AN ABSTRACT OF THE THESIS OF

Eric D. Anderson for the degree of Doctor of Philosophy in Genetics presented on March 17, 1995. Title: Molecular study of the cytopathic mechanisms of infectious hematopoietic necrosis virus *in vivo* and *in vitro.*

Redacted for Privacy

Abstract approved: _____

Jo-Ann C. Leong

Molecular biological approaches were used to study and interfere with the life cycle of infectious hematopoietic necrosis virus (IHNV). These included the control of IHN disease in rainbow trout by genetic immunization or interference *in vitro* by synthesis of sense and antisense expression of the viral nucleocapsid (N) gene, and *in situ* localization of the virus in infected fish.

The design of DNA vaccines was based upon the finding that fish skeletal muscle cells could express transgenes delivered by direct injection using a needle and syringe. Optimum transgene expression occurred seven days post injection with 25 ug of plasmid DNA. The cytomegalovirus immediate early promoter enhancer was the strongest promoter tested. Transgene expression lasted for at least 115 days post injection. The DNA persisted as an unintegrated and non-replicated plasmid. Vaccination of fish with the IHNV glycoprotein (G) encoding plasmid, pCMV4-G, resulted in a protective immune response. Fish vaccinated with pCMV4-G and an IHNV N encoding plasmid, pCMV4-N, produced a stronger anti-G specific immune response than did fish vaccinated with pCMV4-G. However, the percent survival of fish vaccinated either in combination or with pCMV4-G alone was the same, 86%, still well above the 25-35 % survival of fish vaccinated with pCMV4-N or mock vaccinated.

A temporal state of intracellular immunity was induced in cells expressing the IHNV N gene in the sense or antisense orientation. The cells were completely resistant to IHNV 3 months after transfection. However, five months after transfection the antisense transfected cells were 99 % less susceptible to IHNV

plaque formation than control cells, the sense transfected cells were 33% less susceptible.

The sites of IHNV infection during the height of a viral epizootic were determined by *in situ* hybridization using an oligonucleotide complementary to the IHNV N gene. A number of parameters were optimized, the most critical being the amount of probe used and the washing and hybridization stringency. The virus was found systemically. The most conspicuous site of IHNV infection was found in the hematopoietic tissues of the kidney.

# MOLECULAR STUDY OF THE CYTOPATHIC MECHANISMS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS IN VIVO AND IN VITRO

by

Eric D. Anderson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed March 17, 1995

Commencement June 1995

Doctor of Philosophy thesis of Eric D. Anderson presented on March 17, 1995

APPROVED:

Redacted for Privacy

Major Professor representing Genetics

Redacted for Privacy

Director of Genetics Program

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Eric D. Anderson, Author

Dedication

For my wife Kimberley
and
Grandpa

## ACKNOWLEDGMENTS

I would like to thank Jo-Ann C. Leong for giving me the opportunity to learn--for helping me to the top of one of the many long steep trails I have climbed, the only kind worth following.

Everyone in the lab thanks. Particular thanks to Dan, in every aspect your integrity shows.

Thanks and love to my wife Kimberly, she alone knows--there are mountains to set on fire. Thanks to Kristina and Annie for dancing to the edge and saying jump-proper balance is a heritable trait.

## CONTRIBUTIONS OF AUTHORS

Dr. Jo-Ann Leong was involved in all aspects of this work. Dan V. Mourich was involved with experimental design, conducting experiments, interpretation of the data, and critical review of the manuscripts for Chapter 3 and Chapter 4. Scott Fahrenkrug provided the plasmid pCMV4-G used in Chapter 4. Dr. Craig Cary was involved in the experimental design for Chapter 6. Dr. John S. Rohovec provided critical evaluation, and financial support for Chapter 6.

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## CHAPTER 1
## THESIS INTRODUCTION

The rhabdovirus infectious hematopoietic necrosis virus (IHNV) is a pathogen of certain salmonid fish native to the Pacific Rim Countries. Due to the severity, prevalence, and economic cost of the IHN disease, considerable efforts have been made to control the virus. These efforts can be divided into two broad categories: improving animal health practices, and the development of safe and effective chemotherapeutics and vaccines. Both of these are based upon a wealth of information gathered since the viruses identification in the 1950's (Rucker et al., 1953; Watson et al., 1954; Guenther et al., 1959). The work presented in this thesis was conducted to provide a basic understanding of the cytopathic mechanisms of IHNV *in vivo* and *in vitro*. The information, and techniques developed, should be useful both in the applied field of vaccine design, and the basic fields of virology and immunology.

### Genetic Immunization

Genetic immunization, or the use of plasmid DNA to vaccinate, is one of the newest approaches in vaccine design. This approach was developed following the discovery that naked DNA could be delivered to somatic tissues *in vivo,* using a needle and syringe, and the encoded proteins could be expressed (Wolff et al., 1990). Ulmer et al. (1993) were the first to use this technique to vaccinate against a viral pathogen. Since these original reports, numerous groups have developed DNA vaccines for a number of different pathogens (see Table 2.1). Genetic immunization can be applied using any gene which is appropriate. The vaccine can not revert to a virulent phenotype, involves a broad spectrum immune responses, and is easy to prepare. Because of these virtues, we decided to test DNA vaccines for their ability to immunize fish against IHNV.

Prior to the use of DNA vaccines an understanding of the basic principles underlying gene expression following direct DNA injection was undertaken (Chapter 3). In these studies strong promoters were identified, the sites and duration of gene expression, the dose response (amount of plasmid delivered-level of gene expression), and the nature of the DNA *in vivo* was determined. These studies established that genetic immunization is a feasible method of immunization of fish against IHNV.

In chapter 4, DNA vaccines containing the IHNV nucleocapsid (N) gene or the glycoprotein (G) gene were designed. Previous studies using attenuated IHNV(Fryer et al., 1976; Tebbit, 1976), killed IHNV(Amend, 1976; Nishimura et al., 1985; Naganawa et al., 1993), subunit (Gilmore et al., 1988; Oberg et al., 1991) and purified viral protein (Engelking and Leong, 1989a; Engelking and Leong, 1989b) vaccines have demonstrated that fish could be immunized against IHNV. Based upon the previous work and that in Chapter 3, DNA vaccines were tested for their ability to evoke a protective immune response in fish. Both the specificity of the antibody response and the ability to protect fish from an IHNV challenge were measured.
The DNA vaccines proved efficacious and allowed dissection of the viral proteins (N or G) necessary for a protective immune response in fish.

## Intracellular Immunity

The control of cellular gene expression and protein synthesis by complementary RNA has been well characterized in prokaryotes (reviewed in: Inouye, 1988; van der Krol et al, 1988; Helene and Toulme, 1990; Takayama and Inouye, 1990). The control is believed to be the result of hybridization of the complementary RNA with a target sequence followed by degradation of the duplex, or blockage of mRNA translation. In eukaryotes, expression of complementary RNA has also been used to interfere with cellular and viral gene expression. Izant and Weintraub (1984) were the first group to demonstrate that such an approach was feasible in eukaryotes. Since this initial report, antisense and sense expression of viral genes have been used to induce a state of intracellular immunity in a variety of plants and animal models (*in vivo* and in *vitro*). The mechanism(s) by which interfering RNAs inhibit viruses in eukaryotes are for the most part not understood; published reports have relied on biological assays to assess the degree of intracellular immunity.

In chapter 5, sense and antisense expression of the IHNV N gene was used to examine the ability of the N gene to interfere with cellular life-cycle of IHNV. This work was based upon the finding that oligodeoxyribonucleotides complementary to the N gene of the rhabdovirus vesicular stomatitis virus (VSV) could interfere with VSV replication. Both orientations of the N gene were found to impart a partial state of intracellular immunity in fish cells. The mechanisms underlying the immunity were not investigated but the results do suggest that the N protein plays an integral role in regulation of the IHNV life cycle.

## Localization of IHNV *In Vivo*

An understanding of the life cycle of IHNV is critical to the development of methods for the control of IHNV. Amend (1975) was the first to experimentally show that IHNV could be isolated from mature fish infected with IHNV when they were fry. Until recently, there have not been any other reports confirming the work of Amend. However, recently Drolet et al. (1995) used immunohistochemical analysis to demonstrate that IHNV could be detected in mature fish which had survived a viral epizootic as fry. This work showed that IHNV can persist as defective interfering particles in mature fish. However, though the fish were harboring the virus, infectious IHNV could not be isolated.

Chapter 5 deals with the development of *in situ* hybridization as a tool to localize IHNV in infected fish. This technique was developed as a complementary approach to the immunocytochemical analysis developed by Drolet et al. (1994a, 1994b, and 1995). A number of parameters were optimized to improve the sensitivity of viral localization in fish tissue. The most critical were identified to be the hybridization and washing stringencies. Using *in situ* hybridization, the virus was found to be systemic. The most conspicuous site of infection was the kidney. This technique should provide a powerful tool for the study of the life cycle of IHNV. *In situ* may be a particularly well suited, in combination with polymerase chain reaction and immunocytochemistry, for determining the site(s) of replication of the defective interfering viral particles described by Drolet et al. (1995)

# CHAPTER 2
# LITERATURE REVIEW

## Infectious Hematopoietic Necrosis Virus

### Classification

Infectious hematopoietic necrosis virus (IHNV), a viral pathogen of salmon and trout, is a member of the family *Rhabdoviridae* and has been assigned to the genus *Lyssavirus* (Shope and Tesh, 1987). This classification is based upon the similarities of the IHN virion morphology (Amend and Chambers, 1970), the RNA genome (McCain et al., 1974) and the protein molecular weights (McAllister and Wagner, 1975; Hill et al., 1975; Hsu et al., 1986) with the prototypic *Lyssavirus*, rabies virus.

A feature that distinguishes IHNV and two other fish lyssaviruses, hirame rhabdovirus, and viral hemorrhagic septicemia virus, from all other *Rhabdoviridae* including fish vesiculoviruses, is the possession of a sixth gene termed the non-virion gene (Kurath et al., 1985; Kurath et al., 1994). The non-virion gene is located near the 5' end of the viral genome between the glycoprotein and polymerase gene (Kurath et al., 1985).

### Historical perspective

The written history of IHNV is entwined with the advent of intensive salmon farming in the Pacific Northwest of the United States. As fish hatcheries were developed, good animal husbandry was required to maintain and enhance the health of the farmed fish. Accordingly, fish health specialists identified pathogens that occurred in fish, and documented their findings.

The first reports of IHNV infecting fish occurred during the 1950's and early 1960's from hatcheries in Washington (Rucker et al., 1953), Oregon (Wingfield et al., 1969) and California (Ross et al., 1960). These reports distinguished the disease by a rapid onset and high moralities in juvenile sockeye salmon (*Oncorhynchus nerka*)

(Rucker et al., 1953; Watson et al., 1954). The viral etiology of the disease was confirmed by its transmission with cell-free filtrates from infected fish to uninfected fish which subsequently showed signs of viral infection (Watson et al., 1954).

Initially, the disease was described as highly species-specific; infecting only sockeye salmon (Guenther et al. 1959). However, in 1960 a viral epizootic similar to the sockeye salmon disease outbreaks reported in the decade of the fifties occurred in chinook salmon (*Oncorhynchus tshawytscha*) fry (Ross et al., 1960). In 1969, Amend reported a similar viral disease causing high mortalities in rainbow trout (*Oncorhynchus mykiss*) fry. Amend and Chambers (1970a; 1970b) found that the viruses causing disease in sockeye and chinook salmon and rainbow trout had a similar, cytopathic effects on cell culture, pathology exhibited in fish, and appearance when viewed my electron microscopy. As will be described later, the documented distribution and host range of IHNV have expanded considerably in the past 30 years.

IHNV acquired its name from the microscopic pathology it produced in fish (Amend et al., 1969). Necrosis of the hematopoietic tissues of the anterior kidney is the primary internal histological manifestation of the virus. A recent immunohistochemical study of the route of entry and progression of IHNV infection in fish demonstrated that the virus eventually reaches and infects most organs (Drolet et al., 1994).

Currently IHNV is considered to be the most costly viral fish pathogen in the Pacific Northwest of the U.S.A in lost fisheries resources and revenues to sport and commercial fisherman (Leong, 1993). Thus, considerable efforts have been made to control IHNV. These control methods include improving fish husbandry and hatchery management practices, identifying and using chemotherapeutics, and the development and use of efficacious vaccines against IHNV. The successes and failures of these efforts will be reviewed here.

The control of IHNV is based on a wealth of information gathered since the 1950s concerning IHNV geographic distribution, host range, host susceptibility, life cycle, pathogenesis, molecular biology, and basic fish biology. The development of fish cell culture in the 1950's, enabling virus propagation, was a landmark in the study of IHNV (Wolf, 1988). The application of molecular biological techniques has provided one of the most important leaps in our understanding of IHNV in the past 20 years. Clearly, a combination of disciplines will be required to eventually control IHNV.

Geographic distribution

Since the first IHNV isolations in the 1950's and 1960's, the virus has been identified in numerous areas around the world. The virus is considered to be enzootic in the Pacific Northwest of the U.S.A. and Canada. This is likewise true for Hokkaido and Honshu Islands of Japan (Sano et al. 1977). IHNV has also been reported in fish located 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 isolated from fish in France (Laurencin, 1987), Italy (Bovo et al., 1987), and Belgium (Hill, 1992). Recently, IHNV was isolated from several species of salmon in Korea (Park et al., 1994). The spread of IHNV is believed to be the result of shipment of contaminated eggs and fry from the Pacific Northwest of the U.S.A. and Canada around the world.

The isolation of IHNV from feral fish populations was first reported by Williams and Amend (1976) in sockeye salmon in British Columbia. Traxler (1986) reported an epizootic in feral kokanee salmon (*Oncorhynchus nerka*) in Lake Cowichan, British Columbia. The Oregon Department of Fish and Wildlife (1992 and 1993) reported that a natural IHNV epizootic occurred in pre-adult kokanee in Lake Billy Chinook, Oregon (Engelking and Kaufman, 1994). However, in general, the majority of IHNV epizootics have occurred in fish reared in intensive aquaculture operations (Leong, 1993).

Host range and susceptibility

The host range of IHNV is relatively broad. IHNV has been found to naturally infect rainbow and steelhead trout, sockeye salmon , Atlantic salmon (*Salmo salar*), pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*Oncorhynchus keta*), cutthroat trout (*Salmo trutta*), coho salmon (*Oncorhynchus kisutch*), amago salmon (*Oncorhynchus rhodurus*), and yamame salmon (*Oncorhynchus masou*; reviewed in Lapatra, 1989).

Recently Bootland et al. (1994) demonstrated that bull trout (*Salvelinus confluentus*) and mountain whitefish (*Prosopium williamsoni*) could be experimentally infected with IHNV. The marine fish, seabream (*Archosargus rhomboidalis*) and

turbot (*Scopthalmus maximus*) have also been experimentally infected with IHNV (Castric and Jeffroy, 1991). IHNV has also been isolated from leeches (*Piscicola salmositica*; Mulcahy et al. 1990) and mayflies (*Callibaetis* sp.; Shors and Winston, 1989).

The majority of IHNV epizootics have occurred in very young fry (a few months old; Pilcher and Fryer, 1980). However, IHNV epizootics have been reported in year old sockeye (Yasutake, 1978; Burke and Grischowsky 1984), two-year-old kokanee (Traxler, 1986), and year old-rainbow trout (Roberts, 1986). Thus, it appears that age is not the only factor influencing fish susceptibility to IHNV.

Variations in salmonid susceptibility to IHNV have been observed. Virus isolated from sockeye salmon and rainbow trout have reduced virulence for other salmonid species compared to the host from which they were isolated (Pilcher and Fryer, 1980; Wingfield et al., 1969). Lapatra et al. (1989) and Chen (1983) demonstrated that there are IHNV strains which are more virulent for particular species of salmonids. The electropherotype type 1 IHNV is more virulent for kokanee and the type 2 IHNV is more virulent for rainbow trout (Lapatra et al. 1989) Coho salmon have been considered relatively resistant to IHN. This occurs by an unknown mechanism. However, IHNV has been isolated from naturally infected coho (Lapatra et al. 1987) and experimentally infected coho (Lapatra et al. 1989). Various salmonid hybrids generated by genetic manipulation have been shown to be less susceptible to IHNV (Lapatra et al. 1993). The use of hybrid fish has great promise for the production of IHNV resistant stocks, however it may be costly to produce hybrid fish, and may require continued crosses to ensure that the viral resistance is not lost.

## IHNV life cycle

The nature of the life cycle of IHNV remains controversial. Amend (1975) reported those rainbow trout fry infected with IHNV become life-long carriers as evidenced by recovery of IHNV from their reporductive fluids at maturity. Since this observation, numerous studies at Oregon State University has been conducted to confirm this finding, none of which have been successful. However, recently IHNV has been identified in mature rainbow trout experimentally exposed to IHNV as fry. This was shown by a combination of immunohistochemistry using G and N-specific

monoclonal antibodies and N-specific PCR (Drolet et al., 1995), all of which positively identified IHNV in fish tissues. These last results give credence to the possibility of a life long carrier state.

Fish to fish waterborne transmission of IHNV was first reported by Rucker (1953). Mulcahy et al. (1983) demonstrated that IHNV titers in river water taken near spawning fish held at high density contained sufficient virus to infect yearling sockeye salmon held downstream. These and other studies (Wedemeyer et al. 1978) demonstrated that IHNV remains infective in water for several months. IHNV has been recovered from the feces and urine of infected fish (Nishimura et al. 1988). It has been inferred that this may be the source of IHNV transmission in water. There have not been any definitive reports of transmission of IHNV to fish by virus in water sediments. Thus, these studies suggested that maintenance of IHNV in fish populations is due to waterborne transmission of IHNV from infected to uninfected fish, fry, and developing embryos.

Indirect evidence of vertical transmission of IHNV has been demonstrated in several studies. Mulcahy and Pascho (1985) isolated infectious IHNV from dead eggs and fry obtained from IHNV positive parental fish. IHNV can be detected in ovarian fluid collected from spawning salmon (Lapatra et al. 1990). Mulcahy and Batts (1987) found that cells collected from ovarian fluid could be cultured *in vitro* and release IHNV into the culture media. Mulcahy and Pascho (1984) showed that IHNV strongly absorbed to fish sperm. However, Yoshimizu et al. (1989) demonstrated that salmon eggs or sperm incubated with IHNV, or eggs injected with IHNV, did not result in mortalities in the progeny. Yoshimizu et al. (1989) did show that eyed embryos injected with IHNV resulted in 90% mortality after hatching. Thus, it appears that vertical transmission remains an open question.

Several studies have been undertaken to identify a reservoir of IHNV. It has been suggested that resident non-salmonid fish populations may serve as IHNV reservoirs. However, no reports have been published concerning this possibility. As noted previously, IHNV has been recovered from leeches and mayflies. However, while tissue culture infective virus could be recovered from mayflies and leeches it was unclear whether the virus replicates in the invertebrate host or that there was virus transmission from the host to fish.

A part of the work described in this thesis was directed at developing *in situ* hybridization to localize IHNV in whole fish for the purpose of understanding the life cycle of IHNV.

Progression and pathogenesis

Yasutake (1970) has reviewed the microscopic histopathology of IHNV. The virus causes severe necrosis of the head kidney (hematopoietic cells). Necrosis, to a lesser degree, also occurs in the posterior kidney, stomach, and pancreas. Additionally, there is localized necrosis of the kidney during the latter stages of IHNV infection.

A detailed study of the route of entry and progression of IHNV in rainbow trout has recently been described (Drolet et al., 1994). This study revealed that IHNV infection progresses from two major sites: the gills and the gastrointestinal tract. It appears that both routes of infection eventually result in systemic viremia and that connective tissue is the major site of replication.

Young fish experimentally infected with IHNV typically have external signs of infection 3-5 days post exposure (dpe). Mortalities typically begin 4-5 dpe, peak approximately 10 dpe, and decline thereafter so that by 40-50 dpe there are no more mortalities. Infectious virus can be isolated from fish surviving a viral challenge for approximately 40-50 dpe; after this time infectious virus can not be isolated. Recently, Drolet et al. (1995) demonstrated that IHNV can persistent in fish as defective particles for at least two years post infection.

Biology of IHNV in cells culture

There are numerous cell lines both salmonid and non-salmonid, in which IHNV can replicate (Lannan et al., 1984; Wolf and Mann, 1980). A characteristic manifestation of IHNV in fish cells is the formation of a plaque when the cells are overlaid with semi-solid media. The cytopathic effect is a result of both inhibition of cellular transcription and translation. IHNV has also been shown to grow to high titers in mosquito cells and the harvested virus is infectious for fish (Scott et al., 1980). However, unlike fish cells, mosquito cells do not show cytopathic effects due to IHNV.

A multitude of factors influence the rate of growth of IHNV in fish cells. These include, among others, the virus isolate, cell type, incubation temperature, and cell density (Mulcahy et al., 1984). In general IHNV grows optimally at a

temperature of 16-18° C and does not replicate in fish cells at temperatures much above 24° C. Replication of IHNV at temperatures of 5-10° C is isolate specific (Mulcahy et al., 1984). Following infection of cells, there is a lag period of approximately ten hours in which no infectious virus can be recovered, and maximum viral production is 30-90 hours post infection (Mulcahy et al., 1984; Fendrick et al., 1982). Hsu et al. (1985) demonstrated that the IHNV nucleocapsid protein appeared 2-3 hours post infection, the matrix proteins appeared 6-7 hours post infection, and the glycoprotein appeared 9-10 hours post infection. They also found that the polymerase could be detected 30-31 hours post; however, this time is probably indicative of the low levels of polymerase synthesized and not necessarily the actual time of appearance.

Persistent infection of fish cells with IHNV has been reported and has been used as a method for the production of attenuated live IHNV vaccines (Fryer et al., 1976; Fukuda et al., 1989). Engelking and Leong (1981) demonstrated that persistently infected cells were resistant to superinfection by homologous but not heterologous strains of IHNV. They also suggested that persistence was not mediated by interferon. In addition, persistent infection of cells with a temperature sensitive mutant of IHNV resulted in no cytopathicity at 20° C. However, when the cells were shifted to 15° C there was a rapid cytopathicity.

## Control of IHNV

### Overview

Currently there are no licensed vaccines or chemotherapeutics available for the control of IHNV. Avoidance of IHNV is the most practical and practiced method of control (Wolf, 1988). To this end, hatchery reared eggs, fry, and spawning fish are rigorously monitored for IHNV in both private and government facilities. IHNV positive animals are quarantined and, when practical, eliminated. Despite such methods, IHNV remains the most costly of viral pathogens in the Pacific Northwest of the USA.

Chemical control of IHNV Addition of low concentration of iodine to water has been shown to produce a 99.9% reduction in infectious IHNV titers after a 7.5 second exposure (Amend and Pietsch, 1972; Batts et al. 1991). A result of the sensitivity of IHNV to iodine is that now newly fertilized eggs are routinely treated in a dilute solution of iodine to reduce the risk of vertical transmission of IHNV. However, iodine treatment is not foolproof; fry hatched from iodine treated eggs and reared in virus free water can still succumb to IHNV infection, presumably by virus resident within the egg (Mulcahy and Pascho, 1985).

A number of investigators in Japan have reported isolation of anti-IHNV substances from naturally occurring aquatic microbes. Yoshimizu et al. (1991) found that a peptide isolated from *Psuedomonas fluorescens* had a strong antiviral effect on IHNV. Hasobe and Saneyoshi (1985) reported that (S)-DHPA ( 9-(S)-(2,3-dihydroxypropyl) adenine), Chloroquine, and Virazole had significant anti-IHNV activity using an *in vitro* cell culture assay.

Control of IHNV by water treatment

The elevation of water temperature has been suggested as a means of controling IHNV (Amend, 1970; Wingfield and Chen, 1970). Amend (1970) reported that infection of sockeye salmon with IHNV was prevented when fish were held in 18° C water following infection at 15° C. However, it was also found that if infection was allowed to progress at 15° C for several days, followed by the transfer of the fish to 18° C water, the disease was exacerbated. Hetrick et al. (1979), also investigated the effect of temperature on IHN disease in fish, but they held the fish at the same temperature throughout the experiment. From these studies the authors concluded that there was not a significant benefit to elevated water temperature and pointed out that elevated water temperatures could potentiate pathogenic bacterial growth, and further, that elevating water temperatures in hatcheries is not economically feasible.

Ozone and U.V. treatment of water has been shown to be an effective method of control of IHNV *in vitro* ( Wedemeyer et al., 1976). Yoshimizu et al. (1991) demonstrated that U.V. irradiation of river water resulted in a 95% decrease of cumulative mortality in IHNV-infected fish compared to control groups. However, irradiation of water has not been widely used due to the prohibitive cost.

Vaccines

Four types of vaccines have been developed to combat IHNV: Attenuated live virus (Fryer et al., 1976; Tebbit, 1976; Rohovec et al., 1981), killed virus (Amend, 1976; Nishimura et al., 1985), subunit (Gilmore et al., 1988; Oberg et al., 1991), and purified IHNV glycoproteins (Engelking and Leong, 1989a; Engelking and Leong. 1989b). All of the vaccines types have been at least partially successful in conferring a protective immune response to vaccinated fish.

Attenuated viral vaccine Fryer et al. (1976) reported the first attenuated viral vaccine developed for the control of IHNV. The attenuated virus was prepared by continual passage (34 or 41 serial passages) of virulent IHNV, obtained from rainbow trout, on steelhead trout embryo cells (STE 114). The serially passed virus had a 100-fold reduction in virulence compared to the parental isolate. Either immersion or injection of sockeye and kokanee salmon fry with the vaccine resulted in greater than 90% protection from a challenge with high doses of live virus. The vaccinated fish were shown to be protected from subsequent IHNV challenge for at least 110 days post injection (Tebbit, 1976). However, when rainbow trout fry were exposed to the attenuated vaccine approximately 20% of the fry died from the vaccine. Fryer et al. suspended further testing of the vaccine due to a lack of commercial interest and safety concerns.

Killed viral vaccine There have been three reports of the use of inactivated IHNV as a vaccine (Amend, 1976; Nishimura et al., 1985; Naganawa et al., 1993). The report by Naganawa et al. (1993) is interesting in that it appears that formalin-inactivated IHNV administered 24 hours prior to a viral challenge protects fish from IHNV. This finding is indicative of some form of nonspecific protective immune response.

Amend et al. (1976) demonstrated that a β–propiolactone inactivated IHNV vaccine injected intraperitoneally into fish was extremely effective, conferring approximately 94% relative percent protection compared to the control unvaccinated fish. Nishimura et al. (1985) reported a similar result with a formalin inactivated IHNV vaccine injected intraperitoneally into fish. However, fish challenged with

virus following immersion in formalin-inactivated IHNV resulted in 33% greater mortalities than the intraperitoneally vaccinated fish. While inactivated viral vaccines have proven efficacious, they have not been used, primarily due to the cost of the preparation and the possibility that viable, virulent virus remains in the preparation.

Purified glycoprotein vaccine Engelking and Leong (1989a) demonstrated that vaccination of kokanee salmon and rainbow trout by inoculation or immersion with purified IHNV glycoprotein conferred up to as 84% relative percent survival following challenge with a homologous strain of IHNV. The same authors expanded on these studies by demonstrating that vaccination of fish with the IHNV type 1 glycoprotein confers protection to five heterologous strains of IHNV (Engelking and Leong 1989b).

Engelking and Leong (1989a) showed that virus neutralizing antiserum was generated in rabbits receiving the IHNV glycoprotein. Rabbits injected with gel purified IHNV nucleoprotein, or the matrix proteins, generated binding but not neutralizing antibodies to the respective proteins. Both Ristow et al. (1993) and LaPatra et al. (1993) have demonstrated that the humoral immune response of fish to IHNV is relatively restricted; the majority of anti-IHNV antibodies are against the glycoprotein and the matrix proteins. Thus, the purified IHNV glycoprotein is both an effective vaccine and possibly the only viral protein necessary for strong cross-protective immune responses in fish against IHNV.

Subunit vaccines Gilmore et al. (1988) developed an IHNV subunit vaccine by fusing a 329 base pair fragment from the IHNV glycoprotein cDNA with a *trp*-E bacterial expression vector. Immunization of rainbow trout and chinook salmon by immersion in a crude bacterial lysate containing the *trp*-E glycoprotein fusion protein resulted in high degree of protection from a subsequent challenge with IHNV. Oberg et al. (1991) demonstrated that a bacterially expressed nucleoprotein subunit vaccine used in conjunction with the glycoprotein subunit vaccine resulted in an augmented protective immune response in fish. However, the nucleoprotein subunit vaccine used alone did not protect fish from a subsequent IHNV challenge. Currently, large scale preparation of the subunit vaccines are being undertaken and the efficacy of the vaccines is being tested in field trials.

## IHNV Molecular Biology

### Virion Morphology and composition

Electron microscopic analysis of tissues of IHNV infected fish (Amend and Chambers, 1970a; Yamamoto and Clermont, 1990) and tissue culture cells (Amend and Chambers, 1970b), has revealed a bullet-shaped viral particle approximately 90 nm in width and 160 nm in length.

Features of the viral particle include a dense, ether-sensitive lipid and protein coat, protruding glycoprotein spikes, and an inner ribonucleoprotein complex composed of the viral RNA bound intimately with the nucleoprotein, matrix proteins, and RNA-dependent RNA polymerase. Hsu et al. (1985) have estimated that IHNV virions are composed of 43 polymerase, 166 glycoprotein, 766 nucleoprotein, 270 matrix 1 protein, and 192 matrix 2 protein molecules.

### Genome and viral mRNA characteristics

The nucleic acid of IHNV is a contiguous, single-stranded RNA of negative polarity (McCain et al., 1974; McCallister et al., 1974; Hill et al., 1975; Kurath and Leong, 1985). The genome molecular weight has been estimated to be $3.7 \times 10^6$ daltons by electrophoretic separation on glyoxal gels (Kurath and Leong, 1985). The gene order, determined by R-loop mapping, is 3' N-M1-M2-G-NV-L 5' (Kurath et al, 1985). The complete nucleotide sequence has been determined for the 1609 base glycoprotein gene (Koener et al, 1987) and the 1359 base nucleocapsid gene (Gilmore and Leong, 1988).

Six viral mRNAs are synthesized in tissue culture infected cells as determined by glyoxal gel electrophoretic separation of $^3$H-uridine-labeled polyadenylated viral mRNA (Kurath et al., 1985). The molecular weights of the mRNAs are: glycoprotein (G) mRNA, $5.6 \times 10^5$; nucleoprotein (N) mRNA, $4.8 \times 10^5$; matrix proteins one and two (M1, M2) mRNA, $3.0 \times 10^5$; and the non-virion (NV) mRNA, $1.9 \times 10^5$. The *in vitro* synthesis of mRNA from purified IHNV resulted in mRNA species which co-migrated with viral mRNA isolated from tissue culture cells (Kurath and Leong, 1987)

The molar ratio of the mRNAs produced intracellularly in tissue culture cells, normalized to the N mRNA are as follows: (G) mRNA, 0.49; (N) mRNA, 1.0; M1and M2 mRNA's, 2.52; and NV mRNA, 0.41 (Kurath and Leong, 1985).

Proteins

The virion of IHNV is composed of five virally encoded proteins (McCallister and Wagner, 1975, Hill, 1975). In tissue culture cells, there is an additional non-virion protein synthesized with a molecular weight of 12 kilodaltons (kd; Leong and Kurath, 1985). The role of the NV protein is presently unknown.

The virion-associated protein molecular weights are as follows: polymerase (L), 150 kd; G, 66-70 kd; N, 40-44 kd; M1, 25 kd; and M2, 21 kd (Hsu et al., 1986). Hsu and colleges (1986) have classified IHNV into five types according to the electrophoretic profiles of the virion proteins. The typing has been valuable in analyzing the geographic distribution and virulence of IHNV.

The 150 kd L protein is believed to be the primary component of the virion-associated RNA-dependent RNA polymerase that replicates the viral genome, and transcribes the six mRNAs.

The 66-70 kd G protrudes from the external lipid layer of the virion (Drolet et al., manuscript submitted). The protein is believed to be the protein responsible for attachment of the virion to an unidentified receptor on the cell. The G has been shown to be a glycoprotein by incorporation of $^{3}$H-labeled glucosamine (McAllister and Wagner, 1975) and by enzymatic digestion (Engelking and Leong, 1989). The protein has a putative 20 amino acid signal sequence at its N terminus (Koener et al., 1987). There is a hydrophobic segment at the carboxy terminus suggesting its association with the virion lipid membrane (Koener et al., 1987).

Recently, four IHNV neutralization-resistant variants, that had been selected with a G-specific monoclonal antibody (Roberti et al., 1991), were analyzed for their virulence and pathogenicity in rainbow trout (Kim et al., 1994) in relation to the G amino acid (a.a.) sequence. Kim and colleagues (1994) showed that the variant RB-1 was highly attenuated and predominately infected brain tissues. The analysis of the deduced a.a. sequence of RB-1's G showed a change of threonine to an isoluecine at a.a. 78 and glutamic acid to glycine at a.a. 218. The three other moderately attenuated viruses had an important change in the G a.a. sequence at position 276, glutamic acid

to aspartic acid. Thus, there is a strong correlation of the primary structure of G with tissue tropism and virulence.

The 40-44 kd N protein is believed to encapsulate the RNA genome. The carboxy terminal 57 amino acids are highly hydrophilic and contains a large number of arginines (Gilmore and Leong, 1988). This finding has been suggested as evidence that the carboxy terminus of N is involved in RNA binding. The N along with the M1 protein were found to be phosphoproteins by the incorporation of $^{32}$P-orthophosphate (McCallister and Wagner, 1975). The intracellular concentration of the N protein of VSV is postulated to play an important role in signaling the switch from transcription to replication of the viral genome (Banerjee 1983).

Biochemical analysis of the IHNV 25 kd M1 and 21 kd M2 has not been reported for IHNV. It is believed that they play similar roles to their homologues in VSV and rabies virus. These include virus assembly and budding of the maturing virion from the cell as well as interacting with the RNA-dependent RNA polymerase. Recently, the matrix protein of VSV has also been found to mediate many of the cytotoxic effects induced by the virus including down regulation of cellular transcription and translation (Black et al., 1994).

## Genetic Immunization

### Overview

Genetic immunization, or the use of antigen-encoding DNAs to vaccinate, is one of the newest approaches for the development of vaccines (Ulmer et al., 1993; Cox et al. 1993; Xiang et al., 1993; Montgomery et al., 1993; Fynan et al., 1993; Raz et al., 1994; Segedah et al., 1994). In essence, DNA transfection of somatic cells and resultant antigen expression approximates what the immune system sees following infection with a live virus. The result is that genetic immunization activates both a protective cellular and humoral immune response in vaccinated organisms. The cellular immune response is attributed to the capacity of endogenously synthesized antigens
(viral or DNA encoded) processed through the cytoplasmic and endoplasmic reticulum to associate with major histocompatability class 1 molecules which in turn

can be recognized by cytotoxic T lymphocytes (CTL) (Germain and Margulies, 1993). Presumably, the humoral immune response is activated as a result of shedding of the DNA encoded antigen from the transfected cells or by lysis and release of the antigen by CTLs (Ulmer et al., 1993; Xiang et al. 1993). The free antigen is then able to activate MHC class 2-bearing cells (including B cells) that, in turn, produce antigen specific antibodies.

A number of replicating and non-replicating viral vectors have been utilized for DNA vaccination including retroviruses (Brown et al., 1992; Irwin et al., 1994; Hunt et al., 1988), vaccinia virus (reviewed in Hruby, 1993), adenoviurses (Prevec et al., 1991; Graham and Prevec, 1992), fowlpox virus (Baxby and Paoletti, 1992) and adeno-associated virus (Muzyczka, 1992). Drawbacks to these viral vectors are the risk of associated pathology due to the viral component of the vaccine, and more specifically the fact that none of the vectors mentioned have been shown to infect or replicate in fish. The alternative to viral vectors is the use of naked plasmid DNA for genetic immunization. Plasmid DNA as a vaccine has the benefit of being non-infectious agent, requires no assembly of virus particles and very importantly, expression of genes contained in plasmid DNA has been observed following direct injection into fish (Hansen et al., 1991; Rahman and McClean, 1992).

The use of plasmid DNA-based vaccines requires an understanding of gene expression following the direct introduction of DNA into somatic cells. This includes identifying efficient DNA delivery methods, strong promoters, the duration of gene expression, the fate of the injected DNA, and the sites of gene expression. The following section will deal with factors that govern gene expression by direct DNA injection and a review of current DNA vaccines.

## Transgene expression in somatic cells

DNA delivery Two general methods have been used to introduce DNA into somatic cells for vaccination: direct injection using a needle and syringe (Wolf et al., 1990), and DNA bombardment using a gene gun (Tang et al., 1992). A third method which has been proposed but has yet to be used for vaccination is DNA encapsulated in liposomes delivered either by injection or aerosol delivery (Felgner et al., 1987). Of the three methods the simplest and most commonly used for DNA vaccination is the direct injection DNA into striated muscle tissue.

DNA entry into somatic cells The mode of DNA entry into somatic cells following DNA particle bombardment and DNA/liposomes inoculation is relatively straight forward. The gene gun delivery method transfers DNA directly into somatic cells by passing "DNA bullets" through the cell membranes (Cheng et al., 1993; Yang et al., 1993). The entry of DNA/lipid complex is believed to be mediated by fusion of the lipid/DNA vesicle with the cellular membrane followed by release of the DNA into the cytoplasm (Felgner et al., 1987; Zhu et al., 1993).

The mechanism of DNA entry into muscle cells following injection with a needle and syringe is unclear. Wolf et al. (1990) suggested that the injected DNA may enter muscle cells by combining with calcium ions which influx when muscle cells contract. However, Wolff et al. (1991) demonstrated that including calcium chelators along with the injected DNA solution, electrically stimulating muscle contraction, and denervation of muscle cells, had a minimal impact on gene expression. Physical disruption of cell membranes by needle penetration, uptake of the injected DNA, and subsequent patching of the damaged membrane has also been suggested as method of DNA entry into muscle cells (Wolf et al., 1991). However, Davis et al. (1993) found that muscle cells damaged by the needle during injection did not express the transgene DNA. Thus, the mode of entry into muscle cells remains unknown.

Sites of gene expression Striated muscle cells have been considered the only tissues capable of taking up and expressing genes that are transferred by direct injection of plasmid DNA (Davis et al. 1993). However, Fynan et al. (1993) have shown that mice vaccinated with an influenza virus hemagglutinin plasmid by topical administration intranasally, or by intravenous, intradermal, and subcutaneous injection, resulted in significant protection from viral challenge. Thus it appears that many tissues can take up and express plasmid DNA. Unfortunately, Fynan et al. (1993) did not show any data on the levels of gene expression in the various tissues following delivery. Raz et al. (1994) clearly demonstrated that plasmid DNA injected into mice intradermally resulted in expression in keratinocytes, fibroblasts, and dendritic cells. In rats, intraperitoneal injection of calcium phosphate-precipitated DNA results in rapid uptake and expression of the genes in the intestine, lung, spleen, kidney, and heart (Bevenisty and Reshef, 1986). Injection of DNA directly into rat hearts has also been shown to result in efficient gene expression (Kitis et al., 1991). Thus, numerous tissues can efficiently be transfected following direct DNA injection

and the notion that striated muscle are the only tissues capable of taking up and expressing DNA should be dismissed.

Transgene expression in numerous tissues has been reported following DNA bombardment with a gene gun (Cheng et al., 1993; Yang et al., 1993). The only limitation of the sites of gene expression following particle bombardment appears to be those tissues which cannot be exposed to the gene gun by surgery.

Intravenous injection of lipid/DNA complexes into mice results in transfection of virtually all tissues (Zhu et al., 1993). Aerosol delivery of DNA/lipid complexes to the lungs may be a powerful tool for vaccination against pathogens such as influenza virus which enter organisms via the lungs; however, no reports have been published on this approach to date.

Promoter analysis A wide variety of promoters have been used to express genes following the direct injection of naked DNA and DNA/lipid complexes or gene gun delivery of DNA: these include 1) viral promoters, 2) hormone, heavy metal, and virus inducible promoters, 3) and constitutive cellular promoters, from both mammals and fish. A review of the current literature indicates that the cytomegalovirus immediate early enhancer/promoter (CMV IEP) consistently provides the greatest level of reporter gene expression in all tissues examined. It is important to note that the expression of transgenes in striated muscle cells following direct DNA injection is promoter dependent. For instance, the mouse mammary tumor virus LTR promoter expresses poorly in muscle tissue (Yang et al., 1993).

Duration of gene expression and fate of the DNA A suprising feature of transgene expression following delivery to somatic cells by direct DNA injection, DNA/lipid complex injection, and DNA particle bombardment, is the long-lived gene expression. Wolf et al. (1990) showed that high levels of gene expression were recovered from injected mouse muscle tissues for two months. In addition the same authors found that the half-life of the reporter mRNA (luciferase) and protein in mouse muscle was less than 24 hours. Thus, the long-lived reporter protein activity in mouse muscle tissue was the result of continuous gene expression from the injected plasmid DNA. Zhu et al. (1993) found that a single intravenous injection of a DNA/lipid complex resulted in high reporter gene expression in multiple tissues for at

least 9 weeks. Cheng et al. (1993) reported sustained gene expression in mouse dermis for 1.5 years following gene gun delivery of reporter transgenes. However, in tilapia fish injected intramuscularly with a reporter transgene plasmid, reporter protein activity could be detected only two days post injection (Rahman and McClean, 1992). This is not the case for carp, in which reporter protein activity could be detected at least four days after plasmid DNA injection (the last time tested; Hansen et al., 1991).

Wolff et al. (1990) found that DNA recovered from injected muscle tissues two months post injection persisted as a circular, unreplicated plasmid. However, during the first three days post injection, the DNA was heavily degraded, and then degradation ceased (Wolff et al., 1991). Liposome-encapsulated DNA delivered intravenously into mice also persists as a circular, unreplicated plasmid for at least 21 days post administration. There have not been any reports of the fate of transgene DNA following delivery with a gene gun.

## DNA vaccines

Overview The use of vaccines composed of antigen-encoding naked plasmid DNA and antigen-encoding-retroviral vectors is summarized in Table 2.1. Tang et al. (1992) was the first group to show that naked DNA encoding an antigen (human growth hormone), when introduced into mouse somatic cells, could express the encoded antigen and subsequently evoke an immune response. Ulmer et al. (1993) was the first group to report that DNA vaccines could be used to protect animals (mice) from a subsequent challenge with a pathogen (influenza virus). To date, naked DNA vaccines have been employed to combat the disease produced by, influenza virus (Fynan et al., 1993 a, b; Montgomery et al., 1993; Ulmer et al., 1993; Yankauckas et al., 1993; Raz et al., 1994), rabies virus, (Xiang et al., 1994), human immunodeficiency virus (HIV) (Wang et al., 1993 a, b), bovine herpesvirus (Cox et al., 1993), and malaria (Sedegah et al., 1994).

Although numerous DNA vaccines have been developed, they all share some common properties. All of the DNA based vaccines reported to date elicit both cellular and humoral immune responses. The magnitude of the immune response to the expressed antigen is dependent on the amount of the DNA injected. The route of vaccine delivery has a profound effect on the extent of the immune response

Table 2.1 Compilation of results concerning genetic immunization

| Pathogen | Animal | Delivery[1] | DNA Vaccine Gene[2] | DNA Vaccine Promoter[3] | Immune Response Humoral | Immune Response Cellular | Protection | Ref[4] |
|---|---|---|---|---|---|---|---|---|
| Influenza | mouse | IM | NP | CMV | + + | + + + + | + + + | 1 |
| Influenza | mouse | ID | NP | CMV | + + + + | + + + + | + + | 2 |
| | | | | RSV | + + + | n.d. | n.d. | |
| Influenza | mouse | IM | NP | RSV | + + + + | + + + + | n.d | 3 |
| Influenza | chicken | IM | NP | ALV | + | n.d. | 0 | 4 |
| Influenza | mouse | IM | NP | CMV | n.d | n.d. | + + + | 5 |
| | | | HA | | n.d. | n.d. | + + + + | |
| Influenza | mouse | IM | HA | CMV | 0 | n.d. | + + + + | 6 |
| | | IV | | | 0 | n.d. | + + + | |
| | | IN | | | + | n.d. | + + | |
| | | SC | | | n.d. | n.d. | + + | |
| | | IP | | | n.d. | n.d. | 0 | |
| | | gene gun | | | + | n.d. | + + + + | |
| Influenza | chicken | IM | HA | CMV | n.d. | n.d. | + + | 6 |
| | | IT | | | n.d. | n.d. | + + | |
| | | IB | | | n.d. | n.d. | + | |
| | | IO | | | n.d. | n.d. | + | |
| Influenza | chickens | IM | HA | ALV | + | n.d. | ++++ | 7 |
| HIV | mice | IM | gp160 | MMTV | + + + | n.d. | n.d. | 8 |
| | | | tat | | + + + | n.d. | n.d. | |
| | | | rev | | + + + | n.d. | n.d. | |
| HIV | macaques | IM | gp41 | MMTV | + + + + | n.d. | n.d. | 8 |
| HIV | mice | IM | env/rev | CMV | + + + + | + + + + | n.d. | 9 |

Table 2.1 (Continued)

| Pathogen | Animal | Delivery [1] | DNA Vaccine Gene[2] | DNA Vaccine Promoter[3] | Immune Response Humoral | Immune Response Cellular | Protection | Ref[4] |
|---|---|---|---|---|---|---|---|---|
| HIV | baboon | IM | env/rev | CMV | + + + + | + + + + | n.d. | 9 |
| Rabies | mouse | IM | G | SV40 | + + + + | + + + + | + + + + | 10 |
| Bovine Herpes | mouse | IM | G | RSV | + + + + | n.d. | n.d. | 11 |
| Bovine Herpes | cow | IM | G | RSV | + + + | | + ++ | 11 |
| Malaria | mouse | IM | G | CMV | + + | + + + | + + | 12 |

[1] IM, intramuscular; ID, intradermal; IV, intravenous; IN, intranasal; SC, subcutaneous; IT, intratracheal IB, intrabursal

[2] NP, nucleoprotein; HA, hemagluttinin; G, glycoprotein

[3] CMV, cytomegalovirus; RSV, rous sarcoma virus; ALV, avian leukosis virus; MMTV, mouse mammary tumor virus

[4] 1-(Ulmer et al., 1993); 2-(Raz et al., 1994); 3-(Yankauckas et al., 1993); 4-(Brown et al., 1988); 5-(Montgomery et al., 1993); 6-(Fynan et al., 1993); 7-(Hunt et al., 1988); 8-(Wang et al., 1993); 9-(Irwin et al., 1994); 10-(Xiang et al., 1994); 11-(Cox et al., 1993); 12 (Sedegah et al., 1994)

n.d., not done

generated, and the amount of DNA vaccine required. Those DNA vaccines which have been tested for their ability to protect animals from a viral challenge have proven efficacious: protection has ranged from 50-100% compared to negative control groups.

Protection conferred by DNA vaccines Immunization of mice with a influenza nucleoprotein (inf vNP)-encoding plasmid DNA vaccine (pDNAv) resulted in 50% (intradermal injection; Raz et al., 1994) to 100% (intramuscular injection; Ulmer et al., 1993) protection from a lethal dose of influenza virus. Mice vaccinated intramuscularly with the inf vNP pDNAv were also protected from heterologous strains of influenza virus (Ulmer et al., 1993, Montgomery et al., 1994). Intramuscular immunization of mice (Montgomery et al., 1993; Fynan et al, 1993) and chickens (Fynan et al., 1993) with an influenza virus hemagglutinin (inf vHA) encoding pDNAv resulted in 95-100% and 63% protection respectively, against a lethal dose of influenza virus. Fynan et al. (1993) demonstrated that the site of delivery of the injected inf vHA pDNAv influenced the degree of relative protection from a challenge with influenza virus (see Table 1.).

Immunization of mice with a rabies virus glycoprotein (rvG)-encoding pDNAv conferred 100% protection from a challenge with lethal dose of rabies virus (Xiang et al., 1993). A bovine herpesvirus glycoprotein (bhv G)-encoding pDNAv successfully protected cows from a subsequent bovine herpesvirus viral challenge (Cox et al., 1993). Immunization of mice with a malaria glycoprotein (G) encoding pDNAv resulted in a 84.5% reduction of shizonts in the liver (Segedah et al., 1994). In addition, the malaria G pDNAv protected 54% of the immunized mice from blood stage sporozoite infection.

Cellular immune response The first report of genetic immunization by Ulmer et al. (1993) demonstrated that mice vaccinated intramuscularly with an inf v NP pDNAv generated both an inf vNP specific humoral and cellular immune response. While the vaccinated mice had high concentrations of anti-inf vNP antibodies, the antibodies were not neutralizing. The authors suggested that protection from influenza virus was mediated by a cellular immune response. This result is similar to that of Segedah et al. (1994) who used a malaria G pDNAv for which a $CD8^+$ CTL

response was found to be the primary protective immune response induced by intramuscular pDNAv immunization (Segedah et al., 1994). A $CD8^+$ restricted cytotoxic immune response has also been reported following intramuscular immunization with rvG pDNAv, but the contribution of the cellular immune response in relation to the protective immunity was not determined (Xiang et al., 1993). Additionally, Xiang et al. (1994) found that the rvG pDNAv DNA vaccine generated a T-helper type 1($Th_1$) immune response in mice and that the $Th_2$ subset did not contribute to the protective immune response.

In general, the cellular immune response can be enhanced by increasing the dose and the number of boosts of the pDNAv. Xiang et al. (1994) found that generation of a T-cytotoxic immune response in mice following intramuscular immunization with a rvG pDNAv required a minimum of two 10 μg boosts. Xiang et al. (1994) also found that increasing the dose of the rv G pDNAv from 10 μg to 250 μg resulted in a three-fold increase in CTL activity. Interestingly, mice immunized intramuscularly with as little as 10 μg of a inf v NP pDNAv generated anti-NP CD8+ CTL activity roughly equal to mice immunized with 600 μg of inf v NP pDNAv when measured one year after vaccination (Yankauckas et al., 1993). However, increasing the dose of the inf v NP pDNAv (10 μg to 600 μg) increased the number of mice which had high levels of inf v NP-specific CTL activity one year post-vaccination (Yankauckas et al., 1993). Yankauckas et al. (1994) was the first group to show that the cellular immune response was long-lasting (13 months) following pDNAv immunization; they also found that the NP-specific CTL response one year post inoculation was similar to the levels reached by three weeks post inoculation.

It is clear that increasing the dose and number of boost of pDNAv enhances the cellular immune response. However, even a single low dose of pDNAv results in a long lasting immune response. What is not clear is whether or not increasing the cellular immune response by inoculating high doses of pDNAv and increasing the number of boosts of pDNAv is necessary to generate a protective immune response. It may be that a single low dose inoculation of pDNAv is sufficient to induce long lasting protective cellular immunity.

Humoral immune response Like the cellular immune response, the humoral immune response is dependent upon the dose and number of boosts of the pDNAv. However, it generally requires less pDNAv to generate a humoral immune response

than it does a cellular immune response. The humoral immune response has also been found to be dependent on the site of vaccine delivery. Unfortunately, the effect of the site of delivery of DNA vaccines on the cellular immune response has not been reported.

Wang et al.(1993) found that an inoculation of 4 boosts consisting of 100 μg each of a HIV gp160 (membrane bound-envelope glycoprotein) pDNAv was required to seroconvert 90% of mice receiving the pDNAv. Xiang et al. (1994) found that the induction of rabies-virus-neutralizing antibodies increased proportionally to the number of boost (one to three) of 150 μg of rv G pDNAv. Segedah et al. (1994) also found a similar increase in malaria-neutralizing antibodies following boosting of mice with a malaria G pDNAv. They also found that increasing the number of malaria G pDNAv boosts from three to four resulted in a decrease in mouse-malaria-specific antibodies. This last finding is the first report of the potential induction of immunotolerance by repeated boosting with a pDNAv. Cox et al. (1993) found that there was no correlation between the level of neutralizing antibodies induced by inoculation of cows with a bhv G pDNAv and the generation of a protective immune response. This finding suggests that the cellular immune response to the bhv G pDNAv plays an important role in neutralizing the herpes virus.

Raz et al (1994) were the first group to report that the site of pDNAv delivery by direct injection has a profound effect on the quantity of vaccine required to elicit a long term immune response. Intradermal immunization with as little as 0.3 μg of inf vNP pDNAv generated a specific NP antibody response for 70 weeks post vaccination. Immunohistochemical analysis with anti-NP antibodies revealed that intradermal vaccination results in gene expression in keratinocytes, fibroblasts, and dendritic cells of the skin. In addition the immune response was dependent on the promoter driving expression of the inf vNP protein; plasmids containing the inf vNP downstream of the CMV IEP resulted in the strongest immune response (Raz et al. 1994; Montgomery et al., 1993). Gene-gun particle bombardment of mouse dermal cells with as little as 0.1 μg of human growth hormone also generates a strong humoral immune response (Tang et al., 1992; Eisenbraun et al, 1993). Gene-gun immunization of mice with an inf vHA pDNAv required 2500 times less pDNAv to generate a humoral response compared to direct intramuscular DNA injection (Fynan et al., 1993). Thus, the site and method of pDNAv delivery has a dramatic impact on the immune response. Further investigations should lead to more efficient and practical methods of immunization using pDNAv, making possible the large scale vaccination of fish in farming facilities.

## Intracellular Immunization

Overview

Cellular expression of endogenously synthesized (genetically engineered) viral RNA in either the sense or antisense orientation can interfere with the life cycle of viruses (reviewed in: Inouye, 1988; van der Krol et al, 1988; Helene and Toulme, 1990; Takayama and Inouye, 1990) . Interference can occur at a number of steps during the virus life cycle, thus defining a broad unifying mode of action of the interfering RNA is not possible. There have been reports of transgene interference occurring at the level of replication, transcription, translation, RNA processing (reviewed in Takayama and Inouye, 1990), virion uncoating, (Register and Beachy, 1988; Reimann-Phillip and Beachy, 1993; Okuno et al., 1993), virus spread (Wisniewski et al., 1990), cell binding (Federspiel et al., 1989), and trans-dominant viral protein interference (Malim et al., 1989; Trono et al., 1989; Bevec et al., 1992; Mermer et al., 1990; Bahner et al., 1993,). Viral RNA can be degraded by endogenously synthesized ribozymes (Ojwang et al., 1992; Taylor et al., 1992; Chen et al., 1992; Dropulic et al., 1992; Xing et al., 1992; Dropulic and Jeang, 1993; Xing et al., 1993). In addition, transgene RNA can serve as decoy sequences acting as a viral protein sponge (Sullenger et al., 1991; Lee et al., 1992; Lisziewicz et al., 1993). Finally, nucleic acid oligonucleotides can be targeted to specific viral nucleic acid sequences disrupting replication, transcription, translation, or RNA processing (Reviewed in Stein and Cohen, 1988; Crooke, 1992). Therapies based upon the sense and antisense strategy have been broadly categorized as inducing a state of intracellular immunity (Baltimore, 1988).

In this review, inhibition of the life cycle of animal viruses, and in specific instances plant viruses, by endogenously synthesized RNA will be addressed. Also, reports concerning the inhibition of the rhabdovirus vesicular stomatitis virus by oligonucleotides will be included.

Mechanisms of sense and antisense RNA interference

The multiple modes of action of natural antisense RNAs in prokaryotes have been demonstrated to be due to hybridization between the antisense RNA and its target nucleic acid sequence (RNA or DNA; reviewed in Inouye, 1988; Takayama and Inouye, 1990). In eukaryotes it has also been hypothesized that the mode of action of artificial antisense RNA upon cellular and viral gene expression involves *in vivo* hybridization between the antisense RNA and its target sequence. But, other than the nuclease degradation of duplex RNA (which will be reviewed), there have not been any reports concerning the precise mechanism of action of interfering RNAs with animal viruses *in vivo*. Recently, Helene and Toulme (1990) summarized the current information available concerning interference of cellular (eukaryotic) genes by antisense RNA. Inhibition begins with putative duplex formation. The duplexes are then either degraded by nucleases, chemically inactivated by "unwindases" (which have not been characterized), trapped in the nucleus or, unable to be translated by ribosomes. Presumably, any one, or combination of these mechanisms, is involved in viral interference.

There have been only two reports which have identified duplex formation between an antisense RNA and its target in eukaryotic cells (Kim and Wold, 1985; Yokoyama and Imamoto, 1987). The authors showed, using an RNAase protection assay, that at least 95% of the RNA duplexes between an antisense thymidine kinase (TK) RNA and its complementary sense target are confined to the nucleus. Kim and Wold (1985) suggested that the duplexes formed in the nucleus inhibited export of the mRNA to the cytoplasm, accounting for the 90% reduction in cellular TK activity observed.

Indirect evidence of hybrid formation has been reported (Crowley et al., 1985; Kasid et al. 1989). In these studies, interference by the transgene RNA could be detected by a biological assay (measuring the effects of discoidin or Myc gene expression on cell morphology or growth, respectively) but the interfering RNA could not be detected by Northern blotting. The authors concluded that duplexes between the interfering RNA and the target sequence were formed in the nucleus and were then rapidly degraded by specific double-stranded RNAases. Chatterjee et al. (1992) found that HIV type 1 mRNA was not detectable by Northern blotting in HIV-infected cells expressing antisense RNA complementary to the viral LTR. However, in this case, prior to HIV infection the antisense RNA could be detected by Northern

blotting. Again, it was concluded that a duplex formed between the mRNA and the antisense RNA was targeted for degradation by cellular RNAases.

The extent to which viral gene expression can be inhibited by a particular antisense or sense sequence is believed to ultimately depend upon the length and accessibility of the interfering RNA, the availability of the target mRNA, and the ability of the interfering RNA and its target to form duplexes (Shierczinger and Knecht, 1993). Systematic analyses of antisense RNA inhibition of gene expression dependent upon the target site (Kim and Wold, 1985; Chang and Stoltzfus, 1985; Izant and Weintraub, 1985; Rhodes and James, 1990; Rittner and Sczakiel, 1991; Shierczinger and Knecht, 1993 ) and hybridization kinetics (Rittner et al., 1993) have been reported. The result of these studies are contradictory. For instance, Izant and Weintraub (1985) found that the greatest inhibition of thymidine kinase (TK) expression occurred with antisense RNA complementary to the 5'-untranslated region of the TK mRNA. In contrast, Shierczinger and Knecht (1993) found that myosin heavy-chain (MHC) gene expression was inhibited to the greatest extent by antisense RNA complementary to the 3'-portion of the MHC gene; 5'-complementary antisense RNA did not inhibit MHC gene expression. Rittner and Sczakiel (1991) found that of ten antisense RNAs complementary to HIV mRNA, two interfering RNAs complementary to distinct viral mRNAs most effectively inhibited HIV replication. Rittner et al. (1993) found that three of three fast-hybridizing antisense-RNAs specific to the HIV tat and rev coding regions inhibited viral replication. However, the authors also found that one of three slow-hybridizing, antisense-RNAs complementary to the same HIV sequence inhibited viral replication to the same degree as the fast-hybridizing RNA. Thus, it may be that the choice of target sites and hybridization kinetics of an antisense RNA is particular to the system being evaluated.

The quantity of endogenously synthesized antisense RNA necessary to inhibit viral or cellular genes is a controversial issue. It has been suggested that, in general, antisense or sense RNA must be present in excess of the target mRNA to exert an effect (van der Kroll et al., 1988; Helene and Toulme, 1990). This is illustrated by Kim and Wold (1985) who found that antisense TK RNA only inhibited TK activity when the antisense expressing plasmid (containing the dihydrofolate reductase gene) was amplified by increasing concentrations of methotrexate, which concomitantly increased the quantity of cellular antisense RNA 300-fold. On the other hand, Rhodes and James (1990) found that there was no correlation between the abundance of a HIV antisense RNA in the cell and the degree

of HIV inhibition. The strongest evidence against the need for a large excess of interfering sense or antisense RNA comes from studies using plant viruses (reviewed in Lindbo et al., 1993). Dougherty et al. (1994) compared the amount of untranslated potyvirus coat protein (CP) RNA levels in CP expressing transgenic plants with the degree of plant viral resistance. The authors found that plants expressing relatively low levels of cytoplasmic CP RNA were consistently more resistant to the virus than plants with high levels of CP RNA expression. However, the authors found that nuclear levels of the interfering RNA in the resistant plants was relatively high. Again, this is an example of the complexity of sense and antisense RNA interference with viruses and cellular genes, and suggest that each virus or cellular gene may respond to the interfering RNA differently.

Antisense and sense RNAs and have been shown to suppress viral protein synthesis (Chaterjee et al., 1992; Rhodes and James 1990;Sczakiel et al., 1990; Sczakiel et al., 1992; Joshi et al., 1991; Rittner et al., 1991; plant viruses are reviewed in Beachy, 1993). Studies demonstrating that oligonucleotides complementary to the rhabdovirus vesicular stomatitis virus (VSV) mRNAs down-regulate viral protein synthesis. Agris et al. (1986) found that oligodeoxyribonucleoside methylphosphonates complementary to the initiation codon regions of the VSV N, NS, and G mRNAs inhibited viral protein synthesis in mouse L cells. Synthesis of all five viral proteins were inhibited by the oligonucleotides; the G oligonucleotide inhibited total viral protein synthesis by 90%, the N oligonucleotide by 70%, and the NS oligonucleotide by 40%. Interestingly, the oligonucleotides did not inhibit protein synthesis corresponding to there specificity to for a specific mRNA sequence. The complete inhibition of viral protein synthesis was attributed to duplex formation of a viral mRNA with a complementary oligo resulting in blockage of translation of the duplex, causing a viral protein deficit which, assuming each protein has an interconnected function, abrogated further viral protein synthesis. Leonetti et al. (1987) showed that poly-(L-lysine)-conjugated oligos complementary to the initiation codon of N mRNA, inhibited N protein synthesis by 90%. Leonetti et al. also found that oligos complementary to internal sequences of the N gene did not inhibit viral synthesis, demonstrating the importance of choosing the appropriate target site.

Protein interference

Intracellular expression of functional viral proteins can inhibit virus replication. The majority of reports of protein mediated interference for animal viruses come from studies using HIV (Malim et al., 1989; Trono et al., 1989; Mermer et al., 1990; Sczakiel et al., 1990; Bevec et al., 1992; Bahner et al., 1993). In these studies, expression of mutant Tat or Rev proteins interfered with the wild-type protein resulting in interference with HIV replication. This type of interference requires that the mutant protein be trans-dominant and be either oligomeric or capable of saturating the target site, presumably so that it can interfere with the wild-type protein functions. Bahner et al. (1993) culminated the work with the trans-dominant Tat and Rev-mediated inhibition of HIV replication by side-by-side comparison of the two mutant proteins inhibitory effect on HIV replication. The mutant Rev protein consistently inhibited HIV infection while the mutant Tat protein actually produced mild trans-activation. Interestingly, intracellular expression of both Tat and Rev mutant proteins inhibited reporter gene activity (the reporter gene was linked to a Tat or Rev responsive sequence) in a transient assay. This last point is another example of the variable conclusions which can be drawn regarding the effectiveness of strategies of intracellular immunization according to the system being utilized.

Federspiel et al. (1989) found that the expression of avian reticuloedotheliosis virus envelope protein (which serves as a receptor for cell attachment) protected the cells from a subsequent viral challenge. The authors hypothesized that the envelope protein blocked cell surface receptors inhibiting virus entry into the cell. However, there was not direct evidence to support the hypothesis.

Carr and Zaitlin (1993) has reviewed reports concerning the viral resistance of transgenic plants expressing replicases of plant viruses. It is hypothesized that the transgene replicase interferes with the wild-type viral replicase. However, the precise mechanism of interference is unknown. Further, it is not certain whether the replicase protein of mRNA causes viral interference since in a few cases the interfering protein could not be detected in viral resistant transgenic (replicase) plants (Braun and Hemenway, 1992; Golemboski et al., 1990).

Transgenic plants expressing CP genes have been shown to be resistant to viral infection (reviewed in Reimann-Phillip and Beachy, 1993; Lindbo et al., 1993; Gonsalves and Slightom, 1993). The CP is believed to inhibit an early step in the viral life cycle, such as virion uncoating. However, as is the case for replicase-mediated

viral resistance, it is not clear whether the CP mRNA or protein mediates viral resistance. Powell et al. (1990) found that transgenic tobacco plants which synthesize an untranslatable tobacco mosaic virus (TMV) CP mRNA are not resistant to TMV infection; viral resistance required expression of the TMV CP. On the other hand, it has been shown that transgenic plants synthesizing both translated and untranslated tobacco etch (TEV) virus CP mRNA are resistant to viral infection (Lindbo and Dougherty, 1992a; Lindbo and Dougherty 1992b). In this case, the authors hypothesized that the TEV RNA caused viral resistance by duplex formation with viral RNA or, alternatively, induction of an undefined plant mediated immunity.

Intracellular immunity to viruses

A list of eukaryotic cellular and viral genes inhibited by artificial sense and antisense genes for which expression data were available showed inhibition ranging from 0 to 99% (van der Kroll et al., 1988). The variability of inhibition is likely due to different assay systems, gene target sequences, and the type of interfering RNA, all of which complicate comparison between studies. An example of this comes from studies by Sczakiel et al.(1990) and Sczakiel and Pawlita (1990). Sczakiel et al. (1990) found that microinjection of epitheleiod cells with plasmid DNA encoding a 410 bp HIV leader-gag sequence in the sense or antisense orientation along with an infectious HIV clone (at a ratio of 5:1, respectively) reduced the load of HIV antigen produced by 70%. However, when the same HIV sense and antisense plasmids were stably introduced into human T cells and subsequently challenged with HIV, the cell lines containing plasmid DNA in the sense orientation produced the same HIV antigen levels as the control cells. The cell lines containing the antisense plasmid were HIV resistant. The differential behavior of the sense-encoding plasmid was attributed to the different test assays employed.

Degree of intracellular immunity A recurrent theme, using animal virus model systems, is the incomplete inhibition of viral replication by interfering sense and antisense RNA, proteins and oligonucleotides (Chang et al., 1985; Agris et al., 1986; Lemaitre et al., 1987; Leonetti et al., 1988; Rhodes and James, 1990; Sczakiel et al., 1990; Joshi et al., 1991; Rittner and Sczakiel, 1991; Sczakiel and Pawlita, 1991;

Sczakiel et al., 1992; Lisziewicz et al., 1992; Sullivan et al., 1992; Rittner et al., 1993; Junker et al., 1994). One explanation for incomplete inhibition of viral replication is that the interfering RNA or protein is eventually overwhelmed (saturated) by its complementary viral RNA. This is supported by the fact that interfering RNA and trans-dominant proteins have been found to nearly abolish HIV replication for a period of 10-14 days post infection but thereafter HIV replication rises to the same levels as it does in control cells (Rhodes and James, 1990; Sczakiel and Pawlita, 1991; Sczakiel et al., 1992; Bahner et al., 1993). Further, the level of interfering RNA in cells in which HIV had escaped inhibition remained unchanged, indicating that the viral escape did not result from an inhibition of synthesis of the interfering RNA (Sczakiel and Pawlita, 1991). Finally, a number of reports have shown that when a high infective doses of virus is used to infect otherwise intracellular immune cells (when using a low virus infective doses), viral replication is not inhibited (Beachy, 1993)

Another explanation for incomplete viral interference is that not all cells, even though in many cases they are clonal cell lines, express the interfering RNA or protein. Federspiel et al. (1989), found that D17 cell lines (canine cells) expressing the reticuloedotheliosis virus (REV) envelope protein were 25,000-fold more resistant to REV infection than control cells. The authors found that the high degree of REV resistance was dependent upon the uniformity of expression of the REV envelope protein by the cells, but not dependent upon the overall expression level of the envelope protein by the cells. Other than the report by Federspiel et al. (1989), there have not been any reports characterizing the uniformity of expression of interfering RNA or proteins in animal cell culture.

Oligonucleotides complementary to VSV mRNA inhibit but do not completely abolish VSV replication. Further, the degree of inhibition appears temporal and is target dependent. Agris et al. (1986) found that oligodeoxyribonucleoside methylphosphonates complementary to VSV N, NS, and G mRNA reduced the titer of VSV in infected cells by 1.08, 0.52, and 1.18 logs, respectively, six hours post infection. However, by 24 hours post infection the oligonucleotides reduced VSV titers by a half that found at the 6 hour time point. Both Lemaitre et al. (1987) and Leonetti et al. (1988) found that poly(L-lysine)-conjugated oligodeoxyribonucleotides complementary to the initiation codon of the VSV N mRNA reduced VSV titers in infected cells by roughly 21/2 logs compared to a non-specific oligonucleotide. Both groups also found that oligonucleotides complementary to internal regions of the VSV N mRNA did not inhibit viral titers in VSV infected cells. Leonetti et al. (1988) also

found that an oligonucleotide complementary to a conserved intergenic sequence of the VSV viral genome inhibited viral titers in VSV infected cells by 2 logs.

# CHAPTER 3
# GENE EXPRESSION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FOLLOWING INTRAMUSCULAR INJECTION OF DNA

Eric D. Anderson, Dan V. Mourich, and Jo-Ann C. Leong.*
Department of Microbiology and the Center for Salmon Disease Research,
Oregon State University, Corvallis, Oregon 97331, U.S.A.

*Correspondence should be addressed to this author

(for submission to Molecular Marine Biology and Biotechnology)

This paper reports a portion of the work encompassed by a thesis submitted to Oregon State University, Department of Microbiology, in partial fulfillment of that required for the Ph. D. degree for Eric D. Anderson.

## Abstract

Expression of the firefly luciferease gene under the control of viral or fish promoters were observed in fish tissue after direct DNA injection of plasmid DNA. Plasmid DNA containing the firefly luciferase gene was injected into the skeletal muscle of rainbow trout (*Oncorhynchus mykiss*) and levels of luciferase activity were found to be dependent on the controlling promoter and the amount of injected DNA. Plasmids using the cytomegalovirus immediate early enhancer/promoter (CMV-IEP) consistently produced the highest levels of luciferase activity. Maximal activity was observed 5-7 days postinjection with 50 μg of DNA. This activity persisted in the tissues for as long as 115 days postinjection. When the DNA was examined up to two months postinjection, the predominant form was unreplicated, unintegrated DNA in linear or relaxed circular conformation. Expression of injected DNA was predominately found within muscle cells along the injection path and in scattered muscle cells anterior to the injection site.


## Introduction

Direct injection of DNA into the somatic tissues of animals is a powerful strategy for immunization (Ulmer et al., 1993; Xiang et al., 1991) and gene therapy (Raz et al., 1993). The technique also provides an attractive alternative to the creation of transgenic animals that have the transgene incorporated into the genome. Not only is direct injection technologically simple since it can be carried out with a hypodermic needle and syringe, but the biological effects of the gene transfer are relatively immediate. Expression of transgenes such as the cytokines IL-2, IL-4, and TGF-β1 (Raz et al., 1993); human dystrophin (Ascadi et al., 1991), the NP gene of influenza (Raz et al., 1994) and the reporter genes β-galactosidase, firefly luciferase, and chloramphenicol acetyl transferase (CAT) has been demonstrated in animals after direct DNA injection (Wolff et al., 1990). The technique bypasses the time consuming selection of transgene germline carriers over several generations and its potential for effecting measureable phenotypic changes in farmed animals being examined.

In fish, biologically active foreign genes have been delivered by electroporation and microinjection of developing embryos (for review, see Fletcher and Davies, 1991) and sperm (Symonds et al., 1994). These studies have demonstrated that both mammalian and fish promoters/enhancers are effective in promoting the expression of foreign genes in fish. Furthermore, the tissue specificity of expression in fish by these promoters is often similar to that observed in mammalian species (Michard-Vanhee et al., 1994). Direct injection of DNA in the muscle of tilapia fish (*Oreochromis niloticus*) has resulted in the expression of the reporter gene, chloramphenicol acetyl transferase (CAT), driven by the carp β-actin promoter (Rahman and Maclean, 1992). Maximal CAT activity was seen at 48 hours postinjection and declined thereafter, so that by 7 days postinjection, there was essentially no activity in the samples. In carp (*Cyprinus carpio*), expression was higher in small, faster growing fish than in larger fish (Hansen et al., 1991). All of these studies suggest that direct DNA injection in fish may provide a viable strategy for immunizing animals with specific viral and bacterial genes or providing animals with genes for growth enhancement or disease resistance.

As part of an effort to develop practical technologies for immunizing fish against viral pathogens, the parameters that govern gene expression by direct DNA injection in fish were examined. In this report, the firefly luciferase gene was used as the reporter gene for examining expression promoters/enhancers, sites of gene expression, duration and level of gene expression, and the persistence of the plasmid DNA in rainbow trout (*Oncorhynchus mykiss*). The information provided by the analysis will be important for future work in developing vaccines and other products for fish.

## Results

### Determination of the amount and volume of DNA for maximum gene expression

To determine the amount of injected DNA required for maximum luciferase activity, fish (50 g average weight) were injected with 10, 25, or 50 μg of DNA and luciferase activity was measured at 36 hours post injection (Figure 3.1). The promoter/enhancers examined included the glucocorticoid responsive mouse mammary

Figure 3.1 Luciferase activity with increasing quantities of injected DNA. The luciferase activity in muscle tissue from 50 g fish was determined at 36 h postinjection. Each bar represents the average of three fish and the standard error is shown for each data set indicated by the T bars. Four expression vectors were examined at 10, 25, and 50 μg of injected DNA. (A) Luciferase activity recovered from fish injected with pMMTVLuc, pFV4EAL , or PBS. (B) Luciferase activity recovered from fish injected with pCMV4EAL or pCMVL .

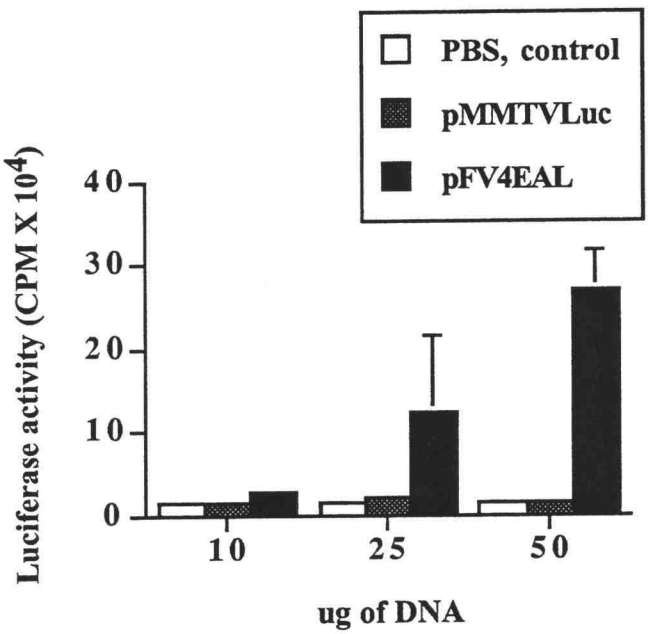


Figure 3.1 a

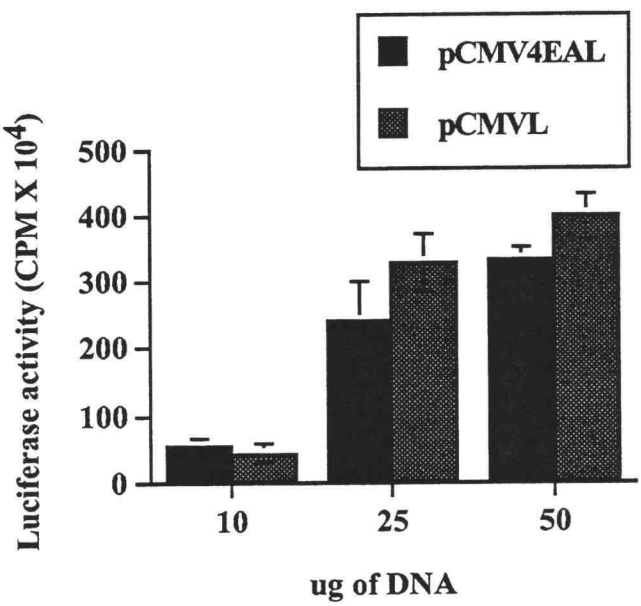


Figure 3.1 b

tumor virus (MMTV) promoter/enhancer, the CMV-IEP + translational enhancer, CMV-IEP enhancer alone, and the carp β-actin promoter. At 25 μg of injected DNA, the maximum luciferase activity was observed for all of the test DNAs. No substantial increase in activity was observed when the amount of DNA injected was increased two-fold to 50 μg.

Tissues from fish injected with the pMMTV-Luc plasmid did not contain much luciferase activity (15,000-19,000 cpm). However, the other plasmids, particularly pCMVL, promoted the expression of large amounts of luciferase activity with 3.2 million relative light units (RLU) at 25 μg DNA and 3.9 million RLU at 50 μg DNA. The fish promoter/enhancer, FV4, for the carp β-actin gene produced intermediate luciferase activity at 124,000 RLU for 25 μg DNA and 269,000 RLU at 50 μg DNA. Luciferase expression from pMMTV was not detectable after 36 hours postinjection.

The effect of the volume of the injected material on the reproducibility of each assay was also examined (Table 3.1). In this study, fish at 1 and 50 g were injected with 25 μg of DNA in 100 and 200 μl volumes. For the fish that received 25 μg of DNA in 100 ul of solution, there was considerable variability in luciferase activity/mg fish tissue. When the volume was increased to 200 μl, reproducible measurements in luciferase activity/mg fish tissue were observed. Thus, the standard conditions for DNA injections was 25 μg of DNA/200 μl of PBS.

Table 3.1 Analysis of gene expression as a function of the volume of injected material. Luciferase activity in 1g and 50 g fish injected with 100 μl or 200 μl of PBS containing 25 μg pCMVL. Luciferase activity was measured 48 hrs post injection. The RLU is the mean of luciferase activity recovered from five individual fish for each measurement.

| | | Luciferase activity | |
|---|---|---|---|
| Weight (g) | Volume (μl) | RLU x $10^4$ | Standard Error x $10^4$ |
| 50 | 100 | 1710 | 579 |
| 50 | 200 | 1207 | 119 |
| 1 | 100 | 1466 | 446 |
| 1 | 200 | 1318 | 44 |

Comparison of the different promoters/enhancers in fish

A comparison of the strengths of the different promoter/enhancers to express luciferase activity in fish was made with 25 μg of DNA injected into 50 g fish.. The luciferase activity was examined at 1, 2, 3, 5, and 7 days postinjection (Figure 3.2). Luciferase activity was observed for all of the plasmid constructs except in pMMTV-luc injected fish which showed low levels of luciferase activity only after seven days. The fish injected with pCMVL and pFV4EAL produced maximum luciferase activity after five days and fish injected with pCMV4EAL showed maximum expression at seven days postinjection. On day 7, luciferase activity for the pCMV4EAL and pCMVL injected fish was approximately 4500-fold greater than the activity found in the tissues of the pFV4EAL injected fish.

The function of the pMMTV-luc plasmid was tested in fish tissue culture cells in transient expression assays. Luciferase activity was increased five-fold over background by dexamethasone treatment (data not shown). When pCMVEAL and pCMVL were tested in transient expression assays in fish tissue culture cells, these plasmids produced a thousand-fold greater luciferase activity than the pMMTV-Luc-transfected cells after dexamethasone induction. Thus, the low expression of pMMTVLuc in vitro, is correlated with the low expression of luciferase produced by pMMTV-Luc in fish skeletal muscle tissue in vivo.

Duration of luciferase activity in DNA-injected fish

Previous reports on the injection of DNA into tilapia suggested that the DNA may be expressed only for 48 hours (Rhaman et al. 1992). If genetic immunization and gene therapy are to be used successfully in fish, the gene must be expressed at relatively high levels for a reasonably prolonged period. Injected fish were monitored for luciferase activity for up to 63 days postinjection (Fig. 3.3) in 50 g fish injected with 25 μg of pCMV4EAL DNA (Figure 3.3 a) and up to 115 days postinjection in 1 g fish injected with 10 μg of the DNA (Figure 3.3 b). Maximum luciferase activity was reached by day 7 for both groups. In the 50 g fish, the activity remained near 80% of this maximum throughout the experiment.

Figure 3.2 Promoter comparison. Fish at 50 g size were injected with 25 μg of plasmid DNA in 200 μl PBS and assayed for luciferase expression 1, 2, 3, 5, and 7 days post injection. The luciferase activity is shown in RLU and is the mean of the activity obtained for three different fish. The standard error is represented by the T bars. (A) Luciferase activity recovered from fish injected with pMMTV, pFV4EAL, or PBS. (B) Luciferase activity recovered from fish injected with pCMV4EAL or pCMVL

Figure 3.3 Duration of expression of luciferase activity. (A) Fish at 50 g size were injected with 25 μg of pCMVEAL DNA in 200 μl PBS. Individual fish were sampled at the indicated days and the luciferase activity in each sample was determined. (B) Fish at 1 g size were injected with 10 μg of DNA in 200 μl PBS and individual fish were sampled to 115 days post injection. For each measurement 3-5 fish were assayed, the standard error is indicated by the T bars.

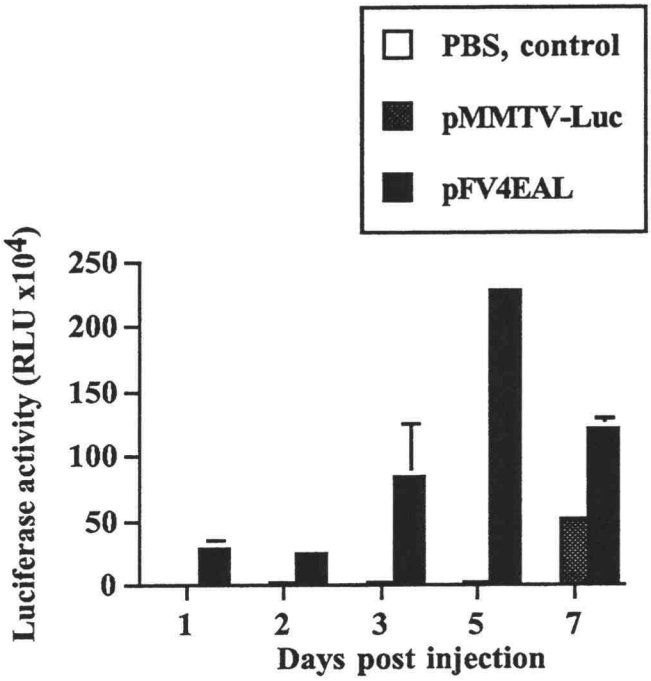


Figure 3.2 a

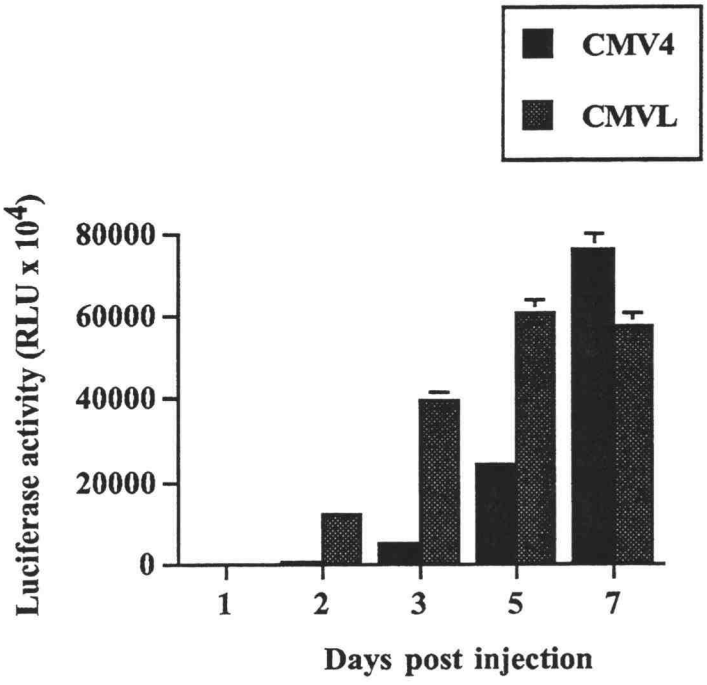


Figure 3.2 b

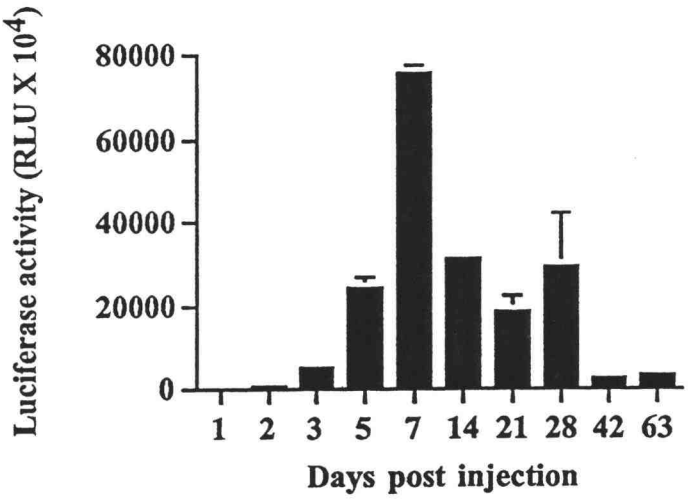


**Figure 3.3 a**

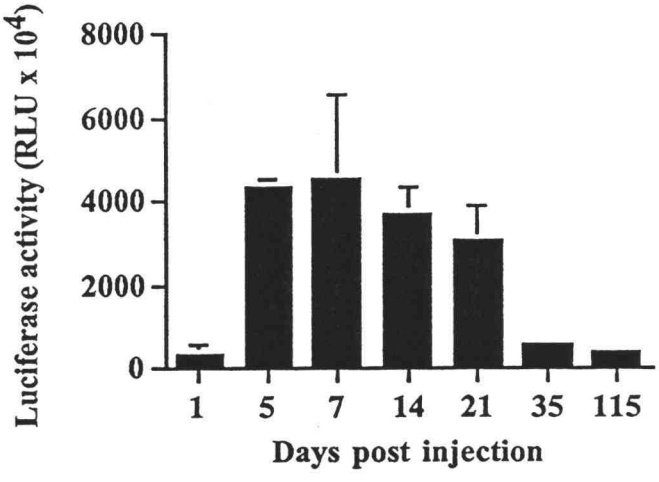


**Figure 3.3 b**

In the study with small fish (1 g), luciferase activity declined over time, and by 15 weeks postinjection luciferase activity had diminished to approximately 10% of the maximum. Although the luciferase activity was comparatively low by day 115, it was still approximately one hundred-fold above background. It is unclear whether the DNA was slowly degraded over time or whether the growth of the tissue at the injection was "diluting" the DNA expression/μg tissue.

Southern blotting and hybridization

The fate of the injected DNA was examined in 50 g fish injected with pCMV4EAL by southern blot analysis. DNA was extracted from fish tissue and digested with the restriction endonuclease HindIII. This enzyme cuts at a single site in the luciferase gene of pCMV4EAL to yield a DNA band of 6.7 kb. When the Southern blots were developed with a radioactively labeled probe to the luciferase gene, a hybridization band at 6.7 kb was observed in samples taken from the injected fish at 2, 14, 28, and 63 days postinjection ( Figure 3.4 a) . Undigested DNA was also analyzed and migrated as linear and relaxed circular DNA on days 2, 14 , and 28 days postinjection (Figure 3.4 b). At 63 days postinjection, the DNA migrated as a single band of approximately 7.0 kb . This change in mobility was more than likely due to differences in the salt concentration of the DNA solution since similar changes in migration patterns can be reproduced by altering the salt concentration of the plasmid DNA solution.

To assess the possibility that the change in DNA at day 63 was the result of DNA replication and/or integration, the DNA taken on day 28 and 63 was digested with HindIII followed by digestion with restriction enzyme isoschizomers DpnI or MboI (Figure 3.4 c). These enzymes recognize the sequence -GATC- and differ in their ability to cut methylated DNA (Lavitrano et al., 1989). Plasmid DNA prepared in *Escherichia coli*, DH5α, is methylated at the N6 position of adenine. The restriction enzyme DpnI requires N6-methylated adenine for activity and the enzyme MboI will not cut DNA with methylated adenines. A Southern blot hybridization of the HindIII-digested DNA showed that the injected DNA was insensitive to MboI digestion and senstivie to DpnI digestion. Thus, it appears that the injected DNA remains in the tissue as unreplicated, unintegrated DNA.

Figure 3.4 Southern blot analysis of DNA isolated from muscle of fish injected with pCMVEAL DNA 2, 14, 28, and 63 days post injection. (A) Approximatley 15 μg of DNA injected muscle was linearized with Hind 111. The linearized DNA comigrates with control plasmid DNA at 6.7 kb. (B) Approximatley 15 μg of undigested DNA was analyzed. Open circular (OC, 7.2 kb), linear (L, 6.7 kb), and supercoiled (SC, 2.3 kb) DNA were identified. (C) Approximatley 15 μg of DNA injected muscle was linearized with Hind 111. The linearized DNA was then digested with Dpn 1 or Mbo1 indicated by the (+) symbol or not digested which is indicated by the (-) symbol.

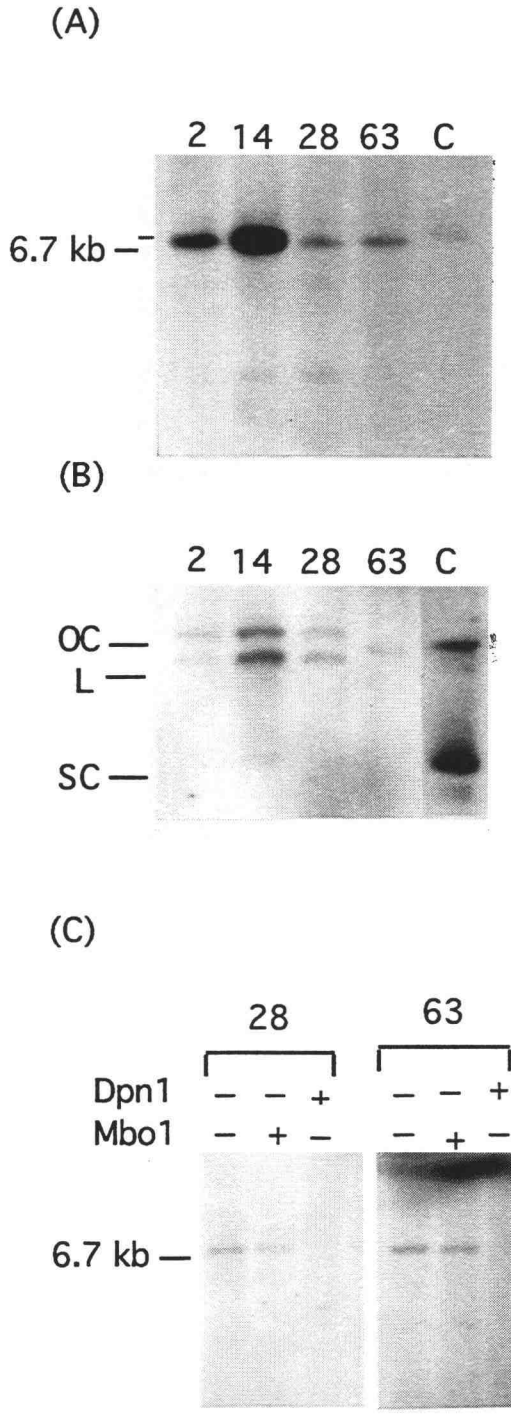


Figure 3.4

Sites of DNA expression

The sites of expression for the injected DNA were determined at 3-5 days postinjection when maximum luciferase acitivity was observed. In these studies, the reported gene, β-galactosidase, was used in the plasmid construct, pCMV-βgal, and this plasmid was injected into the fish muscle tissue. Histochemical analysis of β-galactosidase expression revealed that the active enzyme was synthesized in 1 g fish along the path of injection and in muscle cells at a distance of 5 mm anterior to the path of injection. Because there was a significant amount of β-galactosidase expression throughout the digestive tract in control fish, further analyses were performed using luciferase as the reporter gene.

In 50 g fish luciferase activity remained localized to muscle tissue. In 1 gram fish luciferase activity was recovered from numerous tissues (Figure 3.5 a and 3.5 b). The highest level of luciferase activity in the 1 gram fish was recovered from skeletal muscle along the path of injection. There was also luciferase activity recovered from anterior and posterior kidney, muscle (taken 5 mm from and on the opposite side of the path of injection), heart, liver, spleen, and gill. The luciferase activity in these tissues was variable and was roughly 1000-fold less than that obtained from muscle tissue along the path of injection.

## Discussion

A study of a number of the parameters that govern gene expression following the intramuscular injection of DNA into rainbow trout was conducted to determine the feasibility of using this technology for genetic immunization and gene therapy in fish. The results of this study show that the luciferase gene under the control of CMV-IEP is expressed at high levels for extended periods of time after direct DNA injection into the skeletal muscle of rainbow trout. Gene expression occurs in the cells of skeletal muscle and several other organs and the injected DNA persists as an unintegrated, non-replicated plasmid DNA.

The level of gene expression following the direct injection of DNA was found to be dependent on the the quantity of the injected DNA and the promoter used to drive the foreign gene expression. This finding is consistent with other reports of

Figure 3.5 Sites of gene expression. Fish (1 g) injected with 10 μg of pCMV4EAL in 200 μl of PBS. Tissue from five individual fish were assayed for luciferase activity 5 days post injection. The luciferase activity is reported as RLU-0.1 mg/ tissue. (A) Luciferase activity recovered from the cardiac stomach (c. stomach), pyloric stomach (p. stomach), large intesitine (L. intestine), small intesitne (s. intestine), muscle (distant from the sight of injection), kidney, heart, spleen, liver, and gill. (B) Luciferase activity recovered from muscle tissue along the path of injection.

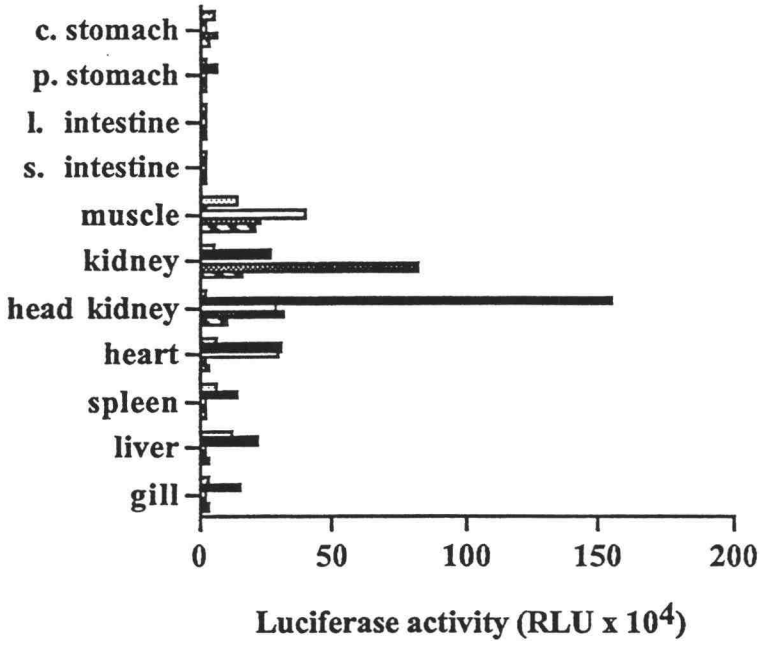


Figure 3.5 a

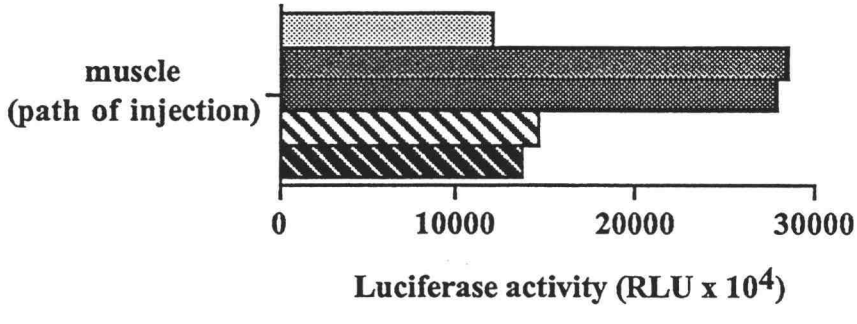


Figure 3.5 b

direct introduction of DNA either by injection in mice (Wolff et al., 1990) and carp (Hansen et al., 1991) or particle bombardment of mice (Cheng et al., 1993). Rainbow trout injected with 10, 25 or 50 μg of the pCMV4EAL produced luciferase activity that reached near maximum activity at 25 μg with a slight increase (1.2-fold increase) when 50 μg of plasmid was injected. Also, like the mammalian studies, rainbow trout cells responded to different promoter/enhancers with differing levels of expression. The MMTV promoter was not a strong promoter in trout tissue and similarly, it is not a strong promoter in mice (Yang et al., 1990). Thus, the direct injection of DNA into fish should provide a powerful and simple approach for functional analysis of untested promoters prior to the creation of transgenic animals.

In carp, the transient expression of the CAT gene after direct DNA injection has been shown to be dependent upon the size of injected fish (Hansen et al., 1991). Expression levels were higher in small, faster growing fish (10 cm in length) compared to larger, slower growing fish (20 cm in length). This was not the case for luciferase expression in rainbow trout where very little difference was observed between the levels of luciferase activity/μg tissue in 1 or 50 g rainbow trout injected with 25 μg of DNA. A significant variability in luciferase expression was observed in fish that were injected with the same amount of DNA in different volumes. Fish that received the larger volume (200 μl) as opposed to the smaller volume (100 μl) showed less variability in expression. Presumably, the larger volume produces changes in tissue turgor and osmotic environment that might lead to greater tissue damage and better uptake of DNA (Davis et al., 1993). Moreover, the larger volumes may disperse the DNA throughout the body cavity.

If genetic immunzation, and particularly gene therapies for growth enhancement and induction of maturation, are going to be developed succesfully for fish, the expression of the introduced DNA must be relatively long-lived. In rainbow trout, luciferase activity was detected for at least 115 days postinjection. The possibility that the luciferase protein or mRNA is stable and is, therefore, responsible for the prolonged activity seems unlikely. The half-life of luciferase has been estimated to be less than 24 h in murine skeletal muscle cells (Wolff et al., 1990). In contrast, the duration of CAT activity from DNA injected in tilapia was limited to a few days (Rhaman et al., 1992). In mice, DNA expression can last as long as several months following injection or intraveneous administration of DNA/lipid complexes (Wolff et al., 1990; Zhu et al., 1993), and 1.5 years following ballistic bombardment (Cheng et al. 1993). The long-lived expression of reporter genes is remarkable since muscle cells expressing foreign proteins should be recognized and attacked in an MHC

Class I restricted manner. This should result in the eventual recognition and elimination of transfected muscle cells by responding lymphocytes (Xiang et al., 1993). Apparently this does not happen and leads one to speculate that not all proteins expressed following DNA injection will be immunogenic, or alternatively, that the predominant cells, muscle cells, which express the reporter gene may not elicit a strong immune response. This long-lived expression also suggests that direct DNA injection with reasonable booster schemes could be used for genes encoding pathogen resistance and growth enhancers (Raz et al., 1991), genes that require continuous production for effectiveness. Also, because the DNA persists, promoters sensitive to stress ( hormone inducible promoters; Kitis et al., 1991) or environmental contaminants (heavy metal inducible promoters) (Cheng et al., 1993) may be used for the analysis of inducible gene expression.

Because a portion of the injected DNA must ultimately find its way into the nucleus, there is the possibility of DNA integration into the host genome. The studies reported here confirm the work of others (Wolff et al., 1990; Zhu et al., 1993), that most of the DNA is not integrated or replicated in vivo. The injected DNA appears to be stable; there was very little plasmid degradation that was detected by Southern blotting. This result is unlike the findings in mice where degradation of the DNA begins within a few hours of injection (Wolff et al., 1991). In this report, the blotting detection limit was approximately 1340 copies of pCMV4EAL. This limit was determined by estimating a detect limit of 1 pg of homologous DNA in 10 μg of genomic DNA. Within these detection limits, we were unable to detect replicated or integrated plasmid DNA.

The sites of DNA expression following intramuscular DNA injection were determined in small (1 g) and large fish (50 g). In the large fish, luciferase activity was confined exclusively to the muscle tissue along or near the path of injection. In the smaller fish, there was detectable expression in numerous tissue of some of the fish. The gene expression in the different tissues was confirmed by histochemical identification of β-galactosidase expressing cells. This result may be explained by the fact that an injection volume as small as 100 μl caused a significant extension of the peritoneal cavity and dorsal fin in the small fish. Thus, the injected DNA may have been distributed throughout the entire fish. The expression of DNA in small fish at a distance from the injection site demonstrates that needle damage is not the only method of DNA entry into cells. A similar finding has been reported for rats injected intraperitoneally with calcium phospate-precipitated DNA (Benvenistry and Reshef,

1986). If the rats were injected with naked DNA, degradation of DNA and accumulation of the degraded DNA in the bladder was observed.

In summary, a study of the parameters which govern gene expression following the intramuscular injection of DNA into rainbow trout was conducted. The results suggest that genetic immunization and gene therapy should be feasible in fish. The direct injection of DNA could be used as an alternative to the production of transgenic fish.

## Material and Methods

### Rearing of Fish

Specific pathogen-free rainbow trout (Shasta strain) at 0.5 g were provided by the Center for Marine and Freshwater Biomedical Research, Food Toxicology and Nutrition Laboratory, at Oregon State University. The fish were reared at the Center for Salmon Disease Research, Salmon Disease Laboratory, in well water at 12° C at a flow rate of 0.5 gal/min. All fish were acclimated at the site for at least one week before any experimental procedures were conducted on the animals.

### Construction of the Expression Plasmids

Four vectors which expressed the firefly luciferase gene (de Wet et.al., 1987) under the regulatory control of one of three mammalian viral promoters or one fish promoter were used in the study. The plasmid, pMMTV-Luc (Clontech, Inc.), contained the luciferase gene under the control of the glucocorticoid responsive mouse mammary tumor virus promoter (Lee et al., 1981). The entire luciferase gene in this plasmid is flanked on the 5' and 3' ends by SmaI restriction enzyme sites. The plasmid, pCMV4EAL, contained the cytomegalovirus immediate early promoter (CMV-IEP) and a translational enhancer fused to the luciferase gene. This plasmid was made by inserting the entire 1.9 kb luciferase SmaI fragment from MMTV-Luc, into the SmaI site of pCMV4 (Andersson et.al., 1989). The plasmid, pCMVL,

contained the CMV-IEP fused to the luciferase gene (P.D.L. Gibbs, 1992). The plasmid, pFV4EAL, contained the carp β-actin promoter fused to the luc gene and was made by inserting the 1.9 kb luc gene Sma1 fragment from MMTV-Luc into the SmaI site of pFV4 (Liu et.al., 1990). In addition, pCMVβgal, which contains the CMV-IEP fused to the β-galactosidase gene was created by inserting the HindIII/BamHI β-galactosidase gene from pSV-β-galactosidase (Promega) into the HindIII/BamHI site of pCDNA-3 (Invitrogen).

Preparation of DNA and DNA delivery

The quantity of plasmid DNA that had been purified by affinity chromatography (Qiagen, Inc. Chatsworth, Calif.) was determined by spectrophotometric measurements at 260 and 280 nm. The DNA in aliquots equivalent to 10, 25, and 50 μg was suspended in 100 or 200 μls of phosphate buffered saline solution ( PBS, 0.14M NaCl, 10mM sodium phosphate, pH 7.4). For DNA delivery, a 27-gauge needle and a 1-ml tuberculin syringe were used. Fish were anesthetized by immersion in 100 μg/ml concentration of tricaine methane sulfonate (MS-222; Argent Chemical Laboratories) for 1-2 min. and then the DNA was injected immediately rostro-ventral to the dorsal fin at a $30^{o}$ degree angle to a depth of 38 mm. Great care was taken to ensure that each fish was injected identically. To eliminate the possible variability due to DNA preparation (Wolff et al., 1991) each experiment was performed with the same preparation of plasmid DNA.

Luciferase assays

Luciferase assays were performed as described for the enhanced luciferase detection system manufactured by Analytical Luminescence Laboratory. The entire fish, in the case of 1 g fish, or individual dissected tissues from 5 g fish were homogenized in 5 volumes of lysis buffer (wt/vol) using a polytron homogenizer (Brinkman) at 13,000 rpm for 20 seconds. For 50 gram fish, 5 g muscle plugs were carefully dissected from the injection site, the skin was removed and discarded, and the tissue was homogenized as previously described. The homogenates were clarified

by centrifugation at 10,000 x g for 10 min. Aliquots of 10 µl of the supernatant material were diluted in lysis buffer and luciferase activity was measured in a Beckman LS 8000 liquid scintillation counter on single photon mode (Nguyen et al., 1988). The resultant counts per minute (cpm) were integrated over a 30 seconds period. Luciferase activity is reported as the mean relative light units (RLU) in cpm/10 µl for three fish. Standardized assays containing 0.01and 1 pg of purified luciferase routinely measured 70,000 cpm (three-fold above background) and 5 million cpm respectively. The maximum reliable count was considered 5 million cpm. Samples from non-injected control fish were usually measured at 15,000 to 20,000 cpm and all measurements below 40,000 cpm (two-fold above background) were considered to be negative for luciferase activity. In this study, cpm is actually a measure of relative light units or RLU since all assays were performed with samples containing 1 mg/ml of tissue unless otherwise indicated.

Southern blotting and hybridization

DNA was extracted from the tissues as described (Maniatis et.al., 1982). Restriction digestions of 10 µg of genomic DNA from mock or DNA-injected fish were carried out overnight and the digested DNAs were electrophoresed in a 0.8% agarose gel. The DNA samples were transferred to nytran membranes (Shleicher and Shuell) according to the manufacturer's recommendations. The 1.9 kb luciferase gene was labeled with $^{32}$P-dCTP (3000 Ci/mmole) to 1-2 x $10^9$ cpm/µg by random priming (Amersham ). Unincorporated nucleotides were removed by chromatographic separation in a G-50 spin column (Boehringer Mannheim). The membranes were prehybridized at 42° C in 5X SSPE (1X SSPE = 150mM NaCl, 10mM $NaH_2PO_4$, 1mM EDTA, pH7.4), 1% SDS, 50% formamide and 1.5% DNA blocking reagent (Boehringer Mannheim) for 1 hr. The probe was then added and hybridization was carried out at 42° C overnight. The membrane was washed in buffer with the highest stringency for 30 minutes in 0.1X SSPE, 1.5% SDS, at 68° C. The membrane was wrapped in polyethylene film before exposure to radiographic film (Hyperfilm-MP, Amersham) at -70° C for 24-48 hours.

Histochemistry

Fish at 0.5- 1 gram were injected with 10 μg of pCMVβgal. At 3-5 days post injection the fish were killed and immersed in 5% formalin, 1% glutaraldeyde for one hr. The fish were rinsed in distilled water for 10 min and then incubated in X-gal solution (0.2% X-gal chromagen, 10mM sodium phosphate, 150mM NaCl, 1mM $MgCl_2$, 3.3mM $K_4Fe(CN)6.3H_2O$, and 3.3mM $K_3Fe(CN)_6$, pH7.0) at 37° C for 16 hours. The fish were rinsed in water for several hours and processed as previously described (Drolet et.al., 1993). Sections of 6 μm were taken from the embedded fish, counterstained briefly in eosin, and examined microscopically for cells containing blue precipitate as an indicator of β-galactosidase expression.

## Acknowledgments

Pinwen Chiou provided the vector construct pCMVβgal. This work was supported by United States Department of Agriculture Grants 91-34123-6124 and 92-37204-7997; and Oregon Sea Grant with fund from the National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerece, under grant no. NA89AA-D-SG108, Project no. R/FSD-16.

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# CHAPTER 4
# GENETIC IMMUNIZATION OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AGAINST INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

Eric D. Anderson[1], Dan V. Mourich[1], S. Fahrenkrug[2] and Jo-Ann C. Leong
[1]Department of Microbiology and the Center for Salmon Disease Research,
Oregon State University, Corvallis, Oregon 97331, U.S.A.
2 Department of Microbiology, University of Minnesota,
St. Paul, Minnesota 55108, U.S.A.

*Correspondence should be addressed to this author

(for submission to Molecular Marine Biology and Biotechnology)

This paper reports a portion of the work encompassed by a thesis submitted to Oregon State University, Department of Microbiology, in partial fulfillment of that required for the Ph. D. degree for Eric D. Anderson.

## Abstract

Plasmid vectors encoding the infectious hematopoietic necrosis virus (IHNV) nucleoprotein gene or the glycoprotein gene were tested for their ability to immunize rainbow trout (*Oncorhynchus mykiss*) against IHNV. The plasmid DNA was injected into the skeletal muscle of 1 g rainbow trout and the specificity of virus-specific antibodies and protection against an IHNV challenge were measured. Fish injected with the glycoprotein encoding plasmid either alone or in combination with the nucleoprotein encoding plasmid generated a glycoprotein-specific antibody response and were protected from a subsequent IHNV challenge. Injection of fish with the nucleoprotein encoding plasmid did not promote a measurable virus-specific antibody response, nor did it immunize fish against IHNV.


## Introduction

The rhabdovirus, IHNV, is a pathogen of salmon and trout that are native to the countries of the North Pacific Rim (reviewed in Wolff, 1988). The severity, prevalence, and ultimately, the economic cost of IHN disease has prompted substantial efforts to develop an effective and safe IHNV vaccine (reviewed in Leong and Fryer, 1993). The effort has led to the development of attenuated (Fryer et al., 1976; Tebbit, 1976), killed (Amend, 1976; Nishimura et al., 1985), and subunit IHNV vaccines (Gilmore et al., 1988; Oberg et al., 1991). These vaccines have all been successful under different immunization regimens in protecting fish against IHNV. Indeed, each vaccine has provided valuable information regarding the fish immune response and the replication and pathogenesis of IHNV. These successes, however, have not resulted in a commercially viable IHNV vaccine because questions have been raised regarding the safety and preparation costs in the case of the attenuated and killed vaccine, and consistent efficacy in the case of the subunit vaccine (Leong and Fryer, 1993)

One of the newest approaches in vaccine design is the use of DNA vaccines. This approach, genetic immunization, is based upon the finding that naked plasmid DNA when injected into skeletal muscle cells is able to express its encoded protein(s) (Wolff et al., 1990). The protein antigen can stimulate a specific immune response

composed of cytotoxic T cells, T-helper cells, and antibodies (Cox et al., 1993; Fynan et al., 1993; Ulmer et al., 1993; Yankauckas et al., 1993; Wang et al., 1993; Xiang et al., 1994; Raz et al., 1994; Segedah et al., 1994). These DNA vaccines have been successful in immunizing animals against influenza virus (Ulmer et al., 1993; Fynan et al., 1993; Montgomery et al., 1993; Raz et al., 1994), rabies virus (Xiang et al., 1993), bovine herpesvirus 1 (Cox et al., 1993), and malaria (Segedah et al., 1994). With the DNA vaccines as well, there is no longer a need to purify the pathogen or immunoprotective antigen for vaccination and there is no possibility of reversion to virulence since the DNA encodes a single viral protein.

In this study, an effort was made to develop a DNA vaccine to IHNV. Initial experiments had shown that rainbow trout will express firefly luciferase and b-galactosidase from naked DNA injected into the skeletal muscle cells (Anderson et al., submitted). The route of injection, the appropriate promoter, the optimal dose of DNA required for expression, and the duration of gene expression had been determined with plasmid DNA containing the firefly luciferase under the control of the cytomegalovirus immediate early promoter (CMV-IEP). The experiments described in this paper demonstrate that plasmid DNA containing the IHNV glycoprotein (G) gene downstream of the CMV-IEP will induce an immune response in fish. Fish injected with plasmid DNA encoding the IHNV G protein or with a combination of plasmid DNAs encoding G and N (nucleoprotein) protein, produced a strong protective immune response to subsequent challenge with a lethal dose of IHNV.

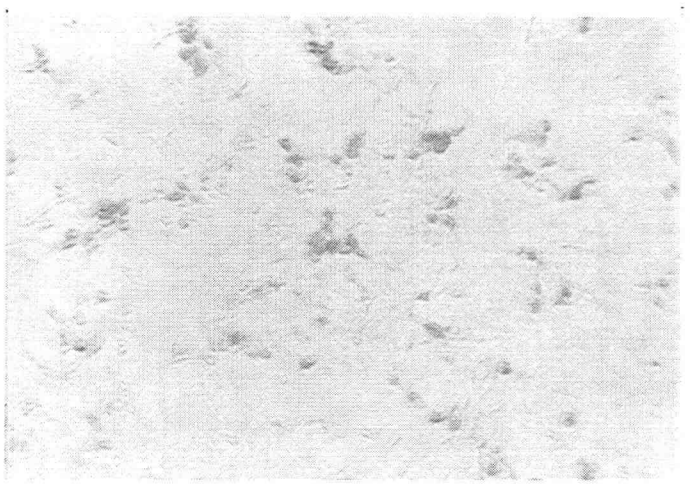
## Results

### Expression of the IHNV nucleoprotein and glycoprotein in transfected cells

Expression of the IHNV G protein and N protein by the plasmids pCMV4-G or pCMV4-N, respectively, was confirmed by immunohistochemical staining of EPC cells transiently transfected with the plasmid DNA (Figure 4.1). The cells were probed 48 hr post transfection with an anti-IHNV G (3GH136J) or N (1NDW14D) specific monoclonal antibody and subsequently stained chemically. The pCMV4-G and pCMV4-N transfected cells reacted specifically with the G or N specific

Figure 4.1 Expression of the IHNV G and N protein in pCMV4-G and pCMV4-N transfected cells. EPC cells were transfected with pCMV4-G (A) or pCMV4-N (B) and then probed with a G-specific (3GH136J) or N-specific (1NDW14D) monoclonal antibody, respectively. The cells expressing the IHNV proteins were subsequently identified using alkaline phosphatase immunocytochemical staining which stains the cells black.

(A)



(B)

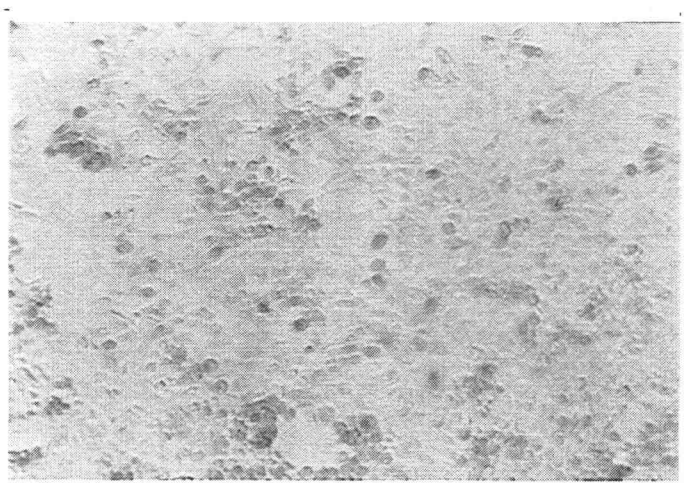


Figure 4.1

antibody, respectively. Both the pCMV4-G and pCMV-N transfected cells were moderately to intensely stained throughout the cytoplasm. In addition, when the pCMV4-G transfected cells were briefly reacted with the color substrate, staining was primarily seen at the cell surface. Thus, both pCMV4-G and pCMV4-N are functional and should direct IHNV protein synthesis in fish skeletal muscle cells transfected by direct injection.

Humoral immune response

IHNV specific antibody activity in serum collected from fish injected with plasmid DNA was measured using an ELISA (Figure 4.2). Fish injected with a combination of pCMV4-G and pCMV4-N first produced measurable antibody activity 4 weeks post injection, the activity peaked at 8 weeks, and remained high throughout the 4-14 week test period. Fish injected with pCMV4-G followed a similar trend as the co-injected fish except that the first measurable anti-IHNV antibody activity was recovered 6 weeks post injection. At peak antibody activity, 8 weeks post injection, the co-injected fish antisera contained 26-fold greater anti-IHNV antibody activity than did the pCMV4-G injected fish antisera. There was not any anti-IHNV antibodies at any point in the experiment when antisera from pCMV4-N injected fish was tested using the ELISA

To obtain enough antisera to perform the ELISA, antiserum was pooled from five vaccinated fish. Thus, it is possible that the time of appearance and intensity of the antibody response was proportional to the number of fish which had seroconverted or, alternatively, that the results were a reflection of the humoral response of individual fish. Whichever is the case, the fish co-injected with pCMV4-N and pCMV4-G plasmids produced measurable anti-IHNV antibodies earlier and more abundantly than the fish injected with pCMV4-G alone. This indicates that pCMV4-N was involved in enhancing the anti-IHNV antibody activity in co-injected fish or, alternatively that the results were due to the limited sample population. However, the enhancing effect of pCMV4-N plasmid was also observed when 350 g rainbow trout were co-injected with pCMV4-G and pCMV4-N (unpublished results).

The specificity of the immune response was measured by Western blot analysis (Figure 4.3) A 1/ 100 dilution of the antiserum collected from the fish co-injected with pCMV4-N and pCMV4-G or with a single injection with pCMV4-G

Figure 4.2 Analysis of anti-IHNV antibody activity recovered from fish injected with plasmid DNA by an ELISA. The IHNV specific antibody response in fish injected with PBS, pCMV4-Luc, pCMV4-G, pCMV4-N, and pCMV4-G + pCMV4-N was determined 4, 6, 8, 12 and 14 weeks post injection. The antibody activity is expressed as the log of realative units (RU) of antibody activity. The RU was calculated by comparison of antiserum collected from a mature fish which had survived a non-lethal experimental infection with IHNV

Figure 4.3 Specificity of anti-IHNV antibody activity recovered from fish injected with plasmid DNA by Western blotting. IHNV (20 μg/lane) was probed with a 1/100 dilution of antiserum recovered from fish injected with: lane 1, pCMV4-G (8 weeks); lane 2, pCMV4-G and pCMV4-N (6 weeks); lane 3, pCMV4-G and pCMV4-N (8 weeks); lane 4, positive control rainbow trout antisera; lane 5, molecular weight markers; lane 6, pCMV4-N (8 weeks); lane 7, pCMV4-Luc (8 weeks); lane 8, PBS (8 weeks). The molecular weight for the purified IHNV is: G 67 kd; N 42 kd; M1 25 kd; M2 21 kd.

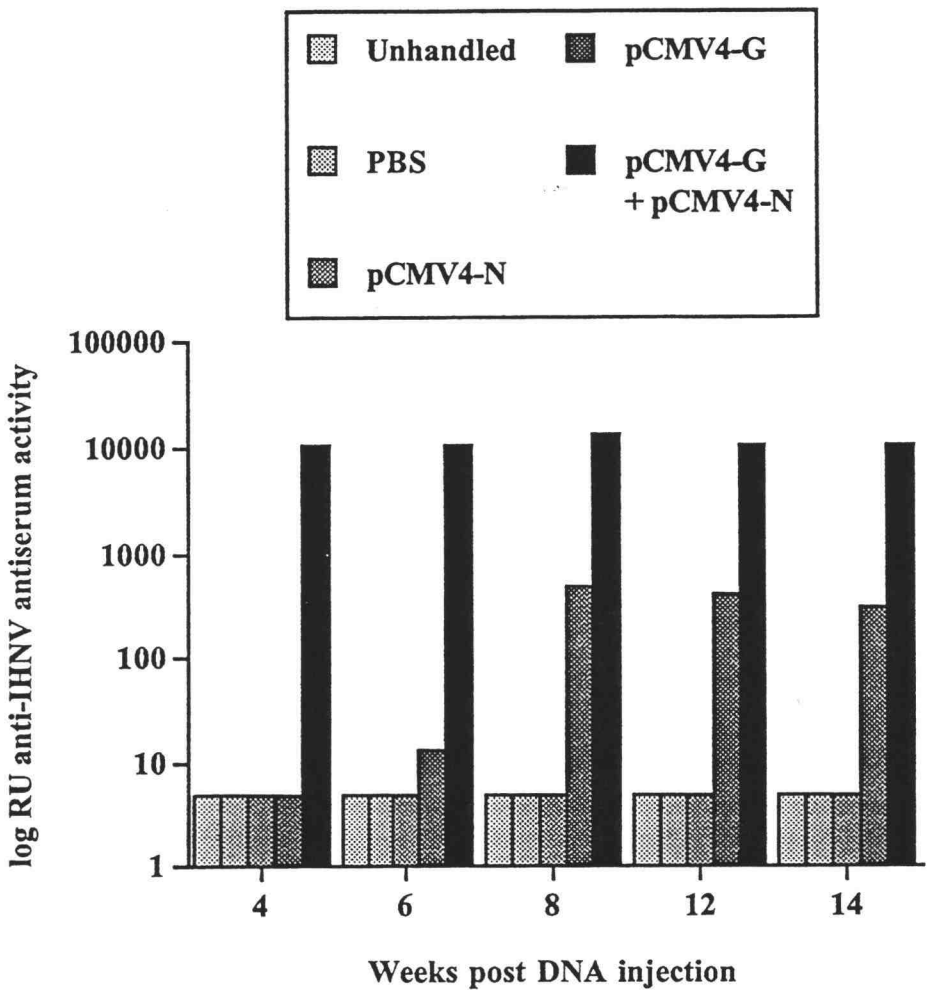

Unhandled
pCMV4-G
PBS
pCMV4-G
+ pCMV4-N
pCMV4-N
log RU anti-IHNV antiserum activity
100000
10000
1000
100
10
1
4
6
8
12
14
Weeks post DNA injection

**Figure 4.2**

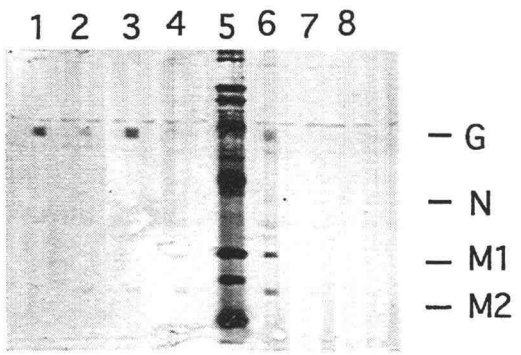



Figure 4.3

was specific to the IHNV G (20 μg/ lane of purified IHNV). There was no detectable anti-N antibodies in serum collected from pCMV4-N or from the co-injected fish.

Immunization of fish with DNA against IHNV

Fish immunized with the pCMV4-N and pCMV4-G together or pCMV4-G alone were significantly protected against a subsequent challenge with $10^8$ PFU / 2L (Rangen isolate) (Figure 4.4 and Table 4.1). This dose of virus killed 65-75% of the pCMV4-N, pCMV4EAL, and PBS injected or unhandled fish during the 30 day assay period (Fig. 4.4). The survival of fish injected with pCMV4-G or pCMV4-N + pCMV4-G was 86% (Table 4.1).

## Discussion

In this study we developed an IHNV DNA vaccines composed of either the IHNV G gene or the N gene. The vaccines were tested for their ability to immunize rainbow trout against the virus and produce an antibody response in vaccinated rainbow trout. The design of the pCMV4-G vaccine was based upon the finding that the purified IHNV glycoprotein is the only viral protein capable of neutralizing IHNV (Engelking and Leong, 1989). In addition, vaccination with the glycoprotein can immunize fish against all five IHNV electropherotypes (Engelking et al., 1991). The pCMV4-N vaccine was included because it has been shown to augment the efficacy of a subunit G vaccine (Oberg et al., 1991). Further, the rabies virus (RV) N protein is able to immunize mice and dogs against RV, though it is not as effective as the RV G protein (Fu et al., 1991; Sumner et al., 1991; Fekadu et al., 1992). While side by side trials were not performed in this study, the efficacy of the pCMV4-G vaccine administered alone or in conjunction with the pCMV4-N vaccine compares favorably with the attenuated, killed, and subunit IHNV vaccines previously reported (reviewed in Leong et al., 1988).

Fish injected with pCMV4-G vaccine generated both anti-G specific antibodies and were protected from a subsequent challenge with IHNV. This is further confirmation that the IHNV glycoprotein alone can confer significant

Table 4.1 Survival of fish injected with plasmid DNA and subsequently challenged with IHNV. Fish of 1 g size were injected or unhandled as indicated. Six weeks post-injection the fish were challenged with $10^4$ PFU/L of IHNV (Rangen isolate). The challenge was conducted for 30 days.

| Treatment | Number of fish/group | Total number of deaths | Percent Survival |
|---|---|---|---|
| Unhandled | 60 | 39 | 35 |
| PBS | 60 | 40 | 33 |
| pCMV4-Luc | 60 | 42 | 30 |
| pCMV4-N | 40 | 30 | 25 |
| pCMV4-G | 60 | 10 | 83 |
| pCMV4-N + pCMV4-G | 60 | 9 | 85 |

Figure 4.4 Mortality rate of fish injected with plasmid DNA. Rainbow trout at an average weight of 1 g were exposed to IHNV (104 PFU/L, Rangen isolate) six weeks after injection with PBS, pCMV4-Luc, pCMV4-N, pCMV4-G, or pCMV4-N + pCMV4. In addition an unhandled (not injected) was included.

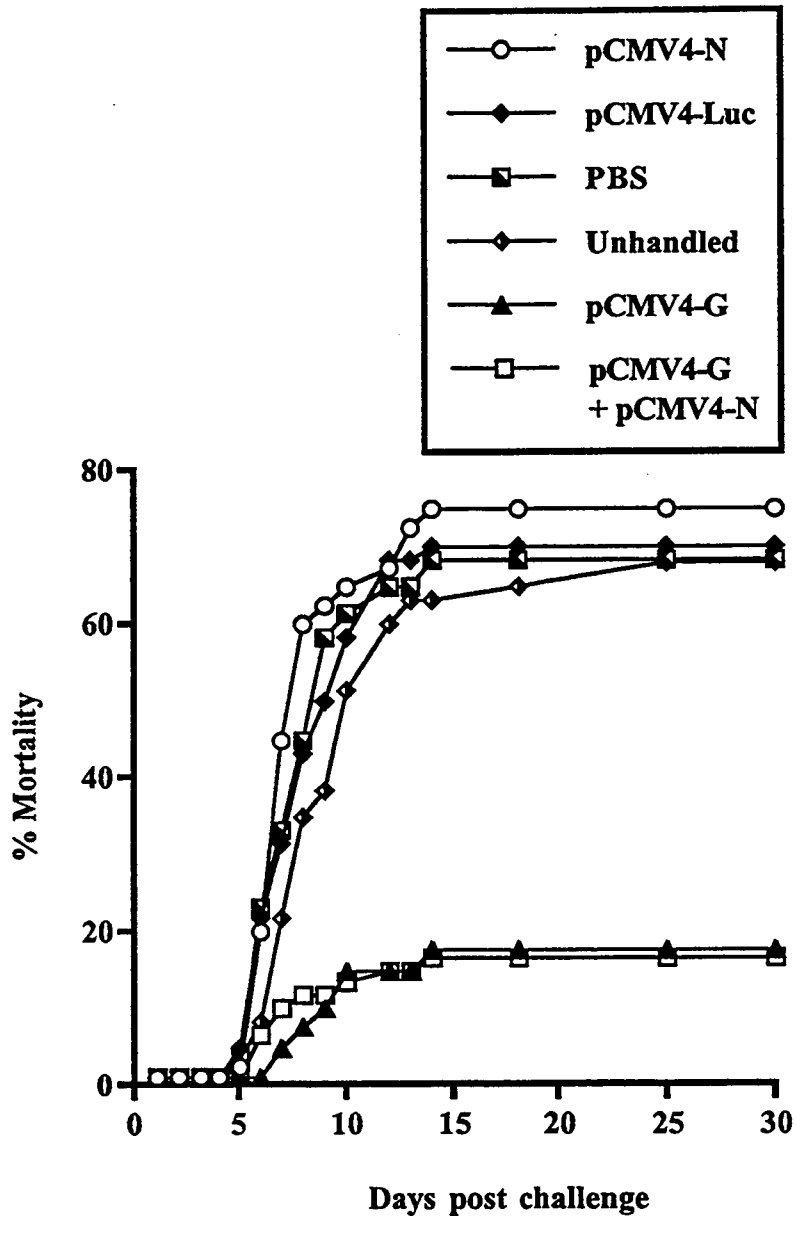

pCMV4-N
pCMV4-Luc
PBS
Unhandled
pCMV4-G
pCMV4-G
+ pCMV4-N
% Mortality
80
60
40
20
0
0
5
10
15
20
25
30
Days post challenge

Figure 4.4

immunity to vaccinated fish against IHNV as has been previously reported (Engelking et al., 1991). These results are similar to those of Xiang et al. (1994) using a DNA vaccine composed of the RV G protein, pSG5rab.gp, to vaccinate mice. The pSG5rab.gp vaccine also produced a strong G-specific T-cytotoxic cell response and lymphokine secretion from T (H1) cells which was both dose and booster dependent. Additional studies have shown that the route and site of administration of DNA vaccination is important in determining the type of immune response generated (Raz et al., 1994; Fynan et al., 1993). Thus, it is clearly possible that further study of the vaccination regimens and delivery methods will improve the efficacy of the pCMV4-G vaccine.

The pCMV4-N vaccine did not protect rainbow trout against IHNV, nor did it evoke a measurable antibody response. It is possible that the N protein is a poor protective immunogen in fish, or is not accessible to immune surveillance, with the strategy employed in our study. However, it has been reported that the N protein subunit vaccine did not immunize fish against IHNV (Oberg et al., 1992). In addition, within a population of IHNV infected fish, only a small proportion contain anti-N antibodies, the majority contain anti-G and M-1 antibodies (Ristow et al;.1993, Lapatra et al., 1993). The possibility that the N protein was aberrantly expressed in fish muscle cells and therefore not available for immune recognition cannot be excluded. Yet, in this study (and unpublished results) it was found that fish co-injected with the N and G DNA vaccines had an earlier and stronger antibody response to G. While the results are tentative, it does suggest that the pCMV4-N vaccine is functional and capable of augmenting the immune response. That pCMV4-N is functional is further supported by the finding that the N protein is synthesized in transfected fish cells. It could be speculated that the augmentation occurs via T-cell activation. The N protein of rabies virus is a major target for T-helper cells (Ertl et al., 1989) and the IHNV N protein contains a number of putative T-helper cell epitopes (Oberg et al., 1991). Activation of T-cells could increase the activity of the B-cells producing anti-G antibodies. This type of cooperation has been observed for both influenza virus (Russell and Liew, 1979) and hepatitis B virus (Milich et al., 1987).

Though fish injected with a combination of pCMV4-N and pCMV4-G had a stronger anti-G specific antibody response than pCMV4-G injected fish, there was not a relative increase in survival following IHNV challenge. Thus, the level of antibody production by DNA vaccinated fish was not a good indication of the degree of protection against IHNV. Zhuang et al. (1992) also found that there was not a relationship between anti-IHNV antibody titer and protection. There are many

explanations for this. It is possible that once a threshold level of antibodies have been reached, increasing the antibody concentration does not enhance the relative degree of protection. It is also probable that the antibody response is not the only immune function conferring protection following genetic immunization.

In summary, the results presented in this report show that fish can be included among the organisms which can be immunized against a viral pathogen using DNA vaccines. Because the DNA vaccines can be delivered simply, the proteins are synthesized indepent of the whole pathogen, and can potentially generate a broad spectrum immune response, genetic immunization should prove to be a powerful tool to study the fish immune repetoire.

## Material/Methods

### Cells and Virus

The chinook salmon embryo cell line CHSE-214 (Lannan et al., 1984) was used for the propagation of IHNV as previously described (Engelking and Leong, 1981). The cells were grown as monolayers in RPMI-1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (Intergen), penicillin 100 I.U./ml, streptomycin 100 μg/ml and 2mM L-glutamine (all from GIBCO Laboratories). The type-2 IHNV isolate used in this study was isolated in 1983 from a juvenile rainbow trout at the Rangen Research Laboratories, Idaho, USA (Hsu et al., 1986). The virus was propagated in fish cells at a multiplicity of infection of 0.001 at 15$^{o}$ C. The cell-free supernatant contained $10^{8}$ plaque forming units (PFU) as determined by a plaque assay (Burke and Mulcahy, 1980).

### Fish immunization and challenge

Rainbow trout (Shasta strain) with no previous history of IHNV were provided by the Food Toxicology and Nutrition Laboratory at Oregon State University. Fish were held at the Oregon State University Fish Disease Laboratory in

12° C fish-pathogen-free well water where subsequent immunization and challenge experiments were conducted. The fish were anesthetized in 50 ppm 3-aminobenzoic acid ethyl ester (Benzocaine; Sigma) followed by intramusclular injection of 10 ug of plasmid DNA as previously described (Anderson et al., submitted). Six weeks post DNA injection, fish were challenged by immersion in 2L of water containing $10^4$ plaque forming units/L of live, infectious type-2 IHNV (Rangen). Dead fish were collected daily for 30 days.

Plasmid Construction

The N and G gene from IHNV were placed down stream of the cytomegalovirus immediate early enhancer/promoter (CMV-IEP) in the plasmid pCMV4 (Andersson et al., 1989) . All of the DNA restriction enzymes used were obtained from Promega. The plasmid pGEM-N which contains the complete cDNA of the IHNV N (Gilmore and Leong, 1988a ) was digested with Hind 111 and the N gene fragment from nucleotides 444-1400 was gel purified using glass beads according to the suppliers protocol (Bio 101 Inc.). The Hind 111 N gene fragment was inserted into the Hind 111 multiple cloning site of plasmid pCMV4 to create pCMV4EAH3N. The plasmid pGEM-N was then digested with Kpn1 and Nar 1 and the N gene sequences from nucleotides 0-841 was gel purified using glass beads. The plasmid pCMV4EAH3N was digested with Kpn1 and Nar 1 and the 6.2 Kb DNA fragment was gel purified using glass beads. The N gene Kpn1/Nar1 fragment was then inserted into pCMV4EAH3N Kpn1/Nar 1 fragment to create a full length N gene plasmid construct named pCMV4-N. The plasmid pG8 containing the complete cDNA of the IHNV G gene (Koener et al., 1987) was digested with Kpn 1 and Hind 111 and the 1600 base pair G gene was gel purified using glass beads. The plasmid pCMV4 was digested with KPN1 and Hind 111 and the gel purified G gene was inserted into the compatible site in the multiple cloning region of pCMV4 to create pCMV4-G. The plasmid pCMV4EAL contains the firefly luciferase gene downstream of the CMV-IEP promoter. The construction of pCMV4 EAL has been previously described (Anderson et al., submitted)

Confirmation of protein synthesis

Protein synthesis from the plasmids pCMV4-N and pCMV4-G was confirmed by transfection of fish cells epithelioma papulosum cyprini (EPC; Fijian et al., 1983) . Fish cells were seeded in a 6 well plate (Corning, 9.4 $cm^2$/ well) at a 50-60% confluencey. The following day the cells at 90% confluencey, were transfected with 1 μg of the appropriate DNA vector using lipofectamine according to the suppliers protocol (GIBCO). The DNA lipid complex was incubated with the cells for 24 hours. Following transfection the lipid DNA complex was rinsed from the cells using RPMI-1640 medium without fetal bovine serum or antibiotics. The cells were incubated throughout the experiments at $20^{o}$ C in a incubator culture chamber (C.B.S. Scientific Co.: model no. M-624) perfused with a blood gas mixture containing 9.9% mol/mol carbon dioxide, 10.2% mol/mol oxygen and 79.9% mol/mol nitrogen (Airco medical gases BOC). Five days post transfection expression of the N and the G in fish cells was confirmed, using the monoclonal antibody (MAb) 1NDW14D and 3GH136J respectively (Ristow and Arnzen de Avila, 1991), as previously described (Drolet et al., 1993).

Immunization

DNA was prepared using qiagen affinity chromatographic columns (Qiagen, Inc.). Ten ug of the appropriate plasmid DNA was suspended in 200 μls of phosphate buffered saline solution (PBS, 0.14 M NaCl, 10mM $Na_2HPO_4 \cdot 7H_20$, pH 7.4). The plasmid DNA was injected into anesthetized fish (50 ppm Benzocaine) as described elsewhere (Manuscript submitted).

Determination of IHNV Ab titers and specificity

Pools of fish blood from five fish was collected 4, 6, 8, 12, and 14 weeks post DNA injection. The blood was obtained by cutting off the caudal fin of anesthetized fish and pressing a heparinized capillary tube to the site. The capillary tubes were

then centrifuged at 5 X g for 15 min and the serum was collected. The activity of anti-IHNV fish antibodies in the collected serum was determined using an enzyme-linked immunosorbent assay (ELISA) . Purified IHNV was prepared as previously described (Engelking and Leong, 1989). Purified IHNV (3ug/ml) in carbonate buffer (15mM $Na_2CO_3$, 35mM $NaHCO_3$, $NaN_3$, pH 9.6) was coated onto a 96 well microtiter plates (Costar E.I.A/R.I.A. certified surface chemistry, Cambridge, MA) overnight at 4° C. The plates were washed with TBS containing 0.5% Tween-20 (TBTS). Serial dilutions of the collected fish antiserum were added and incubated overnight at 17° C . The plates were washed in Tris-buffered saline (TBS, 20mM Tris base, 500mM NaCl, pH 7.5) containing 0.5% Tween-20 (TBTT) followed by a 1 hr in a blocking solution containing 3% bovine serum albumin (Sigma). The plates were then washed in TBTT and MAb 1/14 specific for trout immunoglobulin (Deluca et al., 1983) was added, and incubated for 2 hours at 17° C. The plates were washed in TBTT and then incubated with strepavidin-horseradish peroxidase (Sigma) for 1 hour. The plates were washed again with TBTS and 2,2-azino-di(3-ethylbenzthiazoline sulfonate (ABTS; Boehringer Mannheim) in 0.2% citrate with 0.03% hydrogen peroxide was added as the color development reagent. The color reaction was measured at an optical density of 405 nm using a Titertek Multiscan Plus plate reader (Flow Laboratories). Each plate contained a standard anti-IHNV hyper immune serum titration used for normalization of the data. The antibody activity was determined by a comparison to the dilution of hyperimmune anti-IHNV fish sera which resulted in half maximal activity. The antibody activity was assigned a numeric value accordingly and the data is presented as the log relative units (RU) of ant-IHNV antibody activity.

The specificity of the antibodies produced in response to the expression of plasmids encoding the N or G genes when injected into fish was determined by western blotting. Purified IHNV (20 μg/ lane) was separated on a 12% polyacrylamide gel and transferred to nitrocellulose membranes (Shleicher and Shuell) as previously described (Engelking and Leong, 1989a). Following transfer the membranes were blocked for 1 hr in TBS containing 5% non-fat milk (TBSM). DNA injected fish antisera was incubated with virus at a 1:100 dilution in TBSM at 17°C for 17 hrs. The membranes were washed in TBSM and then incubated for 2 hrs with biotinylated MAb 1/14 in TBSM. The blot was washed in TBSM and then incubated for 30 min with avidin-alkaline phosphatase (Vector Laboratories) in TBSM . The membrane was washed in TBS and developed with a one component developer (Kirkegard and Perry) in the dark for 30 min.

## Acknowledgments

This work was supported by United States Department of Agriculture Grants 91-34123-6124 and 92-37204-7997; and Oregon Sea Grant with fund from the National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerece, under grant no. NA89AA-D-SG108, Project no. R/FSD-16.

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# CHAPTER 5
# INHIBITION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS REPLICATION IN CELL CULTURE BY ENDOGENOUSLY SYNTHESIZED ANTISENSE AND SENSE RNA

Eric D. Anderson and Jo-Ann C. Leong*

Department of Microbiology and the Center for Salmon Disease Research, Oregon State University, Corvallis, Oregon 97331, U.S.A

*Correspondence should be addressed to this author

(for submission to Antisense Research and Development)

This paper reports a portion of the work encompassed by a thesis submitted to Oregon State University, Department of Microbiology, in partial fulfillment of that required for the degree of Ph. D., for Eric D. Anderson.

## Abstract

An inducible expression plasmid pMMTV-neo containing the infectious hematopoietic necrosis (IHNV) nucleoprotein (N) gene in the sense or antisense orientation was tested for their ability to interfere with the IHNV life cycle. Fish cells transfected with the plasmids were resistant to IHNV plaque formation and produced fewer infectious IHNV than control cells. The antisense plasmids conferred a higher degree of cellular viral resistance than the sense plasmids. The degree of viral resistance of the transfected cells decreased with cell passage. This temporal change was observed for both the sense and antisense transfected cell lines.


## Introduction

Intracellular immunity to viruses mediated by cellular expression of viral transgenes has been reported for both plants and animals (for a review, see van der Krol et al., 1988; Beachy, 1993). The interfering transgenes can disrupt the life cycle of the virus at numerous steps during infection. One level of viral interference by the transgene is duplex formation with a viral RNA target sequence resulting in degradation of the duplex, inhibition of viral mRNA translation, or inhibition of mRNA processing (for a review, see Inouye, 1988; Takayama and Inouye, 1990; Helene and Toulme, 1990). Interference can also be achieved by expression of positive polarity viral transgenes. The expressed proteins can disrupt the virus life cycle by receptor interference (Federspiel et al., 1989), blockage of virion uncoating (reviewed in Reimann-Philipp and Beachy, 1993), limiting the systemic spread of the virus (Wisniewski et al., 1990), or functioning as trans dominant repressors (Mermer et al., 1990; Bevec et al., 1992; Malim et al., 1992; Bahner et al., 1993). Intracellular immunity has also been attained using viral specific ribozymes (Weerasinghe et al., 1991), RNA decoys (Sullenger et al., 1991; Lisziewicz et al., 1993; Bevec et al., 1994), and oligonucleotides (reviewed in Stein and Cheng, 1993). In this report the cellular expression of viral transgenes of IHNV was investigated for their ability to disrupt the viruses replicative cycle.

IHNV is a rhabdovirus which causes a severe disease in a number of species of salmonid fish native to the Pacific Rim (Wolf, 1988). The virus establishes a rapid

systemic viremia in infected fish (Drolet et al., 1994), causes extensive necrosis of the hematopoietic tissues (Yasutake, 1970), and in some instances can result in greater than 90% mortalities within a population (Amend, 1969). In cell culture IHNV inhibits both cellular transcription and translation which results in cell death 48-72 hrs post infection. In infected cells the viral nucleoprotein (N) can be detected 2-3 hrs post infection (p.i.), the matrix proteins (M1 and M2) 6-7 hrs p.i., the glycoprotein (G) 9-10 hrs p.i., and the low abundance polymerase (L) 30 hrs p.i (Hsu et al., 1985). The time of appearance of the viral proteins is believed to be a result of sequential transcription of the genome by the viral polymerase complex starting at the 3' end of the negative stranded genome. The IHNV genome gene order been determined to be 3' N-M1-M2-G-NV-L 5' (Kurath et al., 1985).

The N protein of IHNV, by analogy with the rhabdovirus vesicular stomatitis virus (VSV), is thought to be involved in modulating the switch from viral transcription to replication (Banerjee, 1987). Thus, because the N protein is the first and most abundant viral protein synthesized in infected cells, and plays an important role in the regulation of viral synthesis, transgenes targeted to the IHNV N gene may be an effective strategy for disrupting the viruses life cycle. Indeed, antisense oligonucleotides targeted to the N mRNA of vesicular stomatitis virus (VSV) have been shown to interfere with VSV synthesis (Agris et al., 1986; Lemaitre et al., 1987; Leonetti et al., 1988).

In this study, a hormone-inducible, mouse mammary tumor virus plasmid was constructed that contained the IHNV nucleoprotein gene in the sense or antisense orientation. These vectors were used to transfect fish cells and tested for their ability to inhibit IHNV synthesis.

## Materials and Methods

Plasmid DNA construction.

Standard cloning protocols were used as described by Sambrook et al. (1989). The IHNV N gene cDNA (Gilmore and Leong, 1988) was inserted in the sense or antisense orientation downstream of the mouse mammary tumor virus promoter

contained in the plasmid pMMTV-neo (Clontech). The pMMTV-neo plasmid was digested with Sal 1 (Promega) in the multiple cloning site, and blunt ended with the DNA polymerase 1 klenow fragment (Promega). The 1359 base pair IHNV N cDNA contained in pGEM-3Z (Gilmore and Leong, 1988) was released with Pst 1 (Promega), separated from the plasmid DNA by agarose gel electrophoresis, and purified from the gel using glass beads (Bio 101 Inc.). The purified N Pst 1 fragment was blunted using $T_4$ DNA polymerase (GIBCO BRL) and ligated ($T_4$ DNA ligase; GIBCO BRL) to the pMMTV-neo Sal 1 digested and blunted plasmid. Two pMMTV-N vectors were produced, one in the antisense orientation termed pN(-), and the other in the sense orientation termed pN(+). Additionally, plasmid pMMTV-Luc (Clontech) which contains the luciferase gene downstream of the MMTV promoter, and pMMTV-Neo (blank parent plasmid) were used in the experiments.

Transfections of cells

All the plasmids were transfected into the fish cells, *epithelioma papulosum cyprini* (EPC) (Fijian et al., 1983). The fish cells were grown in RPMI-1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Intergen), antibiotics and L-glutamine (penicillin, 100 IU/ml; streptomycin, 100 ug/ml; and L-glutamine, 2mM; all from GIBCO BRL), and rainbow trout embryo extract. The cells were maintained in a incubator culture chamber (C.B.S. Scientific) perfused with a blood-gas mixture composed of 9.9% mol/mol $C0_2$, 10.2% mol/mol $0_2$, and 79.9% mol/mol $N_2$. Transfections were performed on cells in a 6-well plates (Nunc), at 20-22° C. The cells were transfected at a density of $2 \times 10^6$ cells/$cm^2$. All of the cell lines were maintained by a 1:4 passage every 14-17 days.

Two methods were used to transfect the fish cells. The first method was conducted using DNA calcium phosphate precipitation as described by Araki et al. (1991). The second transfection method was performed by incubating cell monolayers for 24 hrs with a solution of 1 ml of opti-MEM (GIBCO BRL) containing 6 ul of lipofectamine (GIBCO BRL) and 1 ug of DNA. Transfected cells were selected using geneticin (G418, .3 mg/ml) supplemented medium. After selection in G418 medium for approximately one month, G418 resistant colonies were pooled to facilitate cell growth.

Viral assays

The IHNV isolate, RB-1, used in this study was isolated from a juvenile rainbow trout in 1976, from Round Butte Hatchery, Oregon, USA. The virus was propagated in EPC cells at a multiplicity of infection of 0.001 at 16° C. The IHNV titer in cell free supernatants taken 5-7 days post infection was determined by a plaque assay (Burke and Mulcahy, 1980).

Viral plaque assays were conducted using transfected and control cell monolayers ($2.5 \times 10^6$ cells/$cm^2$). Prior to IHNV infection, 1.0 uM water-soluble dexamethasone (dex, Sigma) in RPMI-1640 supplemented medium was incubated for 36 hours with a portion of the cells to induce the MMTV promoter. Following dex induction, ten-fold dilutions of IHNV RB-1 suspended in 100 ul of RPMI-1640 (without FBS or antibiotics) was incubated with the cells for five minutes. The cells were washed briefly three times with medium, and then overlaid with complete medium containing 0.75% methylcellulose (Fischer Scientific) and 1.0 uM dex (in the dex induced cells only). The cells were held at 16° C for 10-14 days, and then fixed and stained with crystal violet in formalin (25% formalin, 10% ethanol, 5% acetic acid, 1% w/v crystal violet). The number of viral plaques were enumerated macroscopically and microscopically and standardized to PFU/ml (plaque forming units).

Viral growth was also measured on transfected and control cell monolayers. The appropriate wells were induced with dex as above, and then infected with a multiplicity of infection of one. Infection was allowed to proceed for four days (100% lysis of control cells), the virus in the cell free supernatants was then measured by a plaque assay (Burke and Mulcahy, 1980).

Immunohistochemistry

Transfected and control cells which had been fixed and stained following viral plaque assays were subsequently stained immunohistochemically with an IHNV glycoprotein-specific monoclonal antibody, 3GH136J (Ristow and Arnzen de Avila, 1991). The assay was performed exactly as described by Drolet et al. (1994).

Nucleic acid analysis

Southern analysis of genomic DNA and Northern analysis of cellular RNA extracted from transfected and control cells was performed as described by Sambrook et al. (1989). Prior to southern analysis, the genomic DNA was digested with the restriction enzymes Eco R1 or Hind 111 (Promega). Total cellular RNA was isolated using RNAzol according to the suppliers protocol (TM Cinna Scientific, Inc.). The RNA was electrophoretically separated on a formaldehyde-agarose gel (Sambrook et al. 1989). The southern and northern blots were hybridized with a $^{32}$P-labeled N-specific probe (approximately $10^9$ cpm/ug) which was prepared using a random-primed DNA labeling system (Amersham) according to the suppliers protocol. Following hybridization, the Northern and Southern blots were washed; the highest stringency wash was performed using 0.1 X SSPE (SSPE: NaCl, 149 mM; $Na_2HPO_4$-$H_20$, 10mM; EDTA, 0.99 mM) containing 1% SDS (sodium dodecyl sulfate), for 30 min at 68$^o$ C. The blots were wrapped in Reynolds film and exposed to hyperfilm (Kodak) at -70$^o$ C.

## Results

Transfected cell analysis

The plasmids pMMTV-neo, pMMTV-Luc, pN(+), and pN(-), were transfected into the EPC fish cells. Transfected cells were selected using the antibiotic G418; all of the pMMTV plasmid derivatives contain the neomycin resistance gene downstream of the SV40 promoter allowing G418 selection.

The calcium phosphate precipitation (CPP) method of transfection resulted in a low efficiency of transfection compared to the liposome (L) transfection method. The CPP transfected cells yielded 16-20 individual G418 resistant colonies per 2x $10^7$ cells after one month of G418 selection. It required 2 months of propagation to reach confluency in a 9.4 $cm^2$ dish. There were greater than 500 G418 resistant colonies per 2 X $10^7$ cells using the L transfection method and the cells grew back to complete confluency after one month of G418 selection. For each method of transfection,

isolated G418 resistant colonies could not be expanded. However, pooling of 6-10 G418 resistant colonies and addition of rainbow trout embryo extract to the medium facilitated cell growth. Because the cells were pooled they were likely heterogeneous with regards to the number of copies of plasmid integrations per cell and the level of transgene expression.

Southern blots were performed on high molecular weight DNA extracted from pN(+), and pN(-) CPP transfected G418 resistant cells approximately 5 months post transfection (Figure 5.1 a, b, and c). The DNA was digested with Eco R1 and Hind 111 restriction enzymes. The 7180 base pair Eco R1 fragment contains the pMMTV promoter, the N gene, the SV40 promoter, and the neomycin resistance gene for both plasmids pN(+) and pN(-) (Figure 5.1 a) . Thus, the EcoR1 fragment contains plasmid sequences essential for conferring G418 resistance and expression of the sense and antisense N cDNA. The DNA digested with Hind 111 reveals the positive sense orientation, 2090 and 1907 base pair fragments, and antisense orientation, 2419 and 1578 base pair fragments, of the N gene in the plasmids pN(+) and pN(-). The southern blots also revealed that the plasmid DNA had integrated into multiple sites and/or had integrated as concatamers in the cells genome as evidenced by numerous N probe hybridizing bands of higher and lower molecular weight than predicted by the plasmid restriction map.

Northern blots were performed on RNA extracted from pN(+) and pN(-) CPP transfected G418 resistant cells approximately five months after transfection (Figure 5.2). A portion of the transfected cells were treated with 1.0 uM dex to increase the synthesis of the transgene mRNA by the MMTV promoter (Lee et al., 1981). Hormone induction resulted in a 2-3 fold increase in N mRNA expression (Figure 5.2; lanes 1 and 3) compared to uninduced cells (Figure 5.2; lanes 2 and 4).

## Viral assays

Viral plaque assays were performed on pN(+) and pN(-) transfected cells and non-transfected EPC or pMMTV-Neo ( blank plasmid) transfected cells as negative controls. In the first series of experiments the CPP transfection method was employed to generate cells containing the pN(+), pN(-), of pMMTV-Neo plasmids. Approximately 3 months (5 cell passages) after transfection there were a sufficient number of G418 resistant cells to perform a viral plaque assay. The viral plaque

assays were conducted on transfected and control cells (pMMTV-Neo transfected and non-transfected EPC cells) grown in multi-well plates containing approximately 2.5 X $10^6$ cells/$cm^2$. Prior to infection with IHNV, the appropriate cells were induced with 1 uM dex. The results of the first experiment are presented in Table 5.1. Macroscopic viral plaques appeared only on the control cell lines; there were not any macroscopic IHNV plaques on the uninduced or dex induced pN(+) or pN(-) transfected cells (Figure 5.3). However, at 10 X magnification there were minute plaques visible on the dex uninduced pN(+) and pN(-) transfected cells; there were also foci of infection on the dex induced cells (rounded cells) but there were not any cleared zones indicative of plaque formation.

The viral resistance appeared to be dependent upon the expression of the N transgene. This is supported by the finding that number of plaques formed and free virus particles released using control, dex induced or uninduced pMMTV-Neo or pMMTV-Luc transfected cells, were the same as non-transfected cells. Further, at the time of the assays all the cells lines were growing at similar rates and assayed at the same density. This discounts the possibility that viral resistance of the pN(+) and pN(-) transfected cells was due to the cells' growth characteristics or cell densities as previously described by Mulcahy et al. (1984). Finally, in a separate series of experiments, cell free supernatants taken from the pN(+) and pN(-) cell lines following an IHNV plaque assay tested negative for interferon production (data not shown).

Following the viral plaque assays, the same tissue culture plates were probed with an IHNV glycoprotein specific monoclonal antibody, 3GH136J (Figure 5.4). The microscopic plaques found on the uninduced pN(+) and pN(-) transfected cells reacted with the G-specific antibody as did the viral plaques on the control cells. There were also individual rounded cells which reacted with the G-specific monoclonal antibody on the dex induced pN(+) and pN(-) transfected cells.

The same cells used in the first plaque assay experiment were expanded to provide enough cells to repeat the viral assay (5 months post transfection, 12 passages), and perform southern and northern blots. The viral plaque assay were performed the same as in the first experiment, the results are presented in Table 5.2 (indicated as 2A). The pN(+) and pN(-) transfected cells were less resistant to IHNV plaquing than they were in the initial 3 month assay. In addition, the plaques formed on the pN(+) and pN(-) transfected cells were macroscopically visible (Figure 5.5).

We investigated the possibility that the reduction of viral resistance of the pN(+) and pN(-) transfected cells was the result of a suppression of transgene

Figure 5.1 Southern blot analysis of pN(+) and pN(-) transfected cells. (A) shematic diagram of the pN(+) and pN(-) plasmids and predicted fragments released by EcoR1 or Hind 111 digestion. (B) EcoR1 digested DNA (10 μg), lanes: 1, control pN(+) plasmid (10 pg); 2, pN(-); 3, pN(+). (B).Hind 111 digested DNA (20 μg), lanes: 1, control (EPC-DNA), 2, PN(-); 3, empty; 4, pN(+).

Figure 5.2 Northern blot analysis of pN(+) and pN(-) transfected cells. For the pN(+) and pN(-) transfected cells 30μg of total RNA was loaded/ well; for the IHNV infected cells 0.5 μg of total RNA was loaded/well. Lanes: 1 and 2, dex. induced and uninduced pN(+) transfected cells, respectively; 2 and 4 , dex induced and uninduced pN(-) transfected cells , respectively; 5, positive control IHNV . The label 1400 is the approximate size, in nucleotides, of the band hybridizing with the labelled N cDNA.

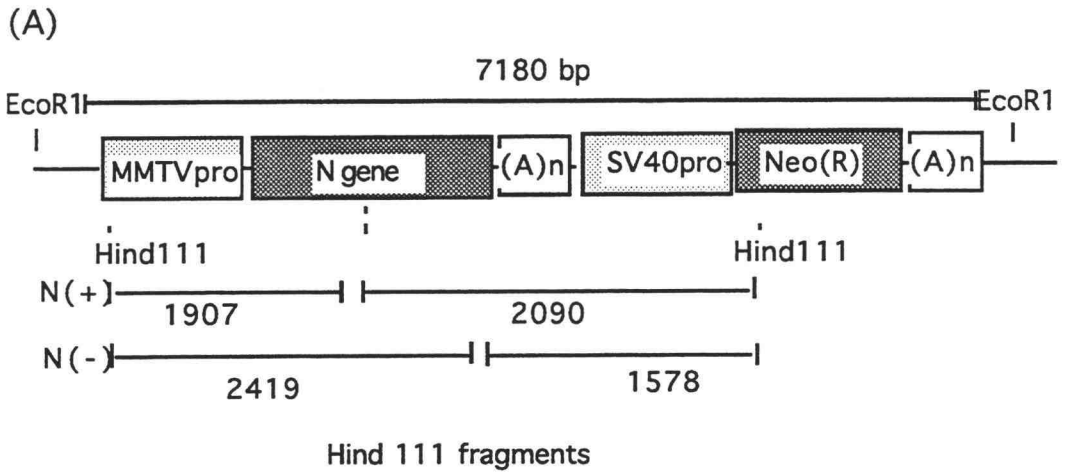

(A)
7180 bp
EcoR1
EcoR1
MMTVpro
N gene
(A)n
SV40pro
Neo(R)
(A)n
Hind111
Hind111
N(+)
1907
2090
N(-)
2419
1578
Hind 111 fragments
(B)
1 2 3
7.1 kb
(C)
1 2 3 4
2.4 kb
1.5 kb
2.0 kb
1.9 kb

Figure 5.1

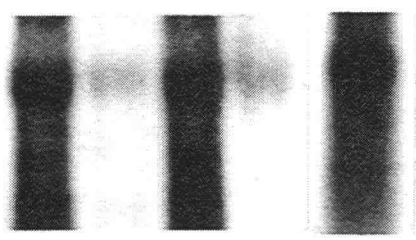



Figure 5.2

assays were conducted on transfected and control cells (pMMTV-Neo transfected and non-transfected EPC cells) grown in multi-well plates containing approximately 2.5 X $10^6$ cells/$cm^2$. Prior to infection with IHNV, the appropriate cells were induced with 1 uM dex. The results of the first experiment are presented in Table 5.1. Macroscopic viral plaques appeared only on the control cell lines; there were not any macroscopic IHNV plaques on the uninduced or dex induced pN(+) or pN(-) transfected cells (Figure 5.3). However, at 10 X magnification there were minute plaques visible on the dex uninduced pN(+) and pN(-) transfected cells; there were also foci of infection on the dex induced cells (rounded cells) but there were not any cleared zones indicative of plaque formation.

The viral resistance appeared to be dependent upon the expression of the N transgene. This is supported by the finding that number of plaques formed and free virus particles released using control, dex induced or uninduced pMMTV-Neo or pMMTV-Luc transfected cells, were the same as non-transfected cells. Further, at the time of the assays all the cells lines were growing at similar rates and assayed at the same density. This discounts the possibility that viral resistance of the pN(+) and pN(-) transfected cells was due to the cells' growth characteristics or cell densities as previously described by Mulcahy et al. (1984). Finally, in a separate series of experiments, cell free supernatants taken from the pN(+) and pN(-) cell lines following an IHNV plaque assay tested negative for interferon production (data not shown).

Following the viral plaque assays, the same tissue culture plates were probed with an IHNV glycoprotein specific monoclonal antibody, 3GH136J (Figure 5.4). The microscopic plaques found on the uninduced pN(+) and pN(-) transfected cells reacted with the G-specific antibody as did the viral plaques on the control cells. There were also individual rounded cells which reacted with the G-specific monoclonal antibody on the dex induced pN(+) and pN(-) transfected cells.

The same cells used in the first plaque assay experiment were expanded to provide enough cells to repeat the viral assay (5 months post transfection, 12 passages), and perform southern and northern blots. The viral plaque assay were performed the same as in the first experiment, the results are presented in Table 5.2 (indicated as 2A). The pN(+) and pN(-) transfected cells were less resistant to IHNV plaquing than they were in the initial 3 month assay. In addition, the plaques formed on the pN(+) and pN(-) transfected cells were macroscopically visible (Figure 5.5).

We investigated the possibility that the reduction of viral resistance of the pN(+) and pN(-) transfected cells was the result of a suppression of transgene

Table 5.1. IHNV plaque assays on control non-transfected EPC, and pMeaN(+) and pMeaN(-) transfected EPC cells ( 5 cell passages).

| | PFU/ml X $10^6$ | | Reduction of PFU/ml | |
|---|---|---|---|---|
| Cell line | (+) dex | (-) dex | (+) dex | (-) dex |
| Control | 24 | 25 | - | - |
| pN(+) | 0 | 25.3 | 100% | 0% |
| pN(-) | 0 | 28.6 | 100% | 0% |

The reduction of PFU/ml was calculated as percentage of control values.
Each test was performed in triplicate, the average PFU/ml is presented.
The standard deviation was less than 7 for all measurements.

Figure 5.3 IHNV plaque formation following infection of control pMMTV-Luc, pN(+) or pN(-) transfected cells ( 5 cell passages). Each well (duplicates are shown) were infected with roughly 250 PFU IHNV.

Figure 5.4 Immunohistochemical analysis of control pMMTV-Luc or p(N-) transfected cells following viral infection (see Table 1 and figure 5.3). The cells were stained with an IHNV G-specific monoclonal antibody; the red stained cells indicate viral infection. (A) control cells, (B) pN(-) transfected cells without dex, (C) pN(-) transfected cells with dex.

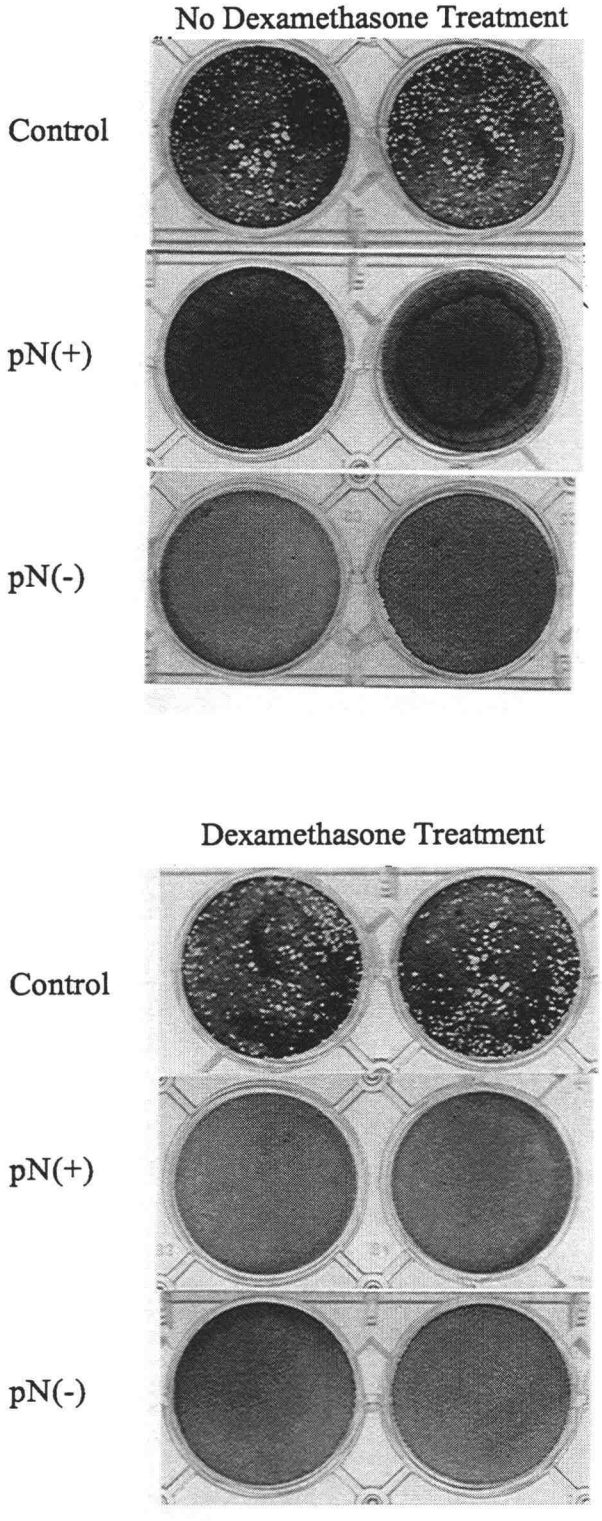
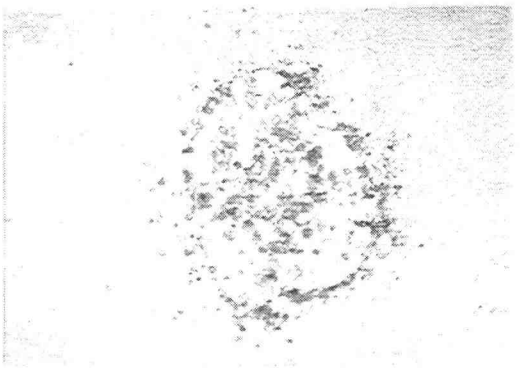
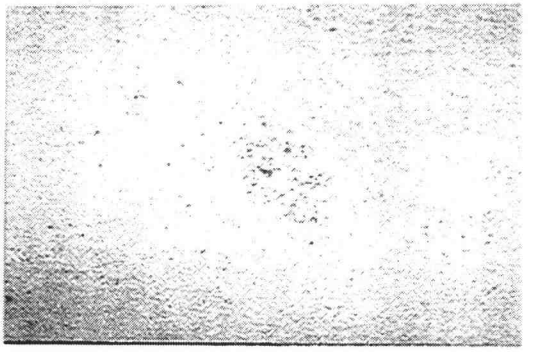

No Dexamethasone Treatment
Control
pN(+)
pN(-)
Dexamethasone Treatment
Control
pN(+)
pN(-)

Figure 5.3

(A)



(B)



(C)

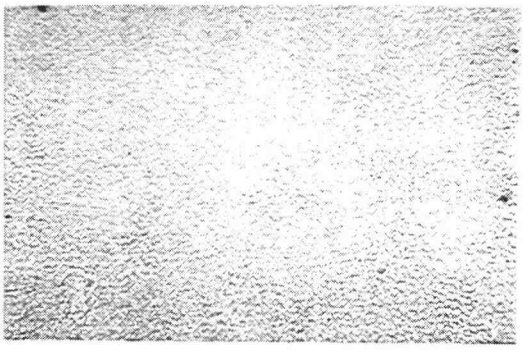


Figure 5.4

Table 5.2. IHNV plaque assays on control pMMTV-luc transfected cells, and pMeaN(+) and pMeaN(-) transfected EPC cells. The assay was conducted using CPP-transfected cells indicated as 1A (12 cell passages), or L-transfected cells indicated as 2A following (3 passages).

| | PFU/ml X $10^6$ | | Reduction of PFU/ml | |
|---|---|---|---|---|
| Cell line | (+) dex | (-) dex | (+) dex | (-) dex |
| Control | | | | |
| 1A | 55.5 | 51.5 | _ | _ |
| 2A | 17.7 | 16.1 | _ | _ |
| pN(+) | | | | |
| 1A | 38.1 | 28.5* | 31% | 45% |
| 2A | 11.3 | 11.9 | 36% | 26% |
| pN(-) | | | | |
| 1A | 1 | 23 | 99% | 54% |
| 2A | 8.4 | 11.1 | 52% | 31% |

The reduction of PFU/ml was calculated as percentage of control values. Each test was done in triplicate, the average PFU/ml is shown. The standard deviation was less than 7 for all measurements except *which was 13.

Figure 5.5 IHNV plaque formation following infection of control pMMTV-Luc, pN(+) or pN(-) transfected cells (12 cell passages). Each well (duplicates are shown) were infected with roughly 450 PFU IHNV.

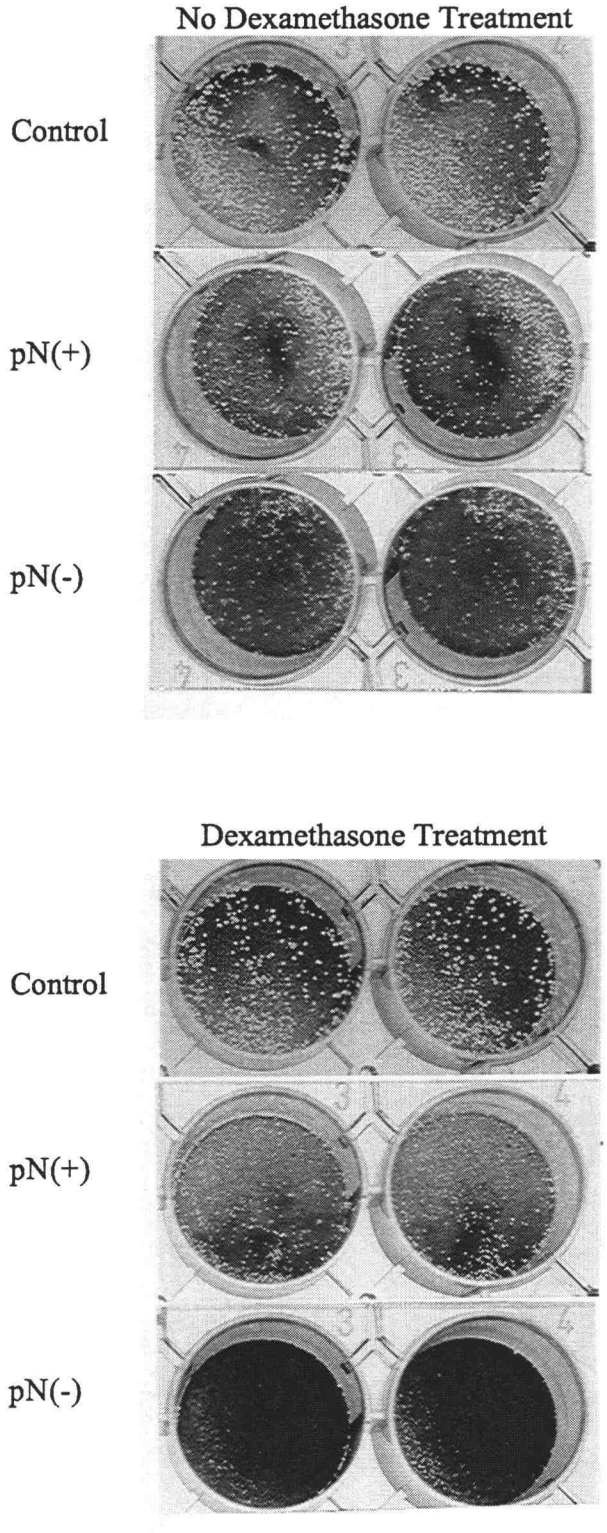

No Dexamethasone Treatment
Control
pN(+)
pN(-)
Dexamethasone Treatment
Control
pN(+)
pN(-)

Figure 5.5

expression due to reduced activity of the MMTV promoter by methylation. However, treatment of the pN(+) and pN(-) transfected cells (6 months post transfection, 15 passages) with 5-aza-2'-deoxycytidine which causes hypomethylation, Friedman et al., 1989), at different concentrations for different lengths of time did not increase the viral resistance of the cells (data not shown).

Because the degree of viral resistance of the pN(+) and pN(-) transfected cells changed with time we generated another series of cell lines using lipofection mediated transfection. During the course of this study we found that this technique enhanced the number of transfected cells and reduced the time required to generate enough cells to perform the plaque assays. The results of the plaque assay performed on L transfected pN(+) and pN(-) positive cells (2 months post transfection, 3 cell passages) are also presented in Table 5.2 (indicated as 2B). The experiments conducted with the L transfected cells included as a negative control cells transfected with pMMTV-Luc. The L transfected cells were less resistant to IHNV plaquing than the CPP pN(+) and pN(-) transfected cells assayed at 3 and 5 months. However, both the CPP and L transfected pN(+) and pN(-) cells inhibited IHNV plaquing. Additionally, the CPP and L generated pN(-) cells were more resistant than the CPP and L generated pN(+) cells to IHNV plaquing. Though the degree of resistance was not identical for CPP and L transfected cells, the results demonstrated that pN(+) and pN(-) cells were IHNV resistant.

The growth of IHNV in L transfected pMMTV-Luc control cells, and pN(+) and pN(-) transfected cells was measured. The cells were infected with IHNV at an m.o.i of 1. The cell free supernatants were collected four days post infection (100% lysis of the control cells), and the number of free viral particles was enumerated by a plaque assay. The results of the experiment are presented in Table 5.3. Consistent with the plaque assay, the pN(-) transfected cells produced less infectious virus than the pN(+) cells both of which produced less virus than the pMMTV-Luc transfected cells. There was little difference in the reduction of viral titers between the dex induced and uninduced pN(+) and pN(-) transfected cells. Infection of pN(+) and pN(-) transfected cells eventually resulted in 100% lysis. However, the time required for 100% lysis of pN(+) and pN(-) transfected cells was delayed approximately 24-36 hrs compared to the pMMTV-Luc transfected cells (data not shown).

Table 5.3. Viral growth on control pMMTV-luc transfected cells, and pMeaN(+) and pMeaN(-) transfected cells (12 cell passages)

| | Viral titre (PFU/ml X $10^6$) | | Reduction of viral titre | |
|---|---|---|---|---|
| Cell line | (+) dex | (-) dex | (+) dex | (-) dex |
| Control | 13.1 | 9.4 | _ | _ |
| pMeaN(+) | 4 | 3.1 | 69% | 66% |
| pMeaN(-) | 2.8 | 3.3 | 78% | 64% |

The reduction of viral titres was calculated as percentage of control values. Each test was performed in triplicate, the average PFU/ml is shown. The standard deviation for each measurement was less than 7.

## Discussion

In this report IHNV N transgenes in the sense and antisense orientation were tested for their ability to interfere with IHNV replication. Two methods were used to measure viral interference; a plaque assay, and direct production of free viral particles. In each case, the presence of pN(+) and pN(-) plasmids in transfected cells correlated with a significant reduction in the number of viral plaques or free virus particles caused by IHNV infection.

While the pN(+) and pN(-) cell lines were resistant to IHNV plaque formation, the degree of resistance changed with cell passage. Several explanations could account for the temporal change. It is possible that cellular expression of the viral transgenes decreased with increasing cell passage. It has been reported that methylation of the MMTV promoter in stably transfected cells occurs with cell passage resulting in reduced gene expression from the promoter (Friedman et al., 1989; Darbe and King, 1987). However, treatment of the pN(+) and pN(-) transfected cells with 5'-aza-2'-deoxycytidine, which has been found to partially reverse the effects of methylation (Friedman et al., 1987; Midgeon et al., 1988), did not increase the viral resistance of the pN(+) or pN(-) transfected cells. It is also possible that during

passage some of the pN(+) and pN(-) transfected cells lost their ability to express the virus inhibiting molecule. Uniform expression of viral interfering molecule by a cell population has previously been shown to be a requirement for successful intracellular immunity to reticuloedotheliosis virus (REV) (Federspiel et al., 1990). However, since the temporal change in the degree of immunity was unexpected, and all the cells were used for expansion (to repeat the plaque assay), appropriate experiments could not be conducted to test the second hypothesis and will be pursued in the future.

Despite the discrepancy in the degree of viral resistance observed over time, the pN(+) and pN(-) were nevertheless viral resistant. Similar results have been reported using oligodeoxyriboducleoside methylphosphonates (oligoMP) (Agris et al., 1986) and poly(L-lysine)-conjugated oligodeoxyribonucleotides (PLO) (Lemaitre et al., 1987; Leonetti et al., 1988) complementary to the N mRNA of the rhabdovirus VSV. In each case the interfering oligonucleotide inhibited the titers of VSV by roughly one to two logs. In addition, Lemaitre et al. (1987) reported that a PLO (positive sense) complementary to the VSV genome intergenic region reduced viral titers by two logs.

Thus, there is precedent evidence for both sense and antisense mediated inhibition of rhabdoviral replication. However, as is the case for many studies, it is difficult to ascribe a mechanism of viral interference since a biological assay was used. Still, several observations can be made which will be useful in giving direction to future experiments. First, antisense mediated viral inhibition is consistently more effective than sense inhibition. This has also been observed previously using a similar strategy with HIV (Sczakiel et al., 1990; Joshi et al., 1991; Junker et al., 1994). It will be important to determine if this is due to differences in the ability of the interfering molecule to block a critical step in the IHNV life-cycle or , as Sczakiel et al. (1990) pointed out, due to differences in the accessibility of the interfering molecule or its viral target. Second, interference is not absolute. This was particularly evident when the number of free infectious IHNV particles released from pN(+) and pN(-) transfected cells was measured. In addition, even when no IHNV plaques were present on pN(+) and pN(-) transfected cells, IHNV could be detected in scattered cells by immunohistochemistry. It will be important to determine if this viral "breakthrough" was due to the virus overwhelming the interfering molecule as has previously been suggested (Rhodes and James, 1990; Joshi et al., 1991; Sczakiel et al., 1992;) or rather due to a lack of uniformly expressed interfering molecules.

In summary, the study presented here demonstrates that IHNV infection can be interrupted by expression of the antisense N cDNA in the sense or antisense

orientation. This should provide a basis for future worked aimed at understanding the intracellular life-cycle of IHNV. Further, this strategy may be valuable in gaining further insight of the role and importance of IHNV proteins and RNA needed for viral replication.

## Acknowledgments

This work was supported by United States Department of Agriculture Grants 91-34123-6124 and 92-37204-7997; and Oregon Sea Grant with fund from the National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerece, under grant no. NA89AA-D-SG108, Project no. R/FSD-16.

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# CHAPTER 6
# DETECTION OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS mRNA BY *IN SITU* HYBRIDIZATION

Eric D. Anderson, Craig Cary, J. S. Rohovec, and Jo-Ann C. Leong

Deparment of Microbiology
Oregon State University Corvallis, Oregon

Published July 31, 1991

Proceedings of the Second International Symposium
on Viruses of Lower Vertebrates

## Abstract

Infectious hematopoietic necrosis virus (IHNV) mRNA was detected in steelhead fry using nucleic acid *in situ* hybridization. Digoxigenin-labeled oligonucleotide probes specific for the IHNV nucleocapsid mRNA were used. Asymptomatic, moribund, and dead fish were taken from an experimentally induced IHNV epizootic and prepared for *in situ* hybridization. The optimal conditions for probe concentration, hybridization stringency, washing stringency and color development were determined. IHNV was detected in many organs and tissues as has been found by the isolation of virus in organ extracts. In addition, this technique has led to the identification of the specific cell types which were susceptible to infection in each organ.


## Introduction

IHNV is a rhabdovirus that infects certain species of salmonid fish and is found endemically on the Pacific Coast of the North American continent and Japan. The route of transmission of IHNV in nature is unknown. However, experimental studies suggest that the virus can be transmitted both vertically and horizontally. 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 geographical areas (Holway and Smith, 1973; Wolf et al., 1973). Horizontal water-borne transmission of IHNV has also been demonstrated by a number of investigators (Amend, 1975; Wingfield and Chan, 1970; Mulcahy et al., 1983) In these studies, susceptible fish held in the effluent water from infected fish subsequently became infected with IHNV. The relative importance of the different transmission routes in the maintenance of the virus in nature has been the subject of considerable controversy.

One reason for the continued discussion on the role of vertical or horizontal transmission in the life cycle of IHNV is that a carrier state for IHNV may exist (Amend, 1975). After a typical IHNV epizootic, survivors continue to release virus for approximately one month, at which time they become virus negative by virus isolation assays. However, these survivors may return to the hatchery during spawning and release large quantities of virus in the milt or ovarian fluids. It is unclear

whether these returning adults were IHNV carriers or if they were reinfected with IHNV when they returned for spawning. The work that is reported here was carried out to determine whether sensitive techniques like *in situ* hybridization might be used to detect the virus in "carrier" fish.

## Materials and Methods

### Virus and cells

The Round Butte (RB1) strain of IHNV was isolated in 1975 from adult steelhead trout at Round Butte Hatchery in Oregon. The tissue culture studies were conducted on chinook salmon embryo cells (CHSE-214) which were obtained originally from J. L. Fryer, Oregon State University, Corvallis, Oregon. The cells were grown as monolayers in Dulbecco's MEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 ug/ml streptomyocin (all from GIBCO) For the infected cell cultures used in these experiments, the cells were infected at a multiplicity of infection (MOI) of 0.5.

### Labeling of the N gene probe

The nucleocapsid (N) gene specific oligonucleotide, 5'-CTTGGTGAGCTTCTGTCCA-3', and the non-homologous Lactococcus 16 S rRNA specific oligonucleotide 5'-CTTTGAGTGATGCAATTGCATC-3', were prepared by the Central Service Facility of the Center for Gene Research and Biotechnology, Oregon State University. The N gene probe has been found to be specific for IHNV and also conserved in a wide variety of IHNV isolates (Arakawa et al., 1990). The 16 S rRNA probe will specifically detect *Lactococcus lactis* subsp. *cremoris* (Salama et al., 1991). The 3' end of each probe was enzymatically labeled with digoxigenin with digoxigenin-11-dUTP by terminal deoxynucleotidyl transferase as directed by Boehringer Mannheim. The digoxigenin-labeled probe was able to detect 3 pg of

IHNV cDNA in a dot blot. This was equivalent to approximately $10^5$ copies of viral RNA.

Fish

Steelhead trout (*Oncorhynchus mykiss*) were obtained through the Oregon Department of Fish and Wildlife. The fish, in two groups of 2,000 were infected by water-borne challenge with $10^3$ plaque forming units (PFU)/ml IHNV as described by Engelking and Leong (1989). After 80 days, the total cumulative mortality in the population was 42%. Fish were taken at three day intervals until the onset of mortality, when moribund fish were taken at daily intervals for fixation and subsequent examination by *in situ* hybridization.

Preparation of sections for *in situ* hybridization

Fish that died of an IHNV infection and uninfected control fish were fixed in 4 % paraformaldehyde (prepared in PBS, 0.14M NaCl, 10mM sodium phosphate, pH 7.4), 0.005M $MgCl_2$, for at least 24 h. The fish were then decalcified in Calex Decalcifier (Fisher Scientific) for 24 h. After this, the fish were dehydrated and embedded in paraffin. Sagittal sections were cut with a microtome to 6 μm thickness and the sections were stored for up to two weeks at room temperature.

For *in situ* hybridization, thin sections of fish were deparaffinized in xylene followed by rehydration in ethanol and then equilibrated in 2 X SSC (1 X SSC is 0.15 M NaCl, 0.015M sodium citrate, pH 7.4) for 5 min. The samples were then treated with 0.25% (V/V) acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min and then equilibrated in 5 X SSC.

All samples were prepared for hybridization by soaking in a solution of 5 X SSC, 0.5 mg/ml salmon sperm DNA (Sigma), and 0.25 mg/ml yeast tRNA (Sigma) for 1 h. Thirty nanograms of the digoxigenin-labeled probe were mixed in 35 μl of hybridization solution and added to each slide. The hybridization and subsequent washing conditions were determined experimentally. After washing, the slides were equilibrated briefly in Buffer 1 (0.1 M Tris-OH, 0.15 M NaCl, pH 7.5) and then

blocked for 30 min in Buffer 1 containing 2% sheep serum and 0.03% Triton X-100. The slides were rinsed in Buffer 1 and then incubated with a 1:500 dilution of sheep anti-digoxigenin (Boehringer Mannheim) for 1 h in the blocking solution, containing 1 % instead of 2% normal sheep serum. The slides were then washed two times in Buffer 1 and then soaked in Buffer 2 (0.1 M Tris-OH, pH 8.0) for 5 min.

Color Development

Vector color substrate kit 3 (Vector Laboratories) was made in Buffer 2 according to the manufacturer's instruction and 500 µl was applied to the slides. The slides were stored in a light-tight moist chamber and viewed periodically until a suitable color reaction was observed. The slides were rinsed in distilled water and mounted in crystal/mount (Biomeda).

## Results and Discussion

An understanding of the pathogenesis of IHNV at the cellular level has been greatly improved by the development of techniques and specific probes to detect IHNV directly in tissues (Yamamoto et al., 1990; Arakawa et al., 1990). Analysis of nucleic acid extracted from tissue specimens by the polymerase chain reaction has also proven to be very sensitive for detecting low copy numbers of viruses in these materials. However, identification of the specific cell types that were infected is not possible with this procedure. Nucleic acid *in situ* hybridization and immunocytochemistry remain valuable techniques for localizing viruses and viral gene products in individual cells. We report here a method for detecting the IHNV N gene mRNA in infected cells and fish tissue.

In the initial series of experiments, hybridization stringency was investigated to determine the hybridization temperature at which cross-reactivity of the IHNV N mRNA specific probe with uninfected tissues was eliminated. The stringency was controlled either by increasing concentrations of formamide in the hybridization fluid at 42° C or by increasing the temperature of hybridization. When concentrations of formamide above 15 % were used, it was clear that hybridization of the

Table 6.1 Serial sections of IHNV (RB1) infected or control fry were hybridized at 42° C in increasing concentrations of formamide to determine the optimum hybridization stringency

| Fish section | Percent formamide | Hybridization Control | Hybridization Infected |
|---|---|---|---|
| 1 | 0 | + | + |
| 2 | 1 | ND | + |
| 3 | 5 | ND | + |
| 4 | 10 | ND | + |
| 5 | 15 | ND | +/- |
| 6 | 20 | +/- | +/- |
| 7 | 30 | ND | - |
| 8 | 50 | +/- | - |

ND, not done.

oligonucleotide probe was reduced (Table 6.1) The disruption of stable duplexes in the presence of formamide was similar in both infected and uninfected fish. In contrast, there was a clear difference in the temperature at which the duplexes were disrupted when the formamide was omitted from the buffer and the temperature was varied. Hybridization of the non-homologous probe was reduced at 48° C and completely disrupted at 50° C. The IHNV specific probe formed stable duplexes with the IHNV N mRNA at 48° C.

The optimal washing temperature was also determined to ensure that the IHNV N gene probe did not bind nonspecifically to cellular nucleic acid. After several washes in SSC as described in figure 6.1, serial sections of an infected fry were subjected to a wash in 0.1 X SSC at different temperatures for 1 h. Hybridization with the non-homologous probe was decrease at temperatures above 48° C and completely eliminated at 52° C. In contrast, the homologous probe did not exhibit reduced hybridization until the wash was performed at 55° C and there was complete loss of hybridization at 60° C.

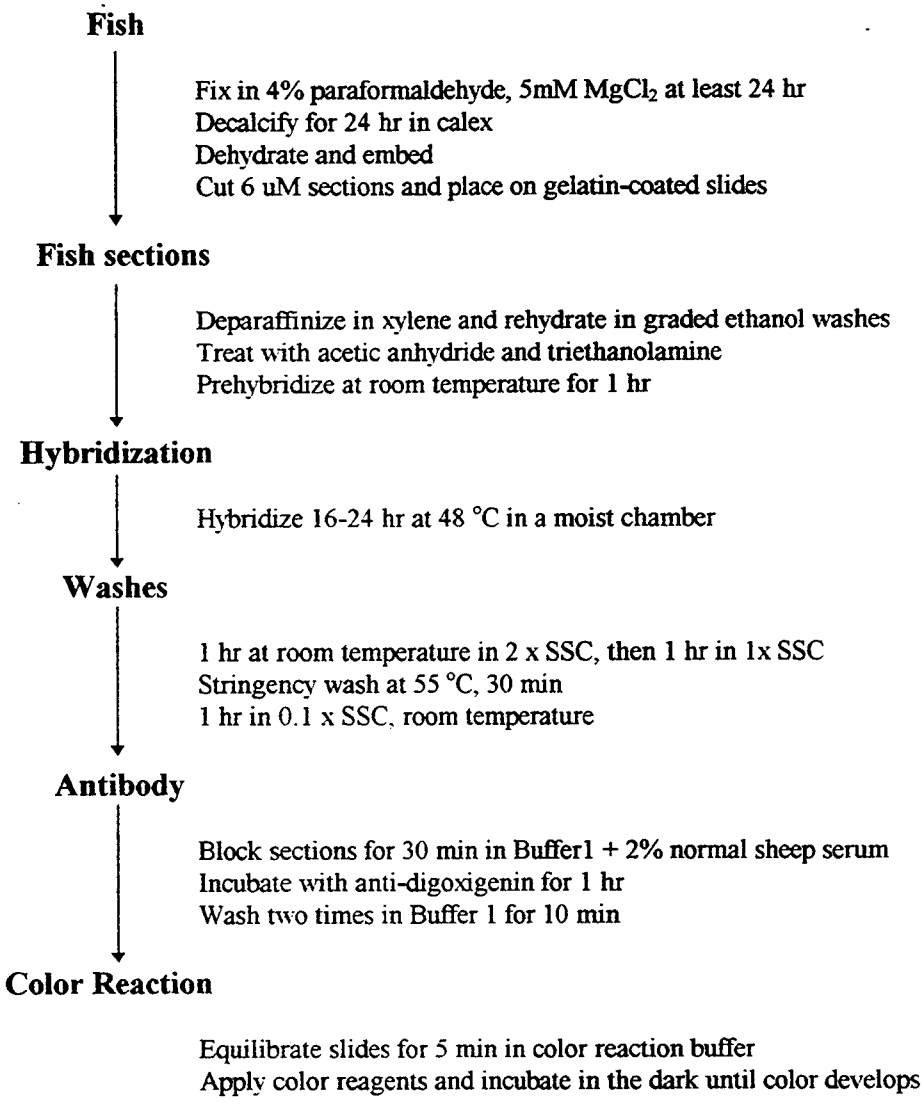



Figure 6.1. Outline of procedure for in situ hybridization

Table 6.2 Serial sections of IHNV (RB1) infected fry were hybridized with a non-homologous Lactococcus 16 S rRNA specific probe or an IHNV N mRNA specific probe to determine the optimum temperature of hybridization. The hybridization buffer contained no formamide.

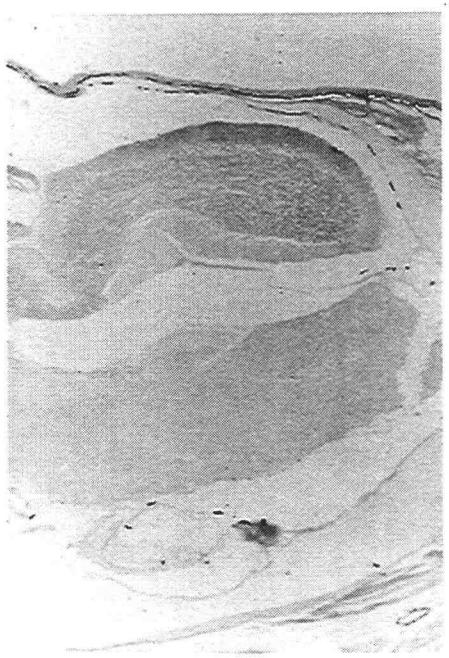
| | | Hybridization | |
|---|---|---|---|
| Section | Temp., °C | Lactococcus | IHNV N |
| 1 | 42 | + | + |
| 2 | 48 | +/- | + |
| 3 | 50 | +/- | + |
| 4 | 52 | - | + |
| 5 | 55 | - | +/- |
| 6 | 60 | - | - |

Table 6.3 Serial sections of IHNV (RB1) infected fry were washed at increasing temperatures in 0.1 x SSC to determine the optimum stringency needed to eliminate non-specific probe hybridization.

| | | Hybridization | |
|---|---|---|---|
| Section | Temp., °C | Lactococcus | IHNV N |
| 1 | 42 | + | + |
| 2 | 48 | + | + |
| 3 | 50 | +/- | + |
| 4 | 52 | +/- | + |
| 5 | 55 | - | +/- |
| 6 | 60 | - | - |

Figure 6.2 Localization of IHNV in fish sections by in situ hybridization. Histological section of the cerebellum and medulla oblongata of a steelhead fry which died 10 d after an IHNV challenge. The non-homologous probe (A) did not hybridize with the brain tissue. The IHNV specific probe (B) hybridized to N mRNA strongly in the molecular layer (upper arrow) of the cerebellum with a weak reaction in the granular layer immediately under the molecular layer. There is also a weak hybridization in the medulla oblongata which is indicated by the lower arrow

(A)



(B)

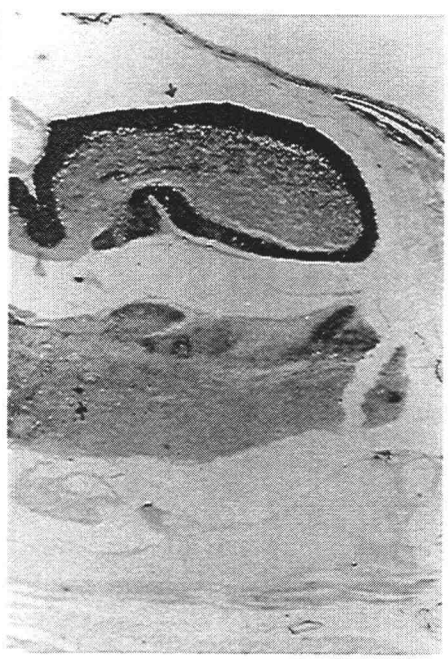


Figure 6.2

These results led to the development of a procedure for detecting the IHNV N mRNA *in situ* by hybridization with an oligonucleotide probe. This procedure is outlined in figure 6.1. The specificity of the reaction was determined by the washing conditions used to disrupt nonspecific hybridization (Table 6.3) The method calls for hybridization of a digoxigenin-labeled oligonucleotide probe to fish sections at 48° C without formamide for 24 h which is followed by a stringency wash at 55° C.

The pathogenesis of IHNV infection were examined by *in situ* hybridization in 10 infected fish taken at the height of an IHNV epizootic. IHNV was located in the brain, gills, eyes, kidney, heart, liver, spleen, pyloric caeca, pancreas, stomach, intestine, skin, and muscle. Reactions were particularly strong in the anterior kidney tubules and hematopoietic tissues. The parenchymal tissue of the liver was positive and the epithelial cells near the surface of the skin were also positive. The most conspicuously positive hematopoietic cells were neutrophils, unidentified lymphocytes and, rarely, erythrocytes. In the brain, the cerebellum was heavily infected in the molecular layer with diffuse infection in the granular layer (Figure 6.2). The brain infection may be one reason for the erratic swimming pattern observed in infected fish. In addition, preliminary results suggest that the medulla oblongata and the spinal cord are also sites of infection. These sites may account for the development of scoliosis which is often associated with survivors of an IHNV epizootic (Busch, 1983)

The fish examined in this study were taken at the height of an IHNV epizootic, and it was possible to discern sites of infection and cell types susceptible to IHNV infection. However, it was not possible to determine the initial sites of infection or the course taken in the spread of the infection in the animal. Those studies are in progress. We are also determining whether survivors of an IHNV epizootic can become lifelong carriers of the virus.

## Acknowledgments

This publication is the result of research sponsored by Bonneville Power Administration Contract DE-PS79-88B92431 (G. R. Bouck and R. Morinaka served as the Contracting Office Technical Representatives on the project); the United States Department of Agriculture to the Western Regional Aquaculture Consortium under grant no. 87-CRSR-2-2319 and 88-38500-4027; Oregon Sea Grant with funds from

the National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerce, under grant no. NA85AA-D-SG095 (project no. R/FSD-13 and R/FSD-15); and from appropriations made by the Oregon State Legislature. We wish to thank Barbara Drolet and Dr. Linda Bootland for confirming the nucleic *in situ* hybridization results with immunohistochemistry and for providing infected fry and tissue culture cells. Oregon Agricultural Experiment Station Technical Paper No. 9685.

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# CHAPTER 7
# THESIS SUMMARY

In this thesis a molecular study of the cytopathic mechanisms of infectious hematopoietic necrosis virus (IHNV) *in vivo* and *in vitro* was reported. The approach was to develop and use methods that interfered with IHNV, or localized IHNV *in vivo*, to better understand the cytopathicity of IHNV. Several new pieces of information regarding IHNV cytopathicity were discovered. In addition, several techniques were developed which should be useful to future scientific endeavors.

## Genetic immunization

The finding that naked plasmid DNA can be easily delivered to somatic cells *in vivo*, and is capable of synthesizing its encoded proteins (Wolff et al., 1990), has led to the use of naked DNA for vaccination. In this thesis the feasibility of using such an approach was investigated. The ultimate goal was to determine if plasmid DNA encoding the IHNV glycoprotein (G) or nucleoprotein (N) could immunize fish against IHNV and if so, determine the importance and interaction of these proteins in evoking an immune response.

In Chapter 4, it was demonstrated that naked DNA delivered by a simple injection protocol could be taken up and efficiently expressed by a number of tissues. It was found that gene expression was of prolonged duration. In addition, it was found that the DNA did not integrate into the genome nor was it replicated. Taken together, these results suggest that in a number of instances, including promoter analysis and functional analysis of protein synthesis, the direct injection of naked DNA could replace or complement the development of transgenic fish. This technique could also be used as an approach toward gene therapy, for instance using cytokines or growth enhancing genes .

Future research on somatic cell delivery of DNA should include the following: 1) Determination of the mechanism(s) of DNA uptake by cells *in vivo*. 2) Determination of the impact of the plasmid DNA on the health of the transfected organism. 3) Development of new DNA delivery systems. 3) Development of

plasmids capable of integrating in a site specific manner. 4) Identifying promoters which express the transgenes in a tissue specific manner.

In Chapter 5, it was reported that the DNA vaccine pCMV4-G injected into fish either alone or in combination with pCMV4-N promoted both a G-specific immune response and immunized fish against IHNV. This work confirms that the IHNV G protein is capable of inducing a protective immune response in fish. The plasmid pCMV4-N did not induce an antibody response nor did it protect fish against IHNV. However, pCMV4-N did appear to augment the humoral immune response against the IHNV G protein in fish injected with a combination of the two DNA vaccines.

Future work on the DNA vaccines should include the following: 1) Determining the role of the N protein in the immune response of fish. 2) Determining whether other vaccination protocols (quantity of DNA, boosters, method of delivery) alter the type of immune response 3) Determine the duration of the immune response. 4) Develop practical methods for the mass vaccination of fish.

## Intracellular immunization

Izant and Weintraub (1984) were the first to show that endogenously synthesized, artificially introduced, expression of antisense RNA could interfere with its complementary RNA in eukaryotic cells. Since this initial report, antisense and sense suppression of viral genes has been successfully employed using both plant and animal model systems.

In Chapter 5, this technology was used to interfere with the life cycle of IHNV. Both sense and antisense expression of the IHNV N gene in transfected fish cells produced a state of intracellular immunity. The immunity was temporal decreasing with cell passage. Cellular antisense expression of N was more efficient than sense expression in providing immunity to IHNV. Taken together these results suggest that viral synthesis of the N mRNA or protein is an integral part of the replication cycle of IHNV.

Areas of future research should include: 1) Comparative study of the other IHNV genes ability to interfere with IHNV replication. 2) A structural analysis of the portion(s) of the antisense and sense N RNA responsible for mediating the

resistance. 3) Development of transient assays allowing the circumvention of the tedious and time consuming process of selecting IHNV resistant cell lines.

In Chapter 5, several techniques were developed which were not available previously to those using fish cell culture. Specifically, a highly efficient transfection protocol was established. Using lipofection , transient transfection efficiency approaching 30% were achieved. by optimizing both cell culture conditions and ratios of lipid (Lipofectamine, Gibco) and DNA. Prior to this technology, transfection efficiencies of 1% or less were common. In addition , protocols for the maintenance and long term culture of transfected, antibiotic resistant cells, was established.

## Localization of IHNV *In Vivo*

It is unclear whether fish surviving an IHNV infection become life long carriers or recover and thereafter are virus free. There has been only one report showing that fish could become life long carriers of IHNV (Amend et al. 1976) However, numerous reports have demonstrated that infectious virus can not be isolated from fry surviving an IHNV epizootic after 40-50 days. Nevertheless, there is a possibility that IHNV persist at levels below that detectable by tissue culture assays.

Thus, the development of *in situ* hybridization, reported in Chapter 6, should provide an important tool for the study of the IHNV life cycle. The technique is sensitive and can be used to determine which cell types are infected with IHNV. Using a similar technique, *in situ* immunohistochemistry, Drolet et al. (1995) have shown that IHNV can persist in fish. It is still uncertain in which tissues IHNV is replicated during a persistent infection. This question could likely be answered using *in situ* hybridization since it allows identification of both viral genome RNA and mRNA.

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