Leong, 1981). IHNV is a rhabdovirus with structural similarity to vesicular stomatitis virus (VSV)

and rabies virus (RV). The IHNV genome encodes five virion proteins, including nucleocapsid protein (N), phosphoprotein (M1 or P), matrix protein (M2 or M), glycoprotein (G) and RNA polymerase (L). In addition, the genome encodes a nonvirion protein (NV), which is not associated with the mature virion (Kurath et al., 1985). Although the pathogenesis of IHNV has been studied extensively, except for the glycoprotein (Kim et al., 1994), the role(s) of other genes in viral pathogenesis has not been addressed.

The rhabdoviral M (matrix) genes encode multivalent proteins with several functions in virus replication (Coulon et al., 1990). For example, M proteins play a central role in the initiation of virion assembly by forming a bridge between the host plasma membrane and the ribonucleocapsid core (Bergmann and Fusco, 1988). Also, the M protein of VSV is solely responsible for the cytopathic effect (CPE), typically seen as cell rounding of polygonal cells in culture (Blondel et al., 1990). VSV M protein expressed in cells after DNA transfection has been shown to inhibit host-directed transcription of a co-transfected plasmid-encoded target gene, chloramphenicol acetyltransferase (CAT) (Black and Lyles, 1992), and stimulate translation of the CAT mRNA under the same conditions (Black et al., 1994). The combined effect was a greater-than-20-fold inhibition of CAT activity (Black et al., 1994). A recent study has further shown that the VSV M protein was able to reduce proviral transcripts of the human immunodeficiency virus type 1 (HIV-1) (Paik et al., 1995). These results demonstrate that the VSV M protein can inhibit expression from chromosomal DNA as well as plasmid DNA. So far the effect of VSV M protein-induced inhibition has been only examined with viral promoters including simian virus (SV40), human cytomegalovirus (CMV) and HIV. It has not been determined if M protein inhibition of transcription is promoter-specific and whether the M proteins of other negative-stranded RNA virus have the same effect as does the VSV M protein.

The VSV P protein is involved in viral RNA transcription and replication. No other function has been described. The phosphorylated IHNV M1 protein (McAllister, 1975; Hsu et al., 1986) is similar in sequence to the VSV P protein (Ormonde, 1995). The IHNV matrix protein, M2, is similar to all reported rhabdovirus matrix proteins The deduced amino acid sequence of IHNV M2 is highly basic and shares 17% and 20% identity with the matrix proteins of RV and VSV, respectively (Ormonde, 1995). M2 protein, like VSV M protein, is localized in the nucleus and cytoplasm of infected cells (Chiou et al., Chapter 3). The IHNV M2 protein likely performs a function similar to VSV M protein in inhibition of host gene expression.

It has been suggested that other viral proteins may be involved in the shutdown of host protein synthesis (Black and Lyles, 1992). A possible candidate is the NV protein, which is expressed in low quantities in infected cells, suggesting that the protein might function as a cofactor in a viral function (Chiou et al., see Chapter 3 and 4). The NV protein is also localized in the nucleus as well as cytoplasm of infected cells (Chiou et al., see Chapter 3). We report here a study of the roles of the M1, M2 and NV proteins of IHNV by transient expression of their genes, individually, in fish cells.

Materials and Methods

Cells and Viruses

The chinook salmon embryonic cell line (CHSE-214) (Fryer et al., 1965; Lannan et al., 1984) and *epithelioma papulosum cyprini* cell line (EPC)(Fijian et al., 1983) were used in this study. The cells were grown at 17 C in minimum essential medium (MEM) (GibcoBRL) supplemented with 10% fetal bovine serum (FBS) (Intergen), 100 IU/ml of penicillin (GibcoBRL), 100 µg/ml of streptomycin (GibcoBRL) and 2mM of L-glutamine (GibcoBRL). The cells were maintained in an incubator culture chamber (C.B.S. Scientific) perfused with a blood-gas mixture composed of 9.9% mol/mol CO_2, 10.2% mol/mol O_2, and 79.9% mol/mol N_2. Two IHNV isolates were used in the DNA fragmentation assay: RB1, a type 1 isolate, taken from an adult steelhead trout at the Round Butt Hatchery in the central Oregon in 1975 and RA, a type 2 isolate, taken in 1983 from dead rainbow trout fry at the International Aquaculture Research Center (Rangen Research), Hagerman, Idaho.

Plasmid DNA Constructs and DNA Transfection.

IHNV M1, M2 and NV genes were subcloned into an eukaryotic expression vector, pcDNA3 (Invitrogen), as described previously (Ormonde, 1995; Chiou and Leong, see Chapter 4). Expression of these viral genes were placed under the control of the human cytomegalovirus (CMV) immediate early promoter. Plasmids pcDNA3-M1(+) (pM1(+)), and pcDNA3-M1(-) (pM1(-)), contain the M1 gene in the protein-encoding and noncoding orientation, respectively; pcDNA3-M2(+) (pM2(+)) and pcDNA3-M2(-) (pM2(-)), contain

the M2 gene in the protein-encoding and noncoding orientation, respectively; and pcDNA3-NV(+) (pNV(+)) and pcDNA3-NV(-) (pNV(-)), contain the NV gene in the protein-encoding and noncoding orientation, respectively. The pCMV-Luc plasmid contains the firefly luciferase gene under control of the CMV immediate early promoter (Anderson et al., 1995). The p561-Luc plasmid (Bandyopadhyay et al., 1995) harbors the luciferase gene under control of the interferon-inducible 561 promoter, which contains the -134 to +1 nucleotides of the 561 gene. This region contains an ISRE and a GAF element. The plasmid was kindly provided by Dr. G. T. Leonard Jr. at the Case Western Reserve University. The construct, pcDNA3-ßgal (pßgal), containing the ß-galactosidase gene from the pSV-ß-Galactosidase plasmid (Promega), was inserted into the pcDNA3 vector used as a control for the immunofluorescence assay.

Unless otherwise indicated, transfections of thecells were performed in a 6-well plates (Corning). For CHSE-214 cells, monolayers were incubated with a solution of 1 ml of Opti-MEM (Gibco-BRL) containing 18 µg of lipofectamine (GibcoBRL) and 2 µg of total DNA. Transfected cells were incubated for 6 h at 17 C , washed twice with Opti-MEM and then resupplemented with 2 ml of MEM containing 10% FBS. Cells were maintained in an incubator culture chamber perfused with a blood-gas mixture. For EPC cells cotransfected with p561-Luc, monolayers were incubated with a solution of 1 ml of Opti-MEM consisting of 12 µg of lipofectamine and 2 µg of total DNA. Transfected cells were incubated for 6 h at 20 - 22 C, washed and then incubated with MEM without FBS.

Protein Radioactive Labeling and TCA Precipitation

Transfected cells were starved in methionine-free MEM containing 2% dialyzed FBS (GibcoBRL) for 15 min before labeling with [^{35}S]methionine for 1 h. Cells were washed twice with ice-cold phosphate buffered saline (PBS), and scraped from the plate in 2 ml of ice-cold PBS. Suspensions were transferred to conical tube, centrifuged for 5 min at 300 x g at 4 C. Pellets were resuspended in 1 ml of ice-cold PBS, transferred to fresh 1.5-ml microfuge tubes and centrifuged for 15 sec at 14,000 x g. Pellets were resuspended in 0.5 ml of 0.1 mg/ml BSA containing 0.02% NaN_3, and 0.5 ml of ice-cold 20% trichloroacetic acid (TCA). Suspensions were agitated vigorously with a vortex mixer, incubated for 30 min on ice and then centrifuged for 10 min. Pellets were resuspended in 200 ml of solution containing 0.5N NaOH and 0.1% Triton-X100. The total radioactivity in counts per minute

(CPM) of the precipitated proteins were measured in a Beckman LS 1800 liquid scintillation counter.

Luciferase Assays and ß-Galactosidase Assays

Luciferase assays were performed with the enhanced luciferase detection system (Analytical Luminescence Laboratory, MI) according to the manufacturer's instructions. Briefly, transfected cells were rinsed twice with ice-cold Mg^{2+} -free PBS and were then incubated with 200 µl of the manufacturer's lysis buffer at 4 C for 15 min. Cells were scraped from the plate and another 200 µl of the same lysis buffer was added into the plate to remove any residual cell debris. The mixture was transferred to a microfuge tube and centrifuged for 5 - 10 sec after which the supernatant was transferred to a clean microfuge tube. Aliquots of 10 µl of the supernatant were diluted in lysis buffer and luciferase activity was measured and integrated over a 30 sec period in a Beckman LS 8000 liquid scintillation counter on single photon mode. The resultant CPM were converted into the total CPM using the dilution factors.

The ß-galactosidase activity was determined *in situ* as described by Fischer et al. (1988). Briefly, transfected cells were fixed for 15 min in a solution containing 1% glutaraldehyde, 0.1M sodium phosphate buffer (pH 7.0), and 1 mM $MgCl_2$. The fixed cells were then incubated in a solution containing 0.2% X-gal (5-bromo-4-chloro-3-indolyl-ß-galactopyranoside; Promega), 10mM sodium phosphate buffer (pH7.0), 150 mM NaCl, 1mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6.3H_2O$, and 3.3 mM $K_3Fe(CN)_6$ for 3 to 5 h at 37 C, and the blue colored cells were identified under a light microscope.

Southern and Northern Blot Analysis

About 10^7 CHSE-214 cells were cotransfected with either pM2(+)/pCMV-Luc plasmid DNA or pM2(-)/pCMV-Luc plasmid DNA at a 1:1 ratio. At 12 h posttransfection, the cells were washed with PBS and subjected to DNase I treatment. The cells were harvested and lysed in ice-cold lysis buffer (10mM Tris, 0.5% Triton X-100; pH7.5) containing proteinase K (0.6mg/ml) (Sigma) for 30 min at 4 C. Total DNA was extracted by phenol/chloroform and then digested with Sma I (Promega). The DNA samples were electrophoresed on a 1% agarose gel and the separated DNA bands were transferred onto a

Nytran membrane (Schleicher & Schuell, Keene, New Hampshire). A ^{32}P-labeled dsDNA probe was used to detect the luciferase gene. The ^{32}P-labeled DNA probes were generated by random priming reaction using the luciferase gene as the template (Amersham).

Total RNA was extracted from transfected cells with phenol and guanidinium thiocyanate according to the RNAzol (Tel-Test, Inc., Friendwood, Texas) manufacturer's recommendations. Separation of polyA$^+$ RNA from total RNA was carried out with Oligotex-dT (Qiagen, Chatsworth, CA) by hybridizing to oligo dT primers coupled to a solid phase matrix. The RNA samples were electrophoresed on 1% agarose gel after denaturation with glyoxal and dimethyl sulfoxide and then transferred to a Nytran membrane. ^{32}P-labeled dsDNA probes were used to detect the viral or luciferase specific RNAs.

Immunofluorescence and Confocal Microscopy Analysis

CHSE-214 cells were grown in 8-well chamber slides (Fisher) to 70 - 80 % confluency and were transfected with DNA as previously described. At 48 h posttransfection, the transfected cells were washed twice with PBS and fixed with 3.7% formaldehyde (Fluka) in PBS for 10 min at room temperature. For permeabilization, the cells were incubated with 0.5% Triton-X 100 in PBS for 5 min at room temperature. A solution of 1% BSA with 0.1% Tween-20 in PBS was used as a blocking agent and incubated with cells for 30 min at room temperature. At this point, the blocking agent was used as the diluent for the primary and secondary antibody preparations. The samples were incubated with a 1:200 dilution of either rabbit anti-M1 (Engelking and Leong, 1989), or rabbit anti-M2 (Engelking and Leong, 1989) or rabbit anti-NV (Chiou et al., see Chapter 3) for 1 h. The cells were washed and subsequently incubated with goat anti-rabbit Ig conjugated with Texas Red (10 µg/ml, Molecular Probes, Inc.) for 1 h. After washing the samples, 250 nM of the nucleic acid staining dye, DAPI, was added for 5 min. The cells were washed, allowed to air dry, and then mounted in Cytoseal (Stephens Scientific). Confocal images were captured using a Leica TCS 4D confocal microscope and combined using Adobe Photoshop software (Mountainview, CA).

Electron Microscopy

Mock-transfected and pM2(+)-transfected cells were collected, washed in PBS, and pelleted. Cells were fixed in a mixture of glutaraldehyde and osmium tetroxide and processed according to standard protocol. Ultrathin sections were stained with uranyl acatate and lead citrate, and examined in a Philips CM12 transmission electron microscope.

DNA Fragmentation Assay

CHSE-214 cells in a 150 cm^2 plate (approximately 2 x 10^7 cells) were infected with virus at multiplicity of infection (M.O.I.) of 10. The low molecular weight DNA was extracted at 6, 12 and 24 h postinfection (Hinshaw et al., 1994; Takizawa et al., 1993). Briefly, the cells were washed twice in PBS, trypsinized, resuspended in 1.5 ml PBS and then transferred to a 2-ml microfuge tube. Cells were centrifuged for 15 sec at 13,000 x g at 4 C and resuspended in 2 ml PBS. These cells were centrifuged once more and the pelleted cells were lysed in 800 ml of ice-cold lysis buffer, and incubated on ice for 30 min. After centrifugation of the lysates for 10 min at 13,000 x g at 4 C, the supernatant fluids were extracted with buffered phenol followed with buffered phenol-chloroform-isoamylalcohol. DNA was then precipitated with ethanol and treated with RNase A to a final concentration of 1.0 mg/ml for 30 min at 37 C. Aliquots of the DNA sample were electrophoresed through a 2% agarose, and the DNA was stained with ethidium bromide.

Results

Effects of M1, M2 and NV Proteins on Total Protein Synthesis in Transfected Cells

The effects of M1, M2 and NV protein on total protein synthesis were assayed by pulse labeling the transfected cells with radioactively-labeled amino acid. CHSE-214 cells were mock-transfected or transfected with pM1(+), pM2(+) or pNV(+) plasmid DNA. At 48 h posttransfection, the cells were pulse-labeled for 1 h with [^{35}S]methionine (30 µCi/ml) and lysed. Total proteins were then harvested by TCA precipitation. As shown in Figure 5.1, total incorporation of [^{35}S]methionine into the newly synthesized proteins was reduced

in the cells subjected to M2-transfection. Transfection with either M1 or NV gene, however, did not inhibit the [^{35}S]methionine incorporation into the proteins *de novo*. Since not all cells take up DNA, and the nontransfected cells were actually the dominant population in the culture, this preliminary result indicates that M2 protein is a potent inhibitor of protein synthesis. As shown in the Figure 5.1, the sensitivity of this assay is limited because of the interference from the nontransfected cells, and therefore is not sensitive enough to examine the effect caused by a regulator with an influence on gene expression. This difficulty was overcome by employing a cotransfection assay described below, in which the target gene being monitored is expressed only in cells that also express the viral gene of interest. However, the data suggest that IHNV M2 protein plays a very important role in the inhibition of the host gene expression.

M2 Protein Inhibited Host-Directed Expression of Plasmid-Encoded Target Genes

To examine the effect of the viral proteins on host-directed gene expression, CHSE-214 cells were cotransfected with plasmids expressing M1, M2 or NV gene and plasmids expressing luciferase as a reporter. All the genes were under control of the CMV immediate early promoter. Three plasmids, pM1(-), pM2(-) and pNV(-), expressing the viral gene in the antisense were included as negative controls. Cells were cotransfected with viral gene-encoded plasmid and a constant amount of pCMV-Luc plasmid at ratios of 19:1, 10:1, 5:1, 1:1, 0.1:1 and 0:1 (Fig. 5.2). The total amount of plasmid DNA transfected in each sample was kept constant at 2mg per ml by adding the appropriate amount of pcDNA3, the parent plasmid of these viral gene-encoded plasmids. At 24 h posttransfection, as shown in the Figure 5.2A and C, M1 and NV proteins demonstrated no significant differences in the target gene expression; whereas, expression of M2 protein drastically inhibited the luciferase activity in a gene dosage-dependent manner (Fig. 5.2B). The luciferase activity decreased 10-fold when the M2(+) plasmid was cotransfected at a 1:1 ratio to the pCMV-Luc plasmid and reached the maximum inhibition (40-fold) at the ratio of 19:1. Even at a ratio of as low as 0.1:1, there was a 2-fold reduction in the luciferase activity. These results demonstrated that expression of the IHNV M2 protein in the absence of other IHN viral proteins inhibited the host-directed expression of a plasmid-encoded gene and furthermore, neither the M1 nor NV proteins were able to cause a similar effect. However, the results did not rule out the possibility that these and other viral products can function as a cofactor in inhibition of target gene expression.

Figure 5.1 Effect of M1, M2 and NV protein on total protein synthesis. CHSE-214 cells were transfected with either pM1(+), pM2(+) or pNV(+) plasmid. At 24 h posttransfection, cells were labeled with [^{35}S]methionine for 1 h and then lysed. Total cellular proteins were precipitated by TCA and the total CPM of the precipitated proteins was measured in a Beckman LS 1800 liquid scintillation counter. Mock-transfected cells and untreated cells were included as controls.

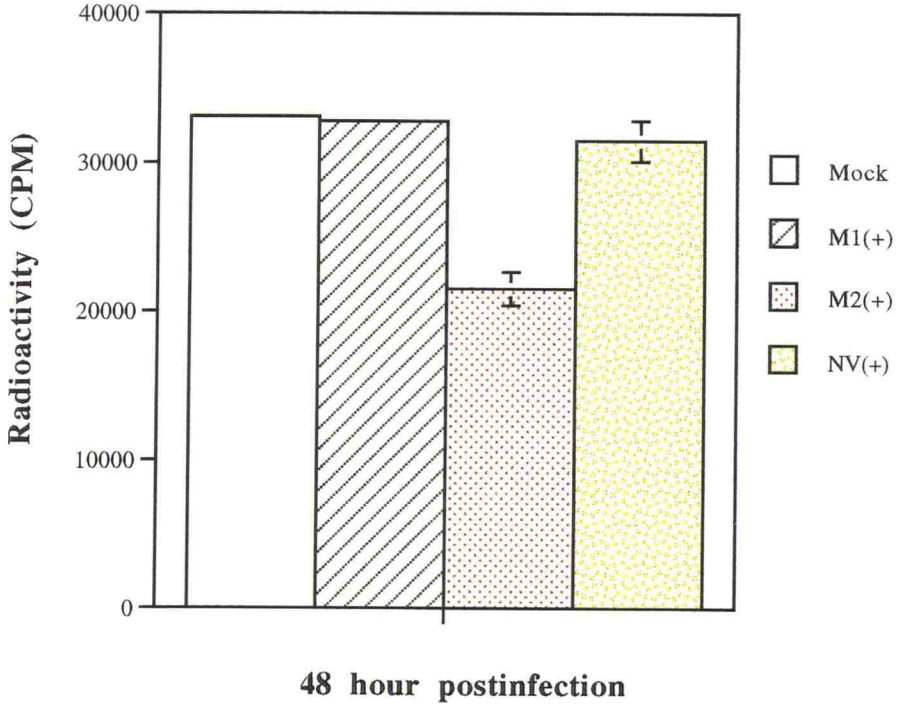
40000
30000
20000
10000
0
Radioactivity (CPM)
Mock
M1(+)
M2(+)
NV(+)
48 hour postinfection

Figure 5.1

Figure 5.2 Analysis of the effect of M1, M2 and NV protein on luciferase gene expression. (A), (B) and (C) CHSE-214 cells were cotransfected with viral gene-encoded plasmid and a constant amount of pCMV-Luc plasmid at ratios of 19:1, 10:1, 5:1, 1:1, 0.1:1 and 0:1. The total amount of plasmid DNA transfected in each sample was kept constant at 2mg per ml by adding the appropriate amount of pcDNA3, the parent plasmid of these viral gene-encoded plasmids. (A) cells cotransfected with pM1(+) or pM1(-); (B) cells cotransfected with pM2(+) or pM2(-); (C) cells cotransfected with pNV(+) or pNV(-). Luciferase activity was assayed at 24 h posttransfection. (D) Time course of M2 protein-induced inhibition of the luciferase gene expression. CHSE-214 cells were cotransfected with pM2(+) or pM2(-) and a constant amount of pCMV-Luc at the ratio of 1.9:0.1. Cells were then lyzed and analyzed at 12, 24, 48 and 100 h posttransfection.

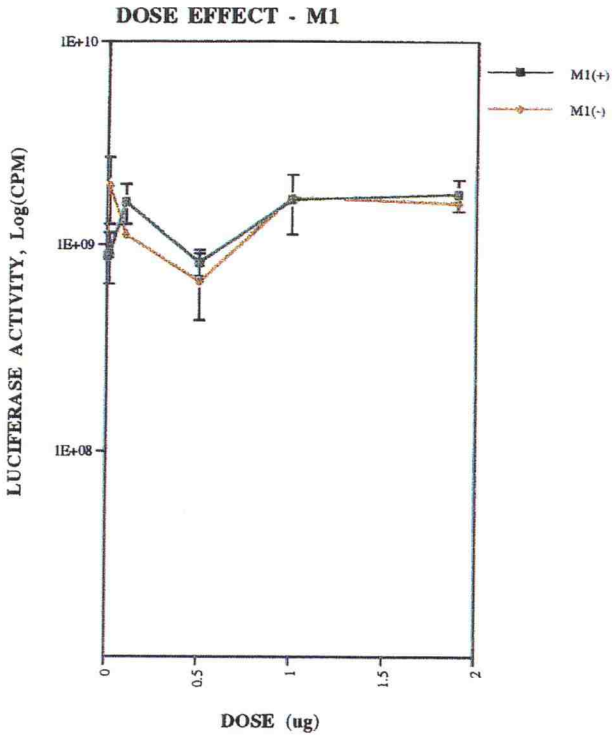

Figure 5.2 (A)

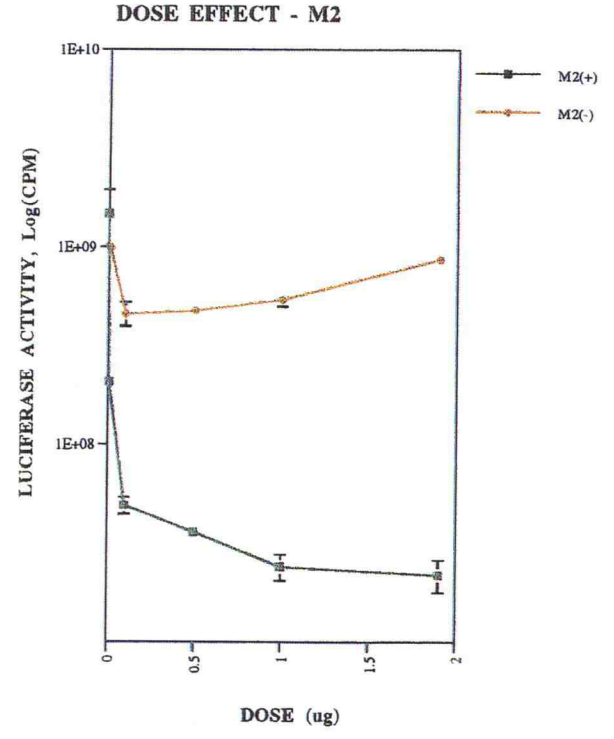

Figure 5.2 (B)

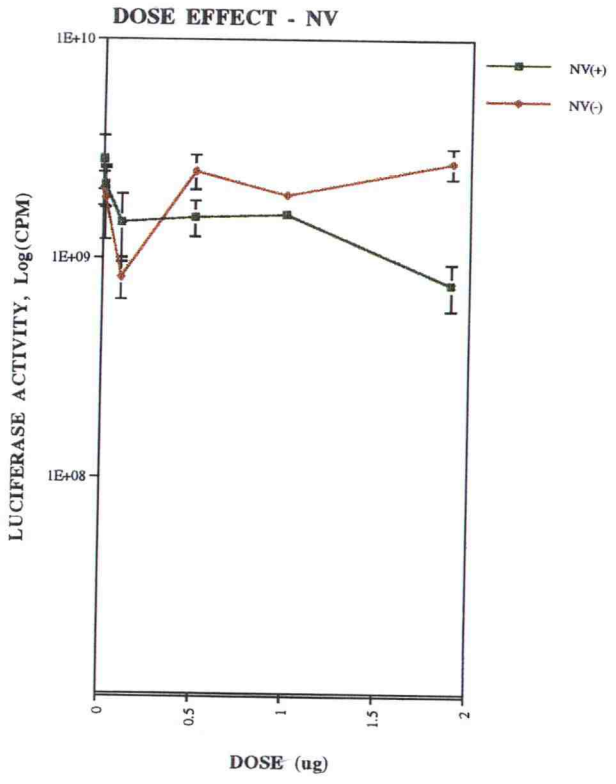

Figure 5.2 (C)

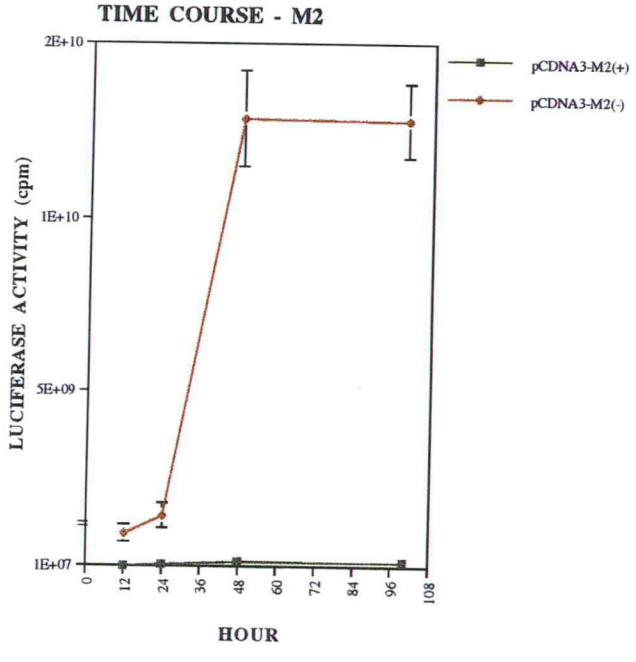

Figure 5.2 (D)

Figure 5.2D shows the time course of M2 protein-induced inhibition of luciferse gene expression. Cells were cotransfected with pM2(+) or pM2(-) and a constant amount of pCMV-Luc at the ratio of 1.9:0.1, since cells transfected at this ratio exhibited the highest inhibition (Fig. 5.2B). The cells were then lysed and analyzed at 12, 24, 48 and 100 h posttransfection. Inhibition of luciferase activity was observed as early as 12 h posttransfection and the maximal inhibition was reached at about 48 h posttransfection with a 100-fold reduction. In this assay, the medium was replaced with fresh medium daily and as shown in Figure 5.2d, the inhibition of the luciferase activity was still observed at 100 h posttransfection with a 100-fold reduction. These results demonstrated that M2 protein inhibited the target gene expression as early as 12 h posttransfection and that this inhibition persistented for up to 100 h.

The extracellular luciferase activity was measured to rule out the possibility that the differences in luciferase activity in the IHNV M2-transfected cells were due to leakage of cellular components through any disruption to the plasma membrane. The culture medium was collected at 24 and 72 h posttransfection and then centrifuged to remove any cellular debris. In the experiment, the culture medium was not replaced daily so that the accumulation of luciferase activity in the medium could be monitored. As shown in Figure 5.3, at 24 h posttransfection approximately the same activity level of luciferase was detected in the culture medium of both the pM2(-)/pLuc-transfected cells and the pLuc-transfected cells. The luciferase activity, however, was inhibited (45-fold) in the medium of the pM2(+)/pLuc transfected cells. This result was parallel to the luciferase activity of the intracellular luciferase. Even at 80 h posttransfection, the extracellular luciferase activity of the pM2(+)/pLuc transfected cells was persistently inhibited (30 to 40-fold) when compared to the other controls. These results demonstrated that the inhibition of the luciferase expression in the pM2(+)-transfected cells was not due to the leakage of cytoplasmic components nor the detachment of the transfected cells. It also suggests that luciferase is transported out of cells at a constant rate in all three transfected cell populations, and indicates that the M2 protein dose not produce significant effect on secretion of intracellular components.

Southern blot analysis was performed to determine whether the differences in the luciferase expression in the pM2(+) cotransfected cells might be due to differences in transfection efficiency. Approximately 10^7 CHSE-214 cells were cotransfected at a 1:1 ratio with either the pM2(+)/pCMV-Luc or the pM2(-)/pCMV-Luc plasmid. At 12 h posttransfection, the cells were washed with PBS and subjected to DNase I treatment. The cells were then harvested and lysed with proteinase K. Total DNA was extracted by

phenol/chloroform and then digested with Sma I, which cleaves the pCMV-Luc plasmid into two fragments of 1.9 kb (the luciferase insert) and 4.9 kb (the vector). The DNA samples were electrophoresed on a 1% agarose gel and the separated DNA bands were transferred onto a Nytran membrane. The luciferase insert was then detected with a ^{32}P-labeled DNA probe specific for the luciferase gene. As shown in Fig. 5.4, lanes 3 and 4 demonstrated the equal amounts of the 1.9-kb luciferase DNA present in the cells cotransfected with pM2(+) and pM2(-). Lane 2, a fragment of the luciferase gene amplified by PCR, was included as a positive control and lane 1 contained a fragment of the M2 gene as a negative control. These results demonstrated that both M2(+) and M2(-) cells were cotransfected with equal amounts of the reporter plasmid and thus indicated that these two cell populations were uniformly transfected. The DNase I treatment eliminated the possibility that the DNA may have been bound to the plasma membrane of the cells rather than internalized by the cells (Figure 5.4). Taken together the differences in the luciferase activity between the pM2(+)/pCMV-Luc and pM2(-)/pCMV-Luc transfected cells were not due to the differences in transfection efficiency of these two populations.

We examined the possibility that the M2 protein-induced inhibition of luciferase gene expression could be a gene-specific event. A plasmid expressing another reporter, ß-galactosidase, under the control of the same CMV promoter was employed. CHSE-214 cells were cotransfected with the pM2(+)/pßgal or the pM2(-)/pßgal plasmid at ratios of 0:1 and 1:1. Five days posttransfection, cells were fixed in glutaraldehyde and incubated with X-gal. The cells expressing ß-galactosidase were then identified by their blue color under a light microscope. The result of the X-gal staining and the relative reduction by M2 protein are shown in Table 5.1. In the cells cotransfected with pM2(+) there was a 96.5% reduction in the number of cells expressing ß-galactosidase compared with the cells not cotransfected with pM2(+). There was a 23.6% reduction of ß-gal expressing cells in the pM2(-)-cotransfected cells. These data provide *in situ* evidence of the M2 protein-induced inhibition of reporter gene expression and demonstrate that the M2 inhibition effect is not a gene-specific event.

Figure 5.3 Analysis of the luciferase activity in the culture medium. CHSE-214 cells were cotransfected with pM2(+) or pM2(-) and a constant amount of pCMV-Luc at the ratio of 1.9:0.1. In this assay, the culture medium was collected at 24 and 72 h posttransfection and then harvested by centrifugation to remove any cellular debris. The culture medium was not replaced daily so that the accumulation of luciferase activity in the medium could be monitored.

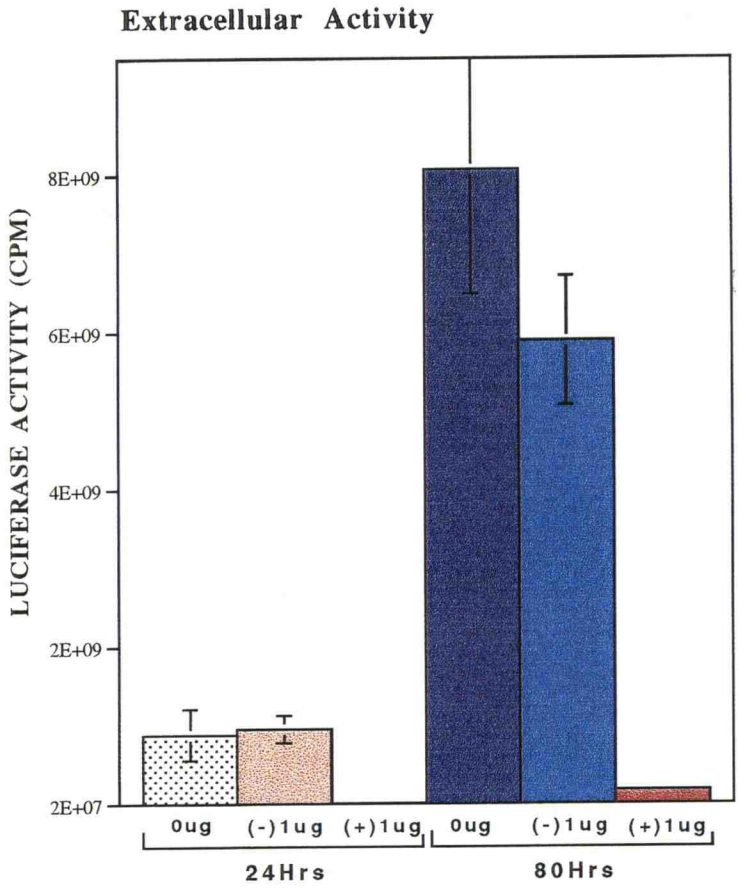
Extracellular Activity
LUCIFERASE ACTIVITY (CPM)
8E+09
6E+09
4E+09
2E+09
2E+07
0ug
(-)1ug
(+)1ug
0ug
(-)1ug
(+)1ug
24Hrs
80Hrs

Figure 5.3

Figure 5.4 Southern blot analysis of transfected plasmids. 10^7 CHSE-214 cells were cotransfected with either pM2(+)/pCMV-Luc plasmids or pM2(-)/pCMV-Luc plasmids at a 1:1 ratio. At 24 h posttransfection, total cellular DNA from transfected cells was extracted, digested with Sma I, and then analyzed by Southern blot. Lane1 contains an M2 fragment from pM2(+); lane 2, a fragment of luciferase gene from the pCMV-Luc DNA by PCR amplification; lane 3, DNA from pM2(-)/pCMV-Luc cotransfected cells; lane 4, DNA from pM2(+)/pCMV-Luc cotransfected cells. Sma I cleaves the pCMV-Luc plasmid into two fragments of 1.9 kb (the luciferase insert) and 4.9 kb (the vector). Molecular sizes in kilobase pairs (kb) are indicated at the left.

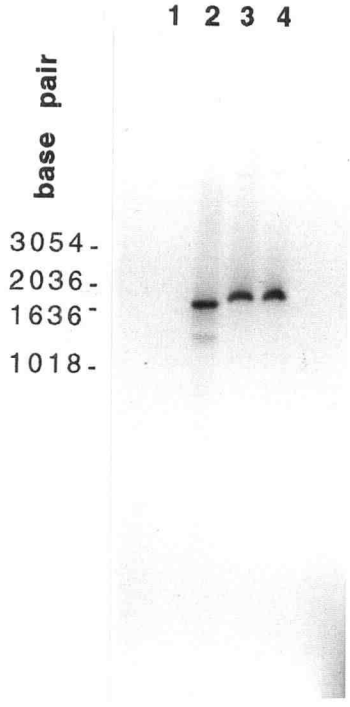

Figure 5.4

Table 5.1 Analysis of the effect of M2 protein on ß-galactosidase expression.

Treatment	Number of positive cells	Reduction %
pM2(+)/pßgal (0µg/1µg)	5544	---
pM2(+)/pßgal (1µg/1µg)	192	96.5
pM2(-)/pßgal (1µg/1µg)	4236	23.6

M2 Protein Inhibited Expression of a Target Gene Controlled by an Interferon-Inducible Promoter

The effect of IHNV M2 protein on the host-directed gene expression was also analyzed on cells cotransfected with the M2 gene and another reporter plasmid, p561-Luc, which contained the luciferase gene under control of the IFN-inducible promoter, 561. This assay was performed for the following easons. All the assays described above were analyzed on the same cell line as well as the same consistitutively-expressed, virally-originated CMV immediate early promoter. A reporter gene controlled by another promoter is thus cotransfected into another cell line to examine the possibility that the IHNV M2 protein-induced inhibition of host-directed gene expression may be a promoter-specific or host-specific case. The 561 promoter was chosen because it is inducibleand nonvirally-originated.

In this assay, EPC cells, a carp cell line, were cotransfected with pM2(+)/p561-Luc plasmids or pM2(-)/p561-Luc plasmids at ratios of 1:1, 0.1:1 and 0:1 (Fig. 5.5). At 12 h posttransfection, the transfected cells were treated with MEM-0 or 100 mg/ml poly IC in MEM-0. Cells were harvested and analyzed after 12 h of poly IC induction. Poly IC ds RNA is a potent artificial inducer of IFN that affects the same region of the beta IFN promoter as viral infection (Goodbourn and Maniatis, 1985). It has been shown in mammalian cells that the induction by ds RNA of the IFN-inducible 561 gene is direct and is not mediated by the intermediate synthesis of IFN (Bandyopadhyay et al., 1995). Fish cells can produce an IFN-like activity and express Mx protein, an IFN-inducible protein, during IHNV infection (Trobridge et al., 1996). Response to poly IC induction was also observed in EPC cells transfected with the p561-Luc plasmid (Marc Johnson, Oregon State University, personal communication).

Figure 5.5 demonstrates poly IC induced luciferase activity in cells cotransfected with pM2(+) or pM2(-) plasmid. The expression of M2 protein abolished the poly IC-induced luciferase activity in a gene dose-dependent fashion (Fig. 5.5) similar to the finding in Fig. 5.2b. The luciferase activity decreased 5-fold when the M2(+) plasmid was cotransfected at a 0.1:1 ratio to the p561-Luc plasmid and decreased 15-fold at the ratio of 1:1. In fact, the luciferase activity of M2(+)-cotransfected cells was shut down almost completely at the ratio of 1:1 as shown in Fig. 5.5. These results demonstrate that the IHNV M2 protein-induced inhibition of host-directed gene expression is neither a promoter-specific nor a host-specific event. The data also indicate that expression of M2 protein can

Figure 5.5 Analysis of the effect of M2 protein on expression of luciferase under control of 561 promoter. EPC cells were cotransfected with pM2(+)/p561-Luc plasmids or pM2(-)/p561-Luc plasmids at ratios of 0:1, 0.1:1 and 1:1. Transfected cells were induced with or without poly IC (100 mg/ml) at 12 h posttransfection. Luciferase activity was analyzed at 12 h after poly IC induction.

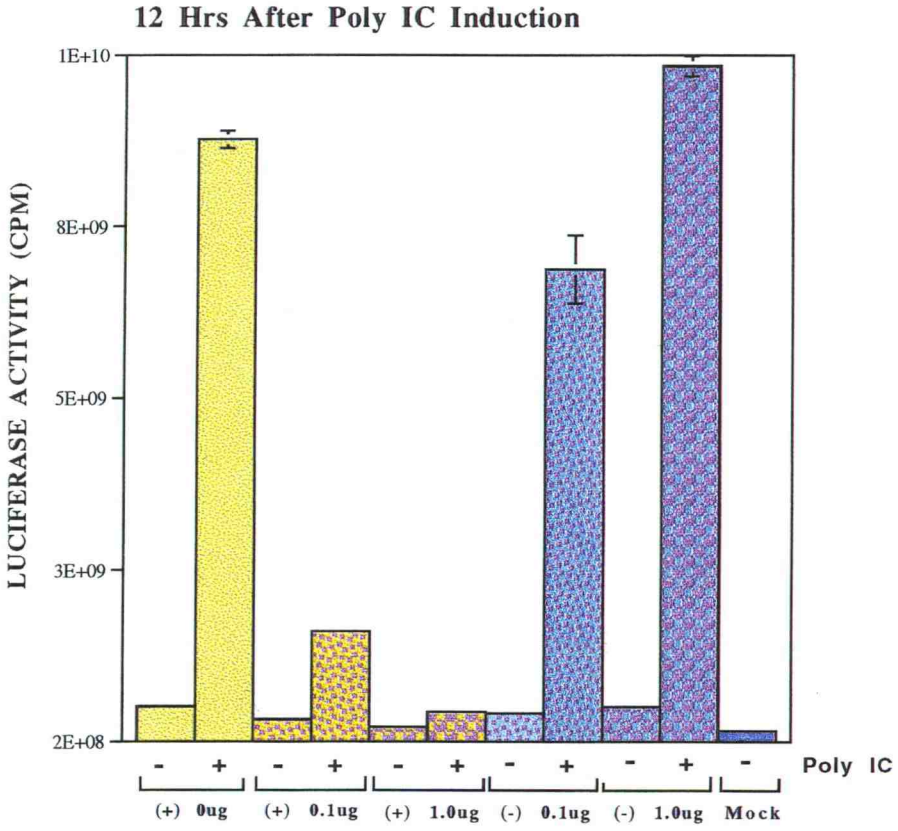
12 Hrs After Poly IC Induction
LUCIFERASE ACTIVITY (CPM)
1E+10
8E+09
5E+09
3E+09
2E+08
- + - + - + - + - + -
Poly IC
(+) 0ug
(+) 0.1ug
(+) 1.0ug
(-) 0.1ug
(-) 1.0ug
Mock

Figure 5.5

Figure 5.6 Northern blot analysis of plasmid-encoded mRNAs. 10^7 CHSE-214 cells were cotransfected at 1:1 ratio with either pM2(+)/pCMV-Luc or pM2(-)/pCMV-Luc plasmids. At 24 h and 72 h posttransfection, polyadenylated RNAs were isolated from the transfected cells and were subjected to Northern blot analysis with a ^{32}P-labeled dsDNA probe specific to luciferase gene (A) or M2 gene (B). Gels in panel (A) and (B) contain equal amount of same RNA samples. Lane1 contains the M2 fragment from pM2(+) plasmid; lane 2, the luciferase fragment from pCMV-Luc; lane 3, RNA from pM2(-)/pCMV-Luc cotransfected cells at 24 h posttransfection; lane 4, pM2(+)/pCMV-Luc, 24 h; lane 5, pM2(-)/pCMV-Luc, 72 h; lane 6, pM2(+)/pCMV-Luc, 72 h. Molecular sizes in kb are indicated at the left.

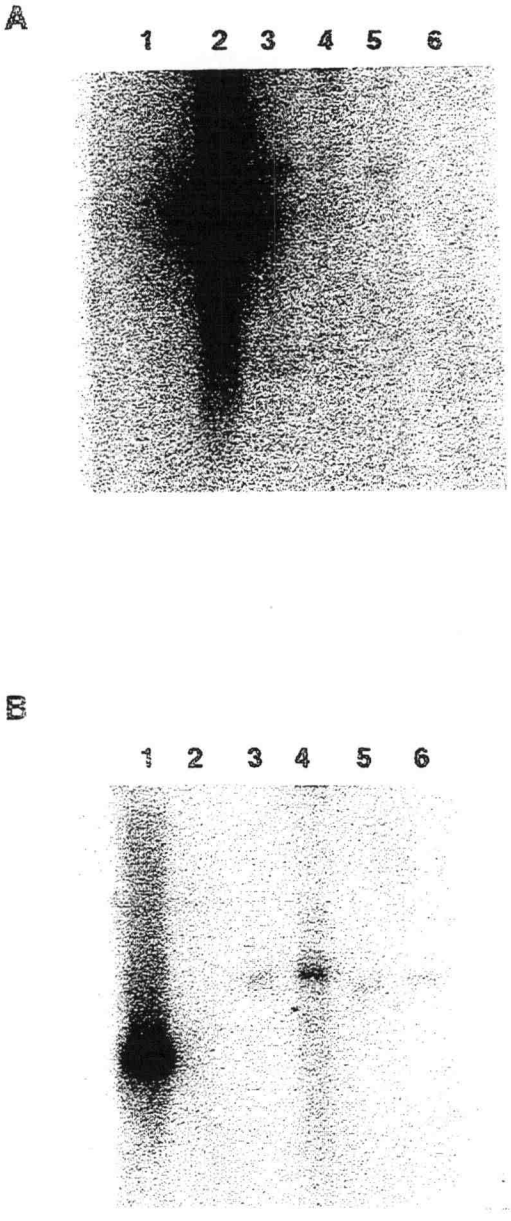

Figure 5.6

inhibit the expression of an IFN-induced early response gene and thus may play an important role in the host defense system during IHNV infection.

Effect of M2 Protein Expression on the Level of Target Gene mRNA.

The quantity of luciferase mRNA was examined by Northern analysis to determine the effect of M2 protein on the expression of the reporter gene. In this assay, about 10^7 CHSE-214 cells were cotransfected with either pM2(+)/pCMV-Luc or pM2(-)/pCMV-Luc plasmids at a 1:1 ratio. At 24 h and 72 h posttransfection, polyadenylated RNAs were isolated from the transfected cells and were subjected to Northern blot analysis with a ^{32}P-labeled DNA probe specific to luciferase gene. Figure 5.6A shows that the amount of luciferase mRNA was inhibited in cells cotransfected with M2(+) gene (lanes 4 and 6) rather than in cells cotransfected with M2(-) gene (lanes 3 and 5) at 24 h and 72 h posttransfection. Luciferase mRNA level in both M2(+)- and M2(-)-cotransfected cells decreased at 72 h posttransfection. Comparison of the signal intensity of luciferase mRNA in a Phosphoimage scanner (Molecular Dynamics) reveals that the luciferase mRNA level in M2(+)-cotransfected cells was decreased 15-fold at 24 h posttransfection and was completely abolished at 72 h posttransfection. The level of luciferase mRNA in cells cotransfected with M2(-) was found to decrease 5-fold from 24h to 72 h posttransfection (Fig. 5.6A). These data suggest that the M2-induced inhibition of luciferase activity (Fig. 5.2B) was at least partially due to the reduction of the luciferase mRNA.

Figure 5.6B shows a Northern blot analysis of polyadenylated M2 RNAs isolated at 24h and 72 h posttransfection detected with a ^{32}P-labeled dsDNA probe, which is able to recognize both the positive- and negative-sense of the M2 gene. Unlike the luciferase mRNA, the amount of polyadenylated M2 RNA did not decrease in the M2(+)-cotransfected cells as did the luciferase mRNA. In fact, comparison of the signal intensity reveals that the amount of the M2 polyadenylated RNA was about 2-fold higher in the M2(+)-cotransfected cells (lane 4 and 6) than in the M2(-)-cotransfected cells (lane 3 and 5) at 24 h as well as 72 h posttransfection. Since these cells were transfected with equal amounts of M2 gene-encoded plasmids, the difference might have been caused by the differences in the hybridization efficiency of the RNAs to the ds probe. It is also possibly due to a higher transcription efficiency or stability of the M2(+) polyadenylated RNA than those of the M2(-) polyadenylated RNA. Alternatively, the higher signal in the M2(+) polyadenylated RNA may be due to the higher incorporation rate of [^{32}P] a-dCTP into its specific probe because

the M2(+)-specific oligomer has a higher ratio of C residue than that of the M2(-)-specific oligomer (198 versus 166 residues). Therefore, it is possible that the M2(+) and M2(-) polyadenylated RNA were synthesized in equal amounts. In any case, the results demonstrate that the expression of IHNV M2 protein did not inhibit expression of its own mRNA under the condition the luciferase mRNA was inhibited.

Transfection of IHNV M2 Gene Causeed Nuclear Fragmentation

To confirm the expression of the viral proteins in the transfected cells and to observe the morphological cytopathic effect which may be caused by the viral proteins, cells expressing the M1, M2 or NV protein were examined by immunofluorescence under a confocal microscope. CHSE-214 cells transfected with pM1(+), pM2(+), or pNV(+) were fixed and hybridized with Texas Red-conjugated antiserum specific to M1, M2 or NV protein. Cells transfected with pbgal were included as negative controls. A UV-excited blue-emitting fluorophore, DAPI (4, 6-diamidino-2-phenylindole) was used to fluorescently label the nuclei of these transfected cells. Confocal microscopy was conducted by capturing single optical sections through the nucleus of the cell. It has been demonstrated that in IHNV-infected cells, M1 protein is confined to the cytoplasm while M2 and NV proteins are expressed in the nucleus and the cytoplasm (Chiou et al., Chapter 3). As shown in Fig. 5.7, in transfected cells, M1 protein was expressed in the cytoplasm and NV protein was found in both the nucleus and the cytoplasm, as they were observed in infected cells. Interestingly, NV protein was frequently identified in cells exhibiting cell rounding, a typical cytopathic effect caused by IHNV infection. This result suggests that NV protein may be responsible for the cell rounding effect caused by IHNV infection. M2 protein was found in both the nucleus and the cytoplasm of transfected cells. In addition, M2 protein was identified in cells undergoing nuclear fragmentation (Fig. 5.7D). The presence of M2 protein was implicated in nuclear fragmention since this phenomenon was not identified in any other cells transfected with either pM1(+), pNV(+) or pbgal (Fig. 5.7B and F).

Fragmented nucleus is one of the manifestations of cells undergoing internally programmed cell death, also called apoptosis. Some virus infections can trigger events that lead to apoptosis, characterized by the appearance of cell shrinkage, plasma membrane blebbing, nuclear condensation and fragmentation, and ultimately appearence of apoptotic bodies. Figure 5.8B shows the ultrastructure of a CHSE-214 cell transfected with pM2(+)

Figure 5.7 Immunofluorescence analysis of the cellular localization of transgenic proteins. In panels B, D, and F, CHSE-214 cells were transfected with pM1(+), pM2(+) and pNV(+) plasmid respectively. Cells transfected with pbgal (A, C and E) were included as controls. At 48 h posttransfection, cells were fixed and labeled with antiserum specific to M1 (A and B), M2 (C and D) or NV (E and F), followed by incubation with goat anti-rabbit Ig conjugated with Texas Red (10 mg/ml), Molecular Probes, Inc.) and counterstaining with 250 nM DAPI. Confocal images were captured using a Leica TCS4 confocal microscope.

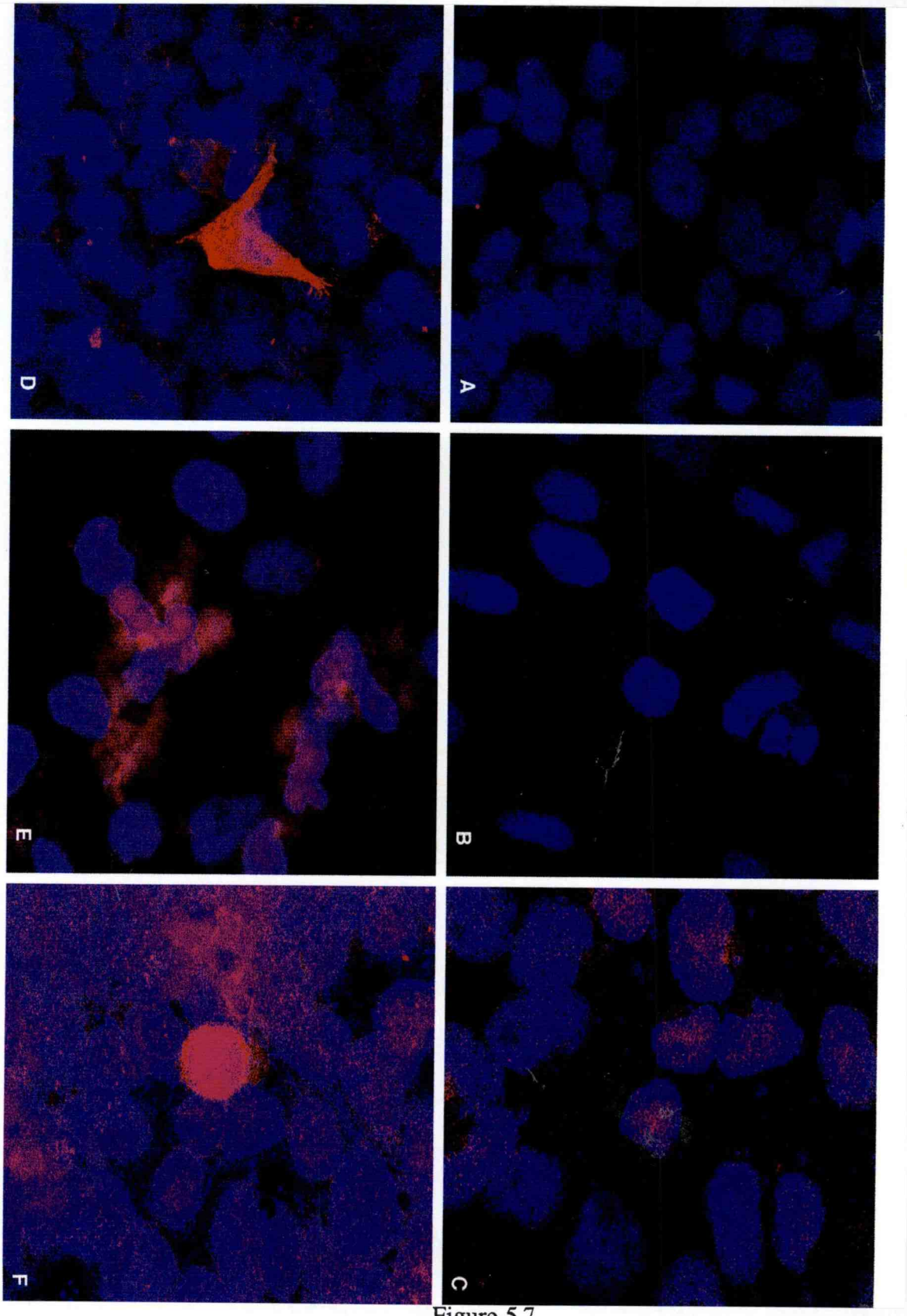

Figure 5.7

Figure 5.8 Electron micrograph of control (A) and M2-transfected CHSE-214 cell (B). CHSE-214 cells were transfected with pM2(+) plasmid and fixed at 24 h posttransfection. Note the fragmented nucleus (arrows), chromatin condensation and cell shrinkage. Magnification: (A), 8,000 x, (B), 6,300 x. Bar, 100 µm.

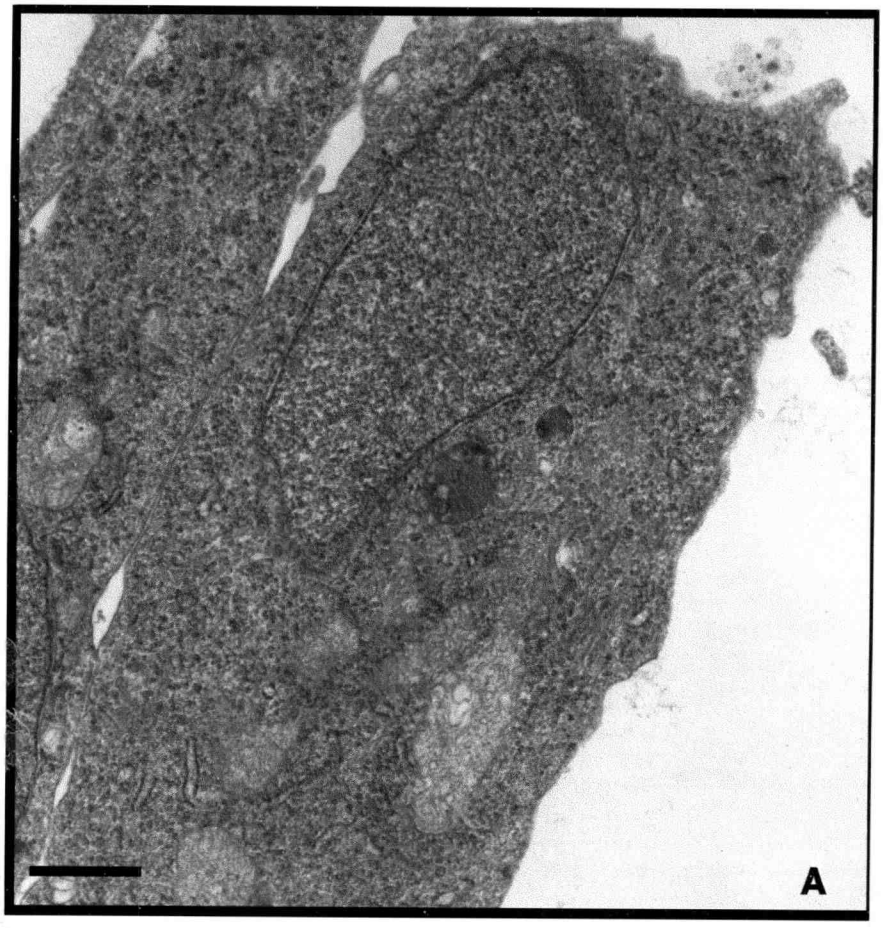

Figure 5.8 (A)

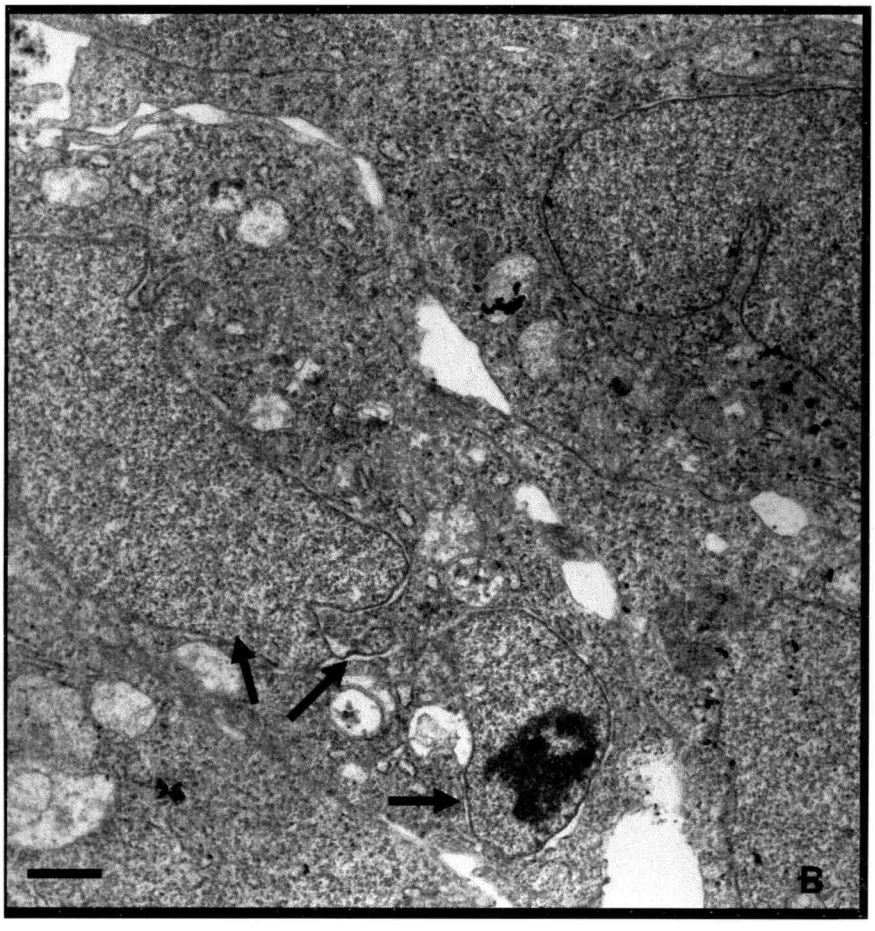

Figure 5.8 (B)

Figure 5.9 DNA fragmentation analysis of IHNV-infected CHSE-214 cells. 2 x 10^7 CHSE-214 cells were infected with virus at M.O.I. of 10, and the low molecular weight DNA was obtained at 6, 12 and 24 h postinfection. DNA was treated with RNase A and then electrophoresed through a 2% agarose. Lane 1 contains the molecular weight marker, 123bp DNA ladder (GibcoBRL); lane 2, 1-kb DNA ladder (GibcoBRL); lane 3, DNA isolated from mock-infected cells at 24 h postinfection; lane 4, IHNV RB-infected cells, 6 h; lane 5, IHNV RB-infected cells, 12 h; lane 6, IHNV RB-infected cells, 24 h; lane 7, IHNV RA-infected cells, 24 h.

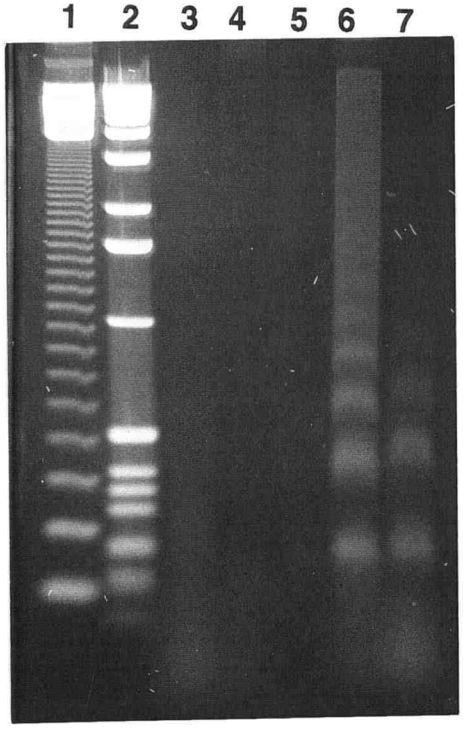

Figure 5.9

plasmid. In this cell, the nucleus was fractionated into three fragments (arrows) and the cell shrinkage was indicated by the ratio of nucleus/cytoplasm. Also, there was some condensation of chromatin DNA found in one fragment, but not dramatic in others. These observed morphological changes, including nuclear fragmentation and cell shrinkage, are indicative of apoptosis. Since it has never been demonstrated in any rhabdovirus infection which could lead to DNA fragmentation or apoptosis, it is possible that the fragmented nucleus (or apoptosis) was a specific outcome caused by overexpressing IHNV M2 protein in cells rather than as the destiny of IHNV infection. To clarify this assumption, IHNV-infected cells were examined for fragmented DNA by the presence of DNA laddering. DNA laddering is a hallmark of fragmented nucleus results from activation of a calcium-dependent endonuclease that fragments chromosomal DNA into oligomers of 180- to 200-bp multiples. In this assay, CHSE-214 cells were mock-infected or infected with IHNV RB1 or RA isolate at the M.O.I. of 10. Cells were harvested and the small molecular weight DNA was isolated at 6, 12 and 24 h postinfection, and then eletrophoresed on a 2% agarose gel. As shown in Figure 5.9, at 24 h postinfection, DNA from RB1-infected CHSE-214 cells as well as RA-infected CHSE-214 cells exhibited the apoptosis-specific pattern of DNA laddering (lanes 6 and 7). This laddering was absence in DNA from mock-infected cells (lane 3) and DNA from RB1-infected cells at 6 and 12 h postinfection (lanes 4 and 5). These results demonstrated that nuclear fragmentation can be triggered by IHNV infection and thus was not a specific consequence caused by M2 protein overexpression. The data also indicate that expression of M2 protein during IHNV infection may initiate the internally programmed cell death in the infected target cells.

Discussion

We have demonstrated in this report that expression of IHNV M2 protein in the absence of other viral proteins inhibited host-directed gene expression. This effect was potent and was not a gene-, promoter-, or host-specific event. Northern analysis revealed that M2-induced inhibition of reporter activity was due to the reduction of the reporter mRNA. VSV M protein can inhibit host-directed plasmid-encoded gene expression at the level of transcription (Black and Lyles, 1992). In addition, it is also capable of inhibiting transcription of chromosomal DNA (Paik et al., 1995). It remains unknown whether the M proteins of other negative-stranded RNA viruses have the same effect as the VSV M protein. IHNV belongs to a group of unclassified fish rhabdoviruses which are genetically closer to

the lyssavirus genus (rabies virus) than to the vesiculovirus genus (VSV). Our results suggest that inhibition of host transcription is a common function of the matrix proteins of the *Rhabdoviridae*.

The mechanism of transcriptional inhibition by the matrix proteins is unknown. Expression from three viral promoters, SV40, CMV and HIV was inhibited by VSV M protein (Black and Lyle, 1992; Paik et al., 1995). The fact that the IHNV M2 can act on both the 561, and CMV host-directed promoters suggests M2 may act on a common factor involved in host-directed transcription. These two promoters have different sequences, and bind different sets of transcriptional regulators (Bandyopadhyay et al., 1995). This supports the suggestion by Paik et al. (1995) that common transcription factors, such as the RNA polymerase II core proteins, may be affected by the matrix proteins. However, unlike the VSV M protein, the IHNV M2 does not inhibit its own transcription, demonstrating some selectivity in its mechanism of action. Further studies are required to determine if this finding is unique for IHNV.

Transient expression of the M2 protein caused nuclear fragmentation in the transfected cells. Further analysis revealed that IHNV infection would also result in nuclear fragmentation, as evidenced by DNA laddering in the infected CHSE-214 cells. Although there is no rhabdoviruses has been reported to be able to cause apoptosis in infected cells so far, IHNV infection was shown to be able to trigger apoptosis in both CHSE-214 and EPC cells (Harry Bjorklund, Abo Akademi University, Finland, personal communication). Many viruses, including DNA and RNA viruses, are known to encode gene products that induce apoptosis (for reviews, see Thompson, 1995; Hale et al., 1996). For example, the E1A proteins of adenovirus type 12 have been shown to block the transcription of MHC class I (Vasavada et al., 1986) and to induce apoptosis in infected cells (Rao et al., 1992). The M2 protein-induced nuclear fragmentation may be important for IHNV replication, or may be an artefact caused by overexpression of M2 protein. Whether or not the M2 protein will induce apoptosis during a natural IHNV infection remains to be determined. It should be noted that fragmented nuclei were only occasionally found in M2 protein-expressing cells. It is also not known whether nuclear fragmentation (or apoptosis) is related to the M2-induced inhibitory effect of host-directed gene expression. It is possible that the M2 protein triggers a programmed cytopathic effect, including inhibition of host gene expression and induction of nuclear fragmentation. Alternatively, it is possible that M2 protein initiates these two effects through separate mechanisms. Further studies need to be carried out to elucidate the relationship between these two effects caused by the IHNV M2 gene.

Another interesting finding was the cypopathic effect caused by transient expression of the NV protein. The NV protein was not involved in the regulation of the host gene expression but was involved in another type of cytopathic effect characterized as the cell rounding. Although the NV genes are highly conserved among different isolates of IHNV as well as among fish lyssa-like rhabdoviruses, the function of the NV protein is still unknown. The cytopathic effect discovered in this work is the first biological function attributed to the NV protein. In VSV, cell rounding has been shown to due to dissocation of cytoskelton (Blondel, 1990). Further studies of the NV function should be carried out to verify whether the NV protein can interfere or damage the cytoskelton in the infected or transfected cells.

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CHAPTER 6
PCR Amplification of Infectious Hematopoietic Necrosis Virus RNA Extracted From Fixed and Embedded Fish Tissue

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Abstract

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that infects salmon and trout, and as a result, causes great economic losses to fish hatcheries. The direct detection of IHNV RNA in infected tissue would be useful in diagnosing the disease and preventing its spread. In this report, we describe a sensitive method for detecting IHNV RNA in formalin-fixed, paraffin-embedded tissues of rainbow trout and steelhead *Oncorhynchus mykiss*. The technique is capable of detecting viral RNA in samples that have remained at room temperature in 10% buffered formalin for over 2 years. The RNA extracted from the deparaffinized tissue sections was phenol and guanidinium thiocyanate was amplified with primers to the viral nucleocapsid (N) gene with a heat stable DNA polymerase capable of reading both DNA and RNA. Prolonged incubation of the first-strand complementary DNA synthesis reaction for 20 min at 70 C was optimal for the synthesis of an N-specific, 252-base-pair product.

Introduction

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus of salmonid fish and causes severe losses to fish hatcheries in western North America (Pilcher and Fryer 1978). Since its discovery, the worldwide incidence of IHNV has increased dramatically. The virus has been transmitted to Japan, Taiwan, Korea, France and Italy more than likely by shipment of infected fish or eggs (Sano 1976; Chen et al. 1983; Laurencin 1987; Bovo et al. 1987). Although vaccines for IHNV are under development, present control measures for the disease still require the testing of hatchery populations for the virus and the destruction of infected populations.

Virus typically is detected by isolating the virus in tissue culture and then confirming its identity via serum neutralization. These methods are sensitive for infectious virus, but are labor intensive and often provides answers long after the epizootic. Recently, new methods for detecting both infectious and noninfectious forms of IHNV have been developed. Viral messenger RNA (mRNA) can be detected in situ by hybridization with a probe to the nucleocapsid (N) gene, and specific infected cells in the major target organs have been identified (Anderson et al. 1991). Immunohistochemical techniques have been used to detect viral proteins in fixed tissue culture cells (Drolet et 1993) and to reveal the route of virus entry during infection and its pathogenic progression in fish (Drolet et al. 1994). A

polymerase chain reaction (PCR) amplification method has been developed to amplify a portion of the N gene of IHNV directly from viral genomic RNA and freshly sampled fish tissues (Arakawa et al. 1990). This method was shown to be rapid, specific and very sensitive.

The PCR method for amplifying very small quantities of viral nucleic acid has been used widely to detect viral infection in animals. In many situations, however, the usefulness of the method has been hampered by the lack of fresh material. A convenient procedure for detecting nucleic acid in archived, fixed and embedded material would be extremely useful. Such techniques have been developed for PCR detection of DNA from formalin-fixed, paraffin-embedded tissues but the amplification of RNA from the same type of tissue material has been more difficult. By virtue of its single-stranded nature, RNA is more susceptible to the ribonucleases that are ubiquitous and stable in these tissues. In addition, the fixatives and the length of fixation time can influence the degradation of RNA (Greer et al. 1991, Rupp and Locker 1988, Ben-Ezra et al. 1991). Most of the reported studies have been developed for human pathogens (Akyol et al.1992, Bresters et al. 1993, Embretson et al. 1993, Rose et al. 1991, Sallie et al. 1992). This is the first report of the successful extraction and amplification of viral RNA from the fixed and embedded tissues of fish.

A member of the Rhabdoviridae, IHNV contains a negative sense genome of approximately 11,500 nucleotides (nt). The genome encodes one nonvirion and five virion proteins in the gene order (from the 3' end) N, matrix phosphoprotein (M1), matrix protein (M2), glycoprotein (G), nonvirion protein (NV), and the viral polymerase (L) (Kurath et al. 1985). As is typical for rhabdoviruses, the IHNV N gene is the most abundantly transcribed RNA in the replication cycle (Kurath and Leong 1985); thus, primers to this gene were selected for the PCR amplification. The nucleocapsid gene encodes a protein of 408 amino acids that is highly conserved antigenically among the IHNV isolates (Hsu and Leong, 1985; J.Winton, National Biological Survey, and S.Nichol, Centers for Disease Control, personal communication). Thus, primers selected for the N gene would detect most isolates of IHNV. In this paper, a simple method for the detection of the N-specific RNA purified from formalin-fixed, paraffin-embedded fish tissues is described. The method was found to be sensitive, specific and it required only 1 d for completion. Researchers could use this technique to detect IHNV in fixed tissues that are more than a year old and confirm virus infection in tissues where electron microscopy has detected rhabdovirus-like structures.

Methods

Virus and Cells

The two viruses used in this study were a type 1 1983 IHNV isolated from diseased fry of steelhead (anadromous rainbow trout *Oncorhynchus mykiss*) at Round Butte Hatchery, Oregon (RB-83), and a type 2 1983 isolated drom dead rainbow trout fry at the International Aquaculture Research Center (Rangen Research), Hagerman, Idaho (RA). The types were determined by electrophoretic migration of viral proteins on sodium dodecyl sulfate-polyacrylamide gels (Hsu et al., 1986) and by reactivity with type-specific monoclonal antibodies (Ristow and Arnzen 1989). For virus growth, RB-83 and RA IHNV were added at a low multiplicity of infection to chinook salmon embryo (CHSE-214) cells. Infected cell monolayers were cultured at 15 C in complete medium containing Eagle's minimum essential medium supplemented with 5% fetal bovine serum, 1,000 IU penicillin G/mL, 1 mg streptomycin/mL, and 2.5 µg amphotericin B/mL and buffered to pH 7.5 with 7.5% sodium bicarbonate.

Sample Preparation

Whole body sagittal sections of infected steelhead fry (1g) showing signs of IHN disease were used in the study. Teelhead were infected with 1 x 10^3 plaque-forming units of IHNV (RB-83, Type 1) per milliliter by static immersion. Thirty steelhead were sampled 7 d afterward (Drolet et al. 1994). Sections taken from two uninfected fish were used as negative controls. Unless otherwise indicated, all of the samples were fixed in buffered formalin (10% formalin, 33 mM sodium phosphate monobasic monohydrate, 46 mM sodium phosphate dibasic heptahydrate) for 24-48 h and then dehydrated in an alcohol-xylene series of washes before embedding in Paraplast paraffin (Oxford Labware, St. Louis, Missouri).

Each sample contained one to four sections (7 µm thick) from blocks of fixed, embedded tissue. Microtome and blade were cleaned with xylene after the completion of sectioning for each block to prevent sample-to-sample contamination. The sections, handled carefully with clean tweezers that had been soaked in xylene and heated in an open flame, were placed into 1.5 mL microfuge tubes. The samples were deparaffinized as described by

Wright and Manos (1990). Briefly, sections were washed twice for 30 min in 1 mL of xylene and twice in 0.5 mL of 100% ethanol at room temperature. Each wash was followed by centrifugation at 11,600 X g in a Beckman microfuge for 5 min. After the last centrifugation step, as much ethanol as possible was removed with aerosol resistant RNAse-free pipets (Laboratory Products Sales, Rochester, New York), and the tubes were drained and left open to dry the pellets. The pellets were then subjected to different RNA extraction methods.

Positive control samples were prepared by adding approximately 1 µg of extracted total RNA from IHNV-infected CHSE-214 cells to the deparaffinized tissue sections of uninfected steelhead. The phenol-guanidium thiocyanate-purified RNA from IHNV-infected cells was initially tested by single-step reverse transcriptase-PCR (RT-PCR) at concentrations ranging from 10 ng to 10 µg of total cellular RNA per 50 µL reaction. There was sufficient viral RNA at all of these concentrations to produce amplification products in a single step PCR. Approximately 0.3% of the total cellular RNA in these samples was IHNV mRNA (unpublished data), or 30 pg of IHNV mRNA in a reaction mixture containing 10 ng of total cellular RNA. We were unable to determine the concentration of the viral genomic RNA in the preparation. Because the PCR amplifies both IHNV messenger and genomic RNA, an accurate determination of the assay's detection limit was not possible.

Extraction of RNA

The RNA was extracted from the fixed tissue sections by either simple proteinase K digestion or by direct extraction with phenol/guanidinium thiocyanate. For proteinase K digestion, the deparaffinized pellets were resuspended in 100 µL of digestion buffer containing 200 µg/mL of proteinase K (Sigma). The digestion buffer consisted of 50 mM Tris (either pH 7.5 or pH 8.5), 1 mM EDTA (pH 8.0), and 0.5% Tween 20. The digestions were carried out at pH 7.5 or 8.5 because subsequent RNA extraction with phenol and guanidinium thiocyanate might be affected by pH. Most RNA extraction procedures call for low pH prior to phenol extraction (Wallace 1987). All reagents were dissolved in water distilled and treated with diethyl pyrocarbonate. Digestion was carried out either at 55 C for 3.5 h or at 37 C for 16h. The samples were then heated at 95 C for 8 min to inactivate the proteinase K.

The RNA extraction with phenol and guanidinium thiocyanate was carried out with RNAzol™ (Tel-Test, Inc., Friendswood, Texas). The procedure was a modification of the manufacturer's recommendation for fresh tissue. Briefly, deparaffinized pellets in microfuge tubes were suspended in 1 mL RNAzol. Then, 0.1 mL of chloroform was added and the mixture was shaken vigorously for 15 s. The mixture was incubated on ice for 15 min and centrifuged for 3 min at 11,600 X gravity. The supernatant fluids were transferred to fresh tubes, and the nucleic acid was precipitated with an equal volume of isopropanol at -20 C for 45 min. The precipitates were collected by centrifugation and washed twice in 70% ethanol, then dried and resolubilized in 100 µL TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA). Five micrliters of the resuspended precipitates were used for each PCR.

Polymerase Chain Reaction Parameters

The oligonucleotide primers for polymerase chain amplification and the probe for hybridization detection of the PCR product were selected from the nucleocapsid protein gene of the Round Butte strain (RB-75) of IHNV (Gilmore and Leong 1988). These oligonucleotide reagents are specific for IHNV in PCRs involving fresh infected tissue (Arakawa et al., 1990). The first primer (20 nucleotides) in the sense orientation (5'-TTCGCAGATCCCAACAACAA-3') was located at position 319-339 in the coding region of the N gene. The second primer (19 nucleotides) in the antisense orientation (5' -CTTGGTGAGCTTCTGTCCA - 3') was located at position 570-552 of the coding region. A thermostable reverse transcriptase with both RNA-dependent and DNA-dependent polymerase activities, Retrotherm™ RT (Epicentre Technologies, Madison, Wisconsin), was used for both first strand complementary DNA (cDNA) synthesis and subsequent PCR amplification. The reaction conditions were: 10 µM of each primer, 200 nM deoxynucleoside triphosphates, 5 units of Retrotherm™ RT, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.75 mM $MnCl_2$. The mixtures were incubated in an automatic thermal cycler (Coy Laboratory Products, Grass Lake, Michigan) for 5 min at 52 C and 10 to 20 min at 70 C for the first strand cDNA synthesis. The amplification was carried out for 35 to 45 cycles of 1 min at 94 C, 1 min at 52 C, and 4 min at 70 C. The primer-annealing temperatures were calculated according to the $T_{50\text{-}hyb}$ method of McGraw et al. (1990).

When a second amplification step was required, 5 µL of the original PCR solution were reamplified under the same conditions used in the first PCR.

Electrophoretic and Southern Blot Analysis

The amplified products were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. A DNA ladder in increments of 123 base pairs (bp) was run as size markers in every gel (Gibco BRL, Gaithersburg, Maryland). The DNA seperated in agarose gels was transferred to Maximum Strength Nytran Plus (Schleicher & Schuell, Keene, New Hampshire) by standard blotting techniques (Sambrook et al. 1989). Hybridization to an internal N gene probe that should be in the amplified product was used to confirm the N specificity of the PCR product. An internal antisense probe (5' CTTGTTTTGGCAGTATGTGGCC ATCTTGTC - 3'), from position 456-427 of the N coding region was end-labeled with digoxigenin (Boehringer Mannheim,Indianapolis, Indiana) by terminal deoxynucleotidyl transferase (Promega, Madison, Wisconsin). Chemiluminescent detection of the hybridized digoxigenin-labeled probe with anti-digoxigenin was carried out as described by Holtke et al (1992).

Results and Discussion

When IHNV-infected steelhead tissue was deparaffinized and digested with proteinase K in tris buffer at either pH 7.5 or 8.5, the extracted RNA was not usable as a template for PCR. Two pH values were tested because RNA extraction and stability is affected by pH. Even with a second amplification step, no PCR products were detected (Fig. 6.1). With proteinase K digestion for 3.5 h at 55C or 12 h at 37 C, no amplification products were detected (data not shown). In other studies, proteolytic digestion for 16 h at 50 C (Gruber et al. 1993) or as long as 2-3 d at 42 C (Sallie et al. 1992) yielded successful RNA extraction. It is possible that a longer digestion period was needed for the fish tissues; however, even negative tissue samples containing added IHNV-infected cellular RNA gave no amplification products (lanes 2 and 3, Fig. 6.1). Other investigators have shown that PCR-suitable template RNA from formalin-fixed and paraffin-embedded tissues can be prepared by proteinase K digestion if the digested material is then extracted with phenol -chloroform (Sallie et al. 1992, Gruber et al. 1993, Koopmans et al. 1993). In their studies, PCR products were detected after a single amplification step for RNA from fixed and embedded mammalian tissues. For the fish tissues in this study, the effect of phenol/chloroform extraction after proteolytic digestion was not examined because use of the commercial preparation, RNAzol, was convenient and effective.

Figure 6.1 Effect of different RNA extraction methods on polymerase chain amplification of IHNV RNA from formalin-fixed, paraffin-embedded tissues of steelhead. The amplification products shown in this ethidium bromide stained gel were obtained after a second amplification step. The deparaffinized tissue sections were extracted with proteinase K digestion at pH 8.5 (lane 2), proteinase K at pH 7.5 (lanes 3, 5 and 7), or phenol and guanidinium thiocyanate (RNAzol: lanes 4, 6, and 8). Lanes 2-4 contained the RNA from an uninfected control that was mixed with IHNV RNA before the extraction procedure was begun. Lanes 5-6 and 7-8 contained the RNA extracted from different IHNV-infected fish. Lane 1 contained marker DNA and each band represents an increment of 123 bp in a ladder. Lane 9 contained the amplification product obtained with purified total RNA from IHNV-infected cells that had not been treated with subsequent proteinase K or phenol and guanidinium thiocyanate. This RNA was the template RNA that had been added to the samples in lanes 2-4. A bright band at the bottom of each lane represents excess primers and deoxynucleoside triphosphates.

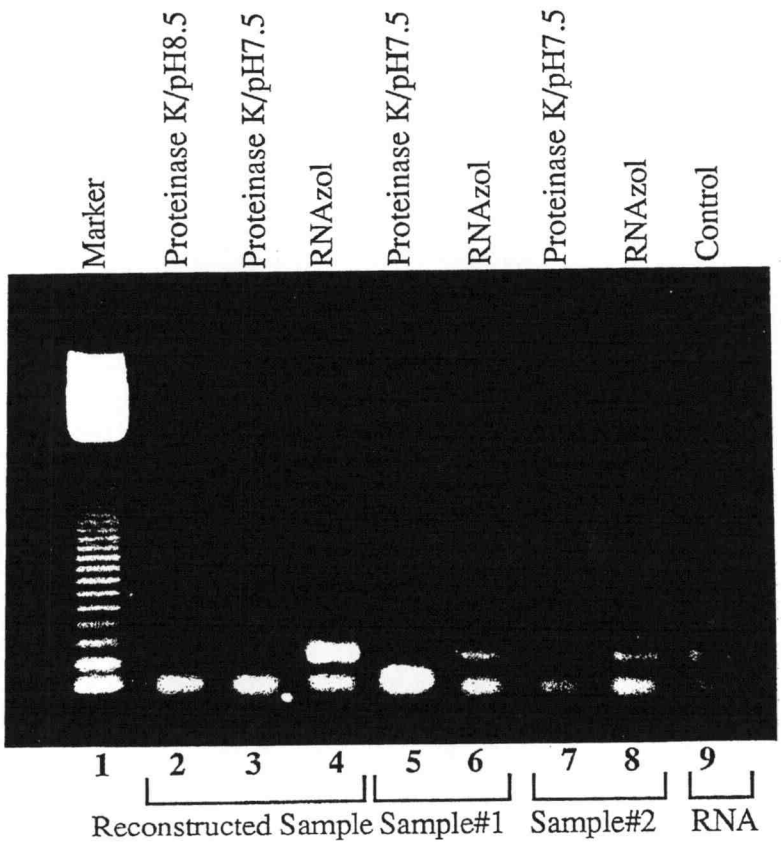
Marker
Proteinase K/pH8.5
Proteinase K/pH7.5
RNAzol
Proteinase K/pH7.5
RNAzol
Proteinase K/pH7.5
RNAzol
Control
1
2
3
4
5
6
7
8
9
Reconstructed Sample
Sample#1
Sample#2
RNA

Figure 6.1

Direct extraction of the reconstructed positive sample with RNAzol produced amplified viral RNA from tissues of fish infected with IHNV (lanes 6 and 8, Fig. 6.1). A second band at approximately 500 bp was detected in the amplified preparations of purified IHNV RNA from infected cells (lane 9, Fig. 6.1). This second band of hybridization was noted by Arakawa et al. (1990) in polymerase chain amplification of RNA from purified virus, and its origin is unknown. Very likely, it is the result of self-priming common to reverse transcriptase (Gubler and Hoffman).

Conventional detection of RNA by PCR relies on an initial reverse transcription step followed by DNA amplification with a thermostable DNA polymerase. In the present study, a thermostable reverse transcriptase, Retrotherm RT, that has both RNA and DNA-dependent polymerase activities was employed. Use of this polymerase simplified the protocol by permitting the amplification process to be completed without changes of buffers and tubes. Although the manufacturer of Retrotherm RT suggests that researchers determine the optimal template:primer ratio for each template:primer combination, it was difficult to determine the quantity of RNA used in each reaction; thus, the initial reactions were carried out with 5 μL of the RNA preparation. The manufacturer's recommendation is at least 1 ng of RNA. After 10 and 15 min, no discernible products were observed after a single step PCR amplification for either RNAzol extracted purified IHNV RNA (lanes 3 and 5, Fig. 6. 2) or the RNAzol extracted reconstituted RNA preparation containing fish tissue RNA and added IHNV RNA (lanes 4 and 6, Fig. 6.2). Amplification products were observed after first strand cDNA synthesis for 20 min at 70 C (lanes 1 and 2, Fig. 6.6). Incubation of the first-strand cDNA synthesis reaction past 20 min was not carried out because of problems with error accumulation in the products.

The primers used in the study were prepared from the sequence of the N gene of the Round Butte (type 1) isolate of IHNV (Gilmore and Leong, 1988). Their efficacy as primers to detect other isolates of IHNV was tested with the purified viral genomic RNA of six different isolates, and their specificity as IHNV-specific probes was verified by their inability to prime specific synthesis with the fish rhabdoviruses, hirame rhabdovirus (HRV) and viral hemorrhagic septicemia virus (VHSV; Arakawa et al. 1990). The primers were selected for a region of the N gene that encoded amino acids with relatively low codon degeneracy. Although the nucleic acid sequence of this region of the N gene is largely conserved (J.Winton, National Biological Survey, and S. Nichol, Centers for Disease Control, personal communication), formalin fixation can alter the secondary structure of nucleic acid in tissues and affect the hybridization properties of the RNA (Rupp and Locker 1988). A few mismatches between PCR primer and viral template might prevent the

Figure 6.2 Effect of time on the first-strand complementary DNA (cDNA) synthesis in the reverse transcriptase-polymerase chain amplification of IHNV RNA. Lanes 1, 3, and 5 contained purified total RNA from IHNV-infected cells. Lanes 2, 4, and 6 contained reconstructed positive fish RNA sample (purified IHNV RNA added to deparaffinized tissue sections from uninfected fish). All RNA extractions were carried out with phenol and guanidinium thiocyanate. The effect on length of time for first-strand cDNA synthesis at 70 C was examined for 10 min (lanes 5 and 6), 15 min (lanes 3 and 4) and 20 min (lanes 1 and 2). After a single amplification step, products were detected only after 20 min.

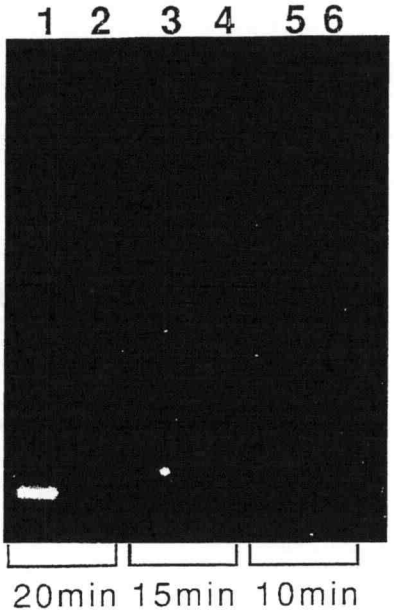

Figure 6.2

Figure 6.3 Hybridization of amplified DNA fragments with an IHNV N gene probe. The specific hybridization of a digoxigenin-labeled probe to the IHNV N gene in two bands (252 and 500 bp) was detected for purified RNA from IHNV-infected cells (RB-83, Type 1: lane 1) and for RNA extracted from a paraffin section of IHNV-infected rainbow trout kidney that had been in 10% formalin for 2 years (Rangen 1983, Type 2: lane 2). The control lane 3 contained the amplification products obtained with RNA taken from an uninfected fish.

Figure 6.3

priming of a nonhomologous virus isolate. Thus, it was important to determine whether the primers would also amplify the viral RNA of a different type of IHNV, such as a type 2 isolate, in paraffin-embedded tissues.

Four sections from a paraffin-embedded kidney tissue of a rainbow trout that had been infected with the Rangen 1983 Type 2 isolate of IHNV were deparaffinized and extracted with RNAzol. The fish had been in 10% buffered formalin for more than 2 years before it was embedded in paraffin. The extracted RNA was amplified as previously described and a visible band at 252 bp was detected in an ethidium bromide stained gel (data not shown). The IHNV specificity of this band was confirmed by hybridization with an N gene-specific probe to a region within the 252 bp amplified N fragment (Fig. 6.3). The 500-bp noted in Figure 6.1 was also detected in the Souther blot of the amplified preparations of purified IHNV RNA from infected cells (lane 1, Fig. 6. 3). Upon longer exposure of the Southern blot, the 500-bp band was detected in the amplified products of RNA from infected fish (lane 2, Fig. 6. 3, longer exposure not shown).

These results show that PCR amplification of RNA from formalin-fixed and paraffin-embedded fish tissues is a useful technique for detecting IHNV. Tissues in fixative for as long as 2 years were satisfactory for this procedure. The recommended procedure, summerized in Figure 6.4, is convenient and provides results in 1 d. It is sensitive and appears to be as specific as the polymerase chain amplification of IHNV N gene sequences of viral genomic RNA from purified virus (Arakawa et al. 1990). The procedure is now being used at Oregon State University to detect IHNV in fish that have survived an IHNV epizootic but no longer yield culturable infectious virus.

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Figure 6.4 Recommended procedure for PCR detection of IHNV RNA in formalin-fixed, paraffin-embedded fish tissues. The TE buffer is 10 mM tris, pH 7.5, and 1 mM EDTA, pH 8.0; bp is base pair; RT-PCR is reverse transcriptase-polymerase chain reaction.

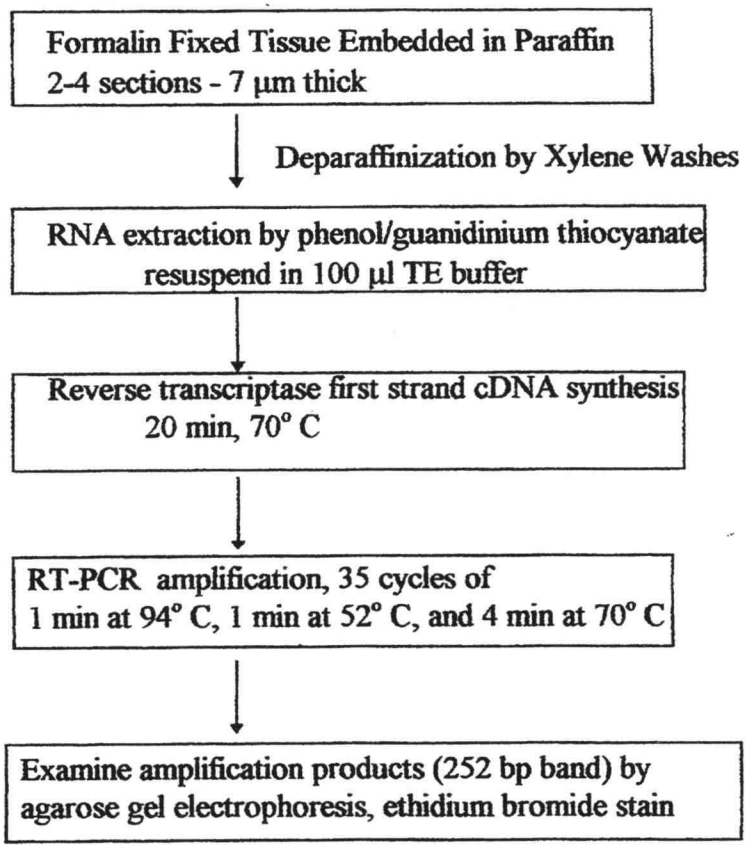
Formalin Fixed Tissue Embedded in Paraffin
2-4 sections - 7 µm thick
Deparaffinization by Xylene Washes
RNA extraction by phenol/guanidinium thiocyanate
resuspend in 100 µl TE buffer
Reverse transcriptase first strand cDNA synthesis
20 min, 70° C
RT-PCR amplification, 35 cycles of
1 min at 94° C, 1 min at 52° C, and 4 min at 70° C
Examine amplification products (252 bp band) by
agarose gel electrophoresis, ethidium bromide stain

Figure 6.4

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CHAPTER 7
THESIS SUMMARY

The work presented in this thesis was conducted to provide a basic understanding of the roles of the viral proteins in the pathogenesis of IHNV at the molecular level. The expression of the viral genes at the protein and RNA level, and the cellular localization of the viral proteins *in situ* was determined. The roles of the M2 and NV proteins in viral pathogenesis were examined by transgenic expression of these proteins individually in cultured fish cells. Finally, a PCR-based method for detection of the viral RNA in archived, fixed, and embedded tissues was developed.

Expression and Cellular Localization of IHNV Proteins

Pulse labeling and immuno-blot analyses confirmed that the sequential expression of the IHN viral genes correlated with their relative location on the viral genome. Further, IHNV replication was inhibited in cells treated with tunicamycin to inhibit glycosylation, demonstrating a strict dependence of viral replication on a functioning host glycosylation system. The study also resulted in the observation of a previously undescribed non-glycosylated protein, S, which appears to be virus encoded.

An extensive survey of NV protein expression was performed. The presence of the NV protein in IHNV-infected tissues was detected by immunofluorescence; however, under several growth conditions, NV protein was either expressed in such low quantities as to be undetectable by pulse labeling, or was extremely labile. The cellular localization of the nucleocapsid protein (N), phosphoprotein (M1), and glycoprotein (G), were confined to the cytoplasm. The matrix protein (M2) and NV were identified in both the nucleus and cytoplasm in infected cells. These findings indicate that interaction between viral proteins and the host nucleus may be important in IHNV replication.

Analysis of RNA Transcripts and Transcription-Related Signals of IHNV

The expression of the NV gene was further analyzed at the transcription level. The nucleotide sequence derived from the NV mRNA and the viral genome were compared.

Only one nucleotide difference, resulting in a deduced single amino acid change was detected between cDNA clones of the original isolate ,where the NV protein was first identified, and progeny virus cloned after passage three times in tissue culture. The NV mRNA appeared in the infected cells as early as 12 h postinfection. The concentration of NV was estimated to be approximately 3×10^{-7} pmole (1.8×10^5 molecules) per infected cell when 100 % of the cells expressed cytopathic effect, and is stable throughout the infection. These results suggest an important role for the NV gene during viral infection, despite the fact that the NV protein product was not detectable by pulse labeling. A unique transcriptional initiation signal and a termination signal for the fish lyssa-like rhabdoviruses was identified. The transcriptional initiation signal, 3'-CGUG-5', located by primer extension analysis of the NV, M1, and M2 mRNAs, is distinctly different from that of the other rhabdoviruses, 3'-UUGU-5'.

The Roles of Viral Proteins in the Cytopathogensis of IHNV

In tissue culture cells, IHNV infection causes shut down of host protein synthesis and cytopathic effect as characterized by the rounding of the infected cells. The roles of the IHNV M1, M2 and NV proteins in these pathogenic effects were examined by transgenic expression of these proteins individually in fish cells. There was no observed evidence for a role for the M1 protein in IHNV cytopathology. However, the expression of the M2 protein alone resulted in inhibition of host-directed gene expression at the transcription level and also induction of nuclear fragmentation. These results indicate that during IHNV infection the M2 protein is responsible for the inhibition of host protein synthesis and induction of apoptosis. The NV protein was not involved in the regulation of the host gene expression but was involved in another type of cytopathic effect characterized as the cell rounding. This is the first biological function attributed to the NV protein.

PCR Amplification of IHNV RNA in Archived, Fixed Fish Tissue

The PCR method for amplifying very small quantities of viral nucleic acid has been used widely to detect viral infection in animals. In many situations, however, the usefulness of PCR has been hampered by lack of fresh material. A sensitive method is developed in this work for detecting IHNV RNA in formalin-fixed, paraffin-embedded fish

tissues. The technique is capable of detecting viral RNA in samples that have been remained at room temperature in 10% buffered formalin for over 2 years. The PCR-based technique should be very useful to detect IHNV infection in fish when fresh samples are impossible to obtain.

Future Studies

A novel transcriptional initiation signal specific to IHNV was identified in this work. To further characterize this unique signal, future studies should include the following: 1) A structural analysis of the motif(s) of the viral polymerase responsible for the recognition of the transcriptional signals. 2) Development a reverse genetic system to verify the functions of these signals. 3) Determination on the mechanism(s) of the interaction between the transcriptional signal and the viral polymerase. In addition, antiviral strategies may be developed using these signal sequences as targets. As an example, ribozymes designed to specifically cleave the nucleotide sequences of these signals may block the replication of IHN virus in infected cells. These ribozymes should be effective for other fish lyssa-like rhabdoviruses since these signal sequences are also present in these viruses.

Two viral proteins were found to be responsible for the IHNV pathogenicity. The NV protein was involved in the rounding of cells, and the M2 protein was capable of inhibiting host-directed gene expression and induction of chromosomal fragmentation. Future studies should include the following: (1) Determination of the component(s) of the M2 and NV protein responsible for these functions. (2) Further characterization of the molecular mechanisms causing these cytopathic effects. (3) Determination of the impact on viral replication by blocking these mechanisms. During IHNV infection, extensive hemorrhaging and tissue damage are attributed as a cause of death (Amend and Smith, 1975; Drolet et al., 1994). Hence, further understanding of the mechanisms of the M2- and NV-induced cytopathic effects may result in treatments to improve the survival of infected fish.

The PCR-based method developed in this work has already been useful in the study of IHNV pathogenesis. Drolet et al. (1995) were able to detect a persistent infection in fish surviving an IHNV epizootic. This technique is especially useful to detect the IHNV infection when fresh samples are not available. As such, this technique can be applied in the study of IHNV in a historical perspective. For example, this technique should be helpful to confirm the etiology of cases reported in the 1950's and 1960's.

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