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Title: Molecular Cloning and Analysis of the Infectious Hematopoietic Necrosis Virus Genome and Development of a Subunit Vaccine.

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Complementary DNA (cDNA) clones were generated from the RNA genome of infectious hematopoietic necrosis virus (IHNV) by using random DNA oligomers to prime first strand synthesis. These clones were mapped to their respective locations on the genome and used to determine the nucleotide sequence of the nucleocapsid (N) gene and the intergenic region between the glycoprotein (G) and the nonvirion (NV) genes. Interesting features of the N gene sequence include short homologies with N genes of other rhabdoviruses at the 5' and 3' non-coding termini of the mRNA, as well as an exceptionally long 5' non-translated region of the mRNA suggesting a leader RNA may be coupled to the N mRNA. The IHNV N protein coding region shows no significant homology with other rhabdovirus N genes at either the nucleotide or amino acid

level. The intergenic region between the G and NV genes also shares some sequence homology with those reported for other negative strand RNA viruses.

In addition, plasmid vectors were constructed which expressed an antigenic determinant of the glycoprotein gene of IHNV as a fusion protein with the trpE protein of Escherichia coli. Insertion of Sau3AI fragments from the IHNV glycoprotein gene into trpE expression plasmids led to a fusion protein containing a hydrophilic segment of 104 amino acids from the middle portion of the viral glycoprotein. After induction with indoleacrylic acid, fusion proteins accumulated stably in the E. coli cells and accounted for approximately 10% of the total protein in the cell. Immunization trials in fish with the crude bacterial lysate containing the fusion protein have indicated that the trpE-glycoprotein fusion protein produced in bacteria does induce protective immunity.

Molecular Cloning and Analysis of the Infectious Hematopoietic Necrosis Virus Genome and Development of a Subunit Vaccine

by

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PREFACE

The contributions of the co-authors of the manuscript "Expression in *E. coli* of an epitope of the glycoprotein of infectious hematopoietic necrosis virus which protects against viral challenge" are as follows. Mark Engelking was responsible for preparation of anti-IHNV sera and anti-IHNV glycoprotein sera, and was instrumental in the fish immunization trials. Scott Manning was responsible for the colony immunoblot assay, as well as some SDS polyacrylamide gels. The author would also like to emphasize that plasmid N2216 used for the sequencing of the nucleocapsid gene was generated by Gael Kurath and Josette Feyereisen.

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INTRODUCTION

Infectious hematopoietic necrosis virus, a pathogen of young salmon and trout, is a member of the Rhabdoviridae family. The virus consists of a single-stranded, nonsegmented RNA genome of negative polarity approximately 11,000 bases long (3,33,43). IHNV transcribes six monocistronic mRNAs encoding five structural proteins and a non-structural protein (32). These genes have been mapped to the genome in the order 3'N-M1-M2-G-NV-L 5' (33), where N is the nucleocapsid protein, M1 and M2 are matrix proteins, G is the surface glycoprotein, NV is the non-virion associated protein of unknown function, and L is the viral polymerase. The morphology and protein structure of IHNV is similar to the prototype rhabdoviruses, vesicular stomatitis virus (VSV) and rabies virus (40,3), although neither VSV or rabies virus has a gene analogous to the NV gene of IHNV.

IHNV is endemic to the Pacific Northwest of North America, and the high mortality rate of viral infected fish results in severe economic problems for the aquaculture industry (23). Contaminated water sources can transmit the virus to fish hatcheries where susceptible fish become

infected, die, and disseminate the virus throughout ponds resulting in an epizootic (48). Presently, there is no treatment for fish when an outbreak occurs, therefore, the method of control is the destruction of all potentially infected fish and decontamination of the ponds (23).

It is known that the virus is transmitted horizontally through water by infected fish (48), and there is strong evidence for vertical transmission from infected parents (12). The most common form of the disease is the onset of rapid and acute mortalities in young fish, and there have been unpublished reports of a chronic type of the disease in older fish. In addition, IHNV isolation is often made from asymptomatic adult fish, suggesting a latent carrier stage of the virus (23). At present, there are no methods to detect such a phase in potential carrier fish, thus the viral life cycle remains unknown.

The studies presented here address these concerns discussed above. By investigating the molecular biology of IHNV, we should gain new insight into the mechanisms of viral replication and protein function. Elucidation of the viral RNA sequence would be valuable in constructing probes to search for nucleic acid of viral origin in fish tissue, possibly providing answers to questions surrounding the IHNV life cycle. Such information may help solve the epidemiological questions surrounding this virus and therefore aid in the prevention of epizootics in hatcheries. In addition to the applied aspects that can be developed, the information garnered from basic study of the molecular biology of IHNV can be used to answer fundamental questions concern- ing the evolutionary relationships of this virus to other similar viruses. This thesis contains data relating to the genomic

structure of IHNV and the application of other molecular studies to develop a vaccine against the disease in fish using recombinant DNA techniques.

LITERATURE REVIEW

Epizootiology

The establishment of a viral agent responsible for the necrosis of hematopoietic tissue in salmonid fish was first reported when the virus was isolated from diseased salmon at an Oregon fish hatchery in 1958 (63). A prior report, describing what is now believed to be the same disease, was published in 1953 citing evidence of a viral pathogen at a salmon hatchery in Washington (53). Since these early observations, this virus has been detected in salmonid fish in Oregon, Washington, California, Alaska, western Canada, Idaho, and Japan. The name infectious hematopoietic necrosis virus was proposed by Amend (2) and is now widely accepted.

Virus can be isolated from adult fish, although the disease state is generally limited to juveniles eventually causing death (64). The disease has, however, been observed as an acute or chronic infection in larger fish including adults. The severity of this disease in larger fish is exemplified by the frequent outbreaks among yearling rainbow trout in the Hagerman Valley of Idaho. An IHNV strain of greater virulence for this species has been attributed as the cause of such epizootics (36). IHNV outbreaks involving yearling fish of other species including sockeye, kokanee, and chinook salmon have been documented (11,60). External signs of IHNV infection include exopthalmia, distension of the abdomen, petechial hemorrhaging at the base of the fins, and erratic swimming patterns, while internally there is massive hemorrhaging and necrosis of the kidney, pancreas, and spleen (64).

The epizootiology of IHNV outbreaks is poorly understood. The virus can be transmitted from infected fish via the water (horizontal transmission) (48), and there is evidence indicating transmission via infected eggs and/or sperm from asymptomatic carriers of the virus (vertical transmission) (2). The viral entry site is unknown and may be different depending on the host fish, the size of the fish, and routes of exposure. In vertical transmission, it is suspected that the presence of virus in the ovarian fluid of the female may be sufficient enough to induce infection in embryos or among newly hatched fry. Subsequently, the dead or dying embryos or fry become potential sources for the emergence of an epizootic (2,49).

The existence of a latent stage of the virus in a carrier fish has been suggested. Studies by Amend showed that mature rainbow trout that survived an IHNV outbreak as alevins and were maintained in virus-free water, expressed virus at spawning (2). However, no studies are available that confirm these results and the question of a host carrier stage in the viral life cycle remains unanswered. Mulcahy and Pascho have shown that prespawning salmon and trout that are negative for virus in tissues and sex fluids show high levels of virus after spawning (49). Whether the appearance of virus is due to expression of latent virus or reinfection is not certain.

Presently, the only method for control is avoidance, as there is no commercially available prophylactic or therapeutic treatment. Strategies for virus control include 1) using gametes from adults determined to be free of virus, 2) egg disinfection by exposure to iodophore, 3) incubation of eggs and fry in virus-free water, 4) elimination or prevention of the migration of infected fish into hatchery water

reservoirs, and 5) strict disinfection procedures for hatchery equipment and facilities. In the advent of an IHNV epizootic, the only method for virus control is the destruction of all potentially infected fish stocks and disinfection of the ponds.

Virus properties

Investigation of the physical properties of the IHN virus showed that it is characterized by a bullet-shaped morphology (3) consisting of a lipid envelope containing a non-segmented, single-stranded RNA genome of negative polarity with a sedimentation coefficient of 42S (26,43), consistent with the properties associated with the Rhabdoviridae. The virion is composed of five structural proteins: a polymerase (L), a surface glycoprotein (G), a nucleocapsid protein (N), and two matrix proteins (M1 and M2), relating the IHN virus more closely to rabies virus than the prototype rhabdovirus, vesicular stomatitis virus (VSV) (40).

IHNV has been shown to be distinct from three known fish rhabdoviruses, spring viremia of carp virus, viral hemorrhagic septicemia virus, and pike fry rhabdovirus, by cross-neutralization comparisons with polyclonal rabbit serum (26). Early serological studies showed differences between IHNV isolates, but failed to demonstrate the presence of distinct serotypes using specific low-titered antisera (42). Arakawa et al., however, found evidence for four distinct groups among six strains of IHNV using two different neutralizing monoclonal antibodies (5). Studies by Hsu et al. have indicated that strains of IHNV can be differentiated by the electrophoretic patterns of the structural proteins (28). Five groups of IHNV strains have been

typified based on the differences in the electrophoretic mobility of the N and G proteins in sodium dodecyl sulfate denaturing polyacrylamide gels. Relationships between the groups have yet to be resolved.

Immunity studies

Though there have been studies concerning the immune response to an IHNV infection in fish, the mechanisms of such a response remains unknown. In experimentally infected trout and salmon, a cellular response was observed by Klontz et al., indicated by an initial accumulation of macrophages and lymphocytes at the injection site, followed by an increase of these cells in the anterior kidney (30). Humoral responses have also been detected. Adult rainbow trout intraperitoneally injected with IHNV developed virus antibodies within 54 days (4). Serum collected from these fish protected juvenile fish from IHNV infection by passive transfer.

An IHNV isolate from rainbow trout adapted in tissue culture was tested for attenuation and vaccine potential in juvenile sockeye salmon by Fryer et al. (19). Protection to a virus challenge was conferred to sockeye salmon using this isolate, however in later studies, it was demonstrated that the virus maintained its virulence for rainbow trout and also failed to protect chinook salmon from subsequent infection.

Recent studies have shown the protective nature of purified IHNV glycoprotein in salmonids to a virus challenge (Engelking and Leong, manuscript submitted). This vaccine was effective when administered either by immersion or injection, and showed cross protection in laboratory challenges to three other strains of IHNV. Although an efficient vaccine, virion-purified glycoprotein is not of practical

application because of the slow growth rate of the IHN virus and its low yield of progeny virus in tissue culture.

Molecular studies

The genomic organization of IHNV consists of an RNA genome of approximately 11,000 bases, encoding six proteins in the order 3'N-M1-M2-G-NV-L 5' (33). NV encodes a non-structural protein of approximately 12,000 daltons and the function is not known (32). The capacity of this virus to encode six proteins, rather than five, makes it significantly different from other rhabdoviruses studied. In vitro transcription systems describing the mRNA species of IHNV have been reported demonstrating the RNA polymerase activity (41, Kurath and Leong in press), and hybrid selection studies determined the respective coding assignments of each mRNA (33).

The IHNV gene order is similar to that of vesicular stomatitis virus (VSV) and rabies virus, i.e. 3' leader RNA-N-M1(NS)-M(2)-G-L 5' (1,7), where NS is for non-structural protein (a misnomer because the NS protein of VSV is now known to complex with the N and L proteins) (17). A leader RNA of 47 bases is transcribed separately from the 3' terminus of the VSV genome and is believed to play a major role in VSV transcription and/or replication (16,8). IHNV leader RNA has not been detected, although there is indirect evidence that it may be transcribed with the N mRNA reported here. It would be unlikely that IHNV does not possess a leader RNA, because similar leader RNAs have been described in rabies virus (34), sonchus yellow net virus (a plant rhabdovirus) (66), and spring viremia of carp virus (52).

Despite the extensive studies on the molecular biology of VSV and

rabies virus, there is still insufficient evidence for a single model of rhabdoviral transcription and replication, although VSV is known to transcribe its genes sequentially (1,7). Currently, there are three models for rhabdoviral transcription, reviewed by Banerjee (8), that are consistent with sequential gene transcription. They are: the cleavage model which involves the formation of single mRNAs by the cleavage of a growing RNA chain initiated at the 3' genomic terminus; the stop-start model, which proposes the initiation of transcription at the 3' end of the genome, and the subsequent reinitiation of transcription for each downstream gene following the termination of transcription of the preceding gene; and the multiple initiation model, which suggests that transcription is initiated at multiple sites on the genome, but the elongation into a mature mRNA depends on the prior transcription of the previous upstream gene.

All molecular studies done on rhabdoviruses report some divergence in the nucleotide and amino acid sequences of genes between species, however RNA leader sequences and intergenic regions, considered to play an integral role in transcription and replication, remain conserved (58,21,66). This may suggest that although the Rhabdoviridae differ widely in their host range and host cell interaction, these viruses share similar mechanisms of transcription and replication.

It is hoped that by determining the genomic base sequences of IHNV, we can use that information to conduct experiments involving genetic probes, development of virus mutants, and expression of viral proteins to gain more insight into the mechanisms of rhabdoviral replication and pathogenesis. This may help solve some of the biological questions posed by this enigmatic virus such as transmission, carrier states,

life cycle, as well as treatment and prevention of the disease.

THE NUCLEOTIDE SEQUENCE OF THE NUCLEOCAPSID GENE AND GLYCOPROTEIN - NONVIRION GENE INTERGENIC REGION OF IHNV

ABSTRACT

cDNA clones were generated from the RNA genome of infectious hematopoietic necrosis virus (IHNV) by using random DNA oligomers to prime first strand synthesis. These clones were mapped to their respective locations on the genome by the use of DNA probes derived from viral mRNAs. The complete nucleotide sequence of the nucleocapsid (N) gene was determined as was the intergenic region between the glycoprotein (G) and non-virion (NV) genes. Interesting features of the IHNV nucleocapsid gene sequence include short homologies with N genes of other rhabdoviruses at the 5' and 3' non-translated termini of the mRNA, as well as an exceptionally long 5' non-coding region of the mRNA, suggesting a leader RNA may be coupled to the N mRNA. The IHNV N protein coding sequence shows no significant homology with other rhabdovirus N genes at either the nucleotide or amino acid level. The intergenic region between the G and NV genes also shares some homology with those reported for other negative strand RNA viruses. The determination of the nucleotide sequences of IHNV genes and intergenic regions will be useful for studying the mechanisms of rhabdoviral transcription and replication.

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) belongs to the family Rhabdoviridae and infects salmonids, usually causing a fatal disease (2). IHNV is similar to other rhabdoviruses with its bullet-shaped structure and single-stranded, negative sense RNA genome of approximately 11,000 bases (3,43,32). The virion consists of a polymerase (L), two matrix proteins (M1 and M2), a surface glycoprotein (G), and a nucleocapsid protein (N) (40). IHNV has an optimal growth temperature of 12-15^{o}C in its piscine host and, having a slow growth rate, produces low yields of progeny virus (38). A significant difference from other rhabdoviruses studied is that IHNV produces six monocistronic mRNAs during transcription. Five of these mRNAs encode the viral structural proteins and a sixth mRNA encodes a unique non-virion protein, NV, the function of which is unknown (33). Although the physical map of the virus has been determined (33), and an in vitro transcription system for IHNV has been described (41, Kurath and Leong, in press), the contributions of these genes in the transcription and replication of IHNV and the regulatory sequences governing these have yet to be determined. Therefore, to further these studies, we have undertaken the cloning of the IHNV genome by using random DNA oligomers to prime cDNA synthesis and generate clones that span the entirety of the genome. We report the complete nucleotide sequence of the N gene including the observation of a long non-coding region of the 5'end of a mRNA clone. We speculate that this region may

be similar to a leader RNA found in other rhabdoviruses. We also report the nucleotide sequence of the intergenic region between the G and NV genes and the comparison to other negative stranded RNA viruses.

MATERIALS AND METHODS

Cells and Virus. The chinook salmon embryo cell line (CHSE-214) used for propagating IHNV was provided by J.L. Fryer, Oregon State University, Corvallis. The cells were grown as monolayers in minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal calf serum (Gibco Laboratories), 2 mM L-glutamine (Gibco Laboratories), 100 IU of penicillin (Gibco Laboratories) per ml, and 100 ug of streptomycin (Gibco Laboratories) per ml. The IHNV used in this study was isolated in 1975 from an adult steelhead trout at the Round Butte Hatchery in Oregon. The fish cells were infected with IHNV at a multiplicity of infection of 0.001 and incubated at 15°C for 7 days. At that time the supernatant was harvested and centrifuged at 2,500 x g for 10 min at 4°C. The cell-free supernatant contained approsimately $1x10^8$ 50% tissue culture infective doses per ml. The virus was purified by sucrose gradient centrifugation as described previously (38).

IHNV genome RNA purification. Purified virions were lysed by suspension in 1 ml STE (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) containing 1% SDS and 1 mg RNAse free pronase (Calbiochem, La Jolla, CA) and incubation at 37°C for 1 h. The mixture was extracted twice with phenol followed by one extraction with chloroform. The RNA was precipitated by addition of 1/10 volume of 2.5 M sodium acetate pH 5.2 and 2.5 volumes of cold 95% ethanol, incubated at -70°C for at least 1

h after which the RNA was collected by centrifugation, washed and dried according to standard procedures (45). The genomic RNA was analyzed for purity and stability by observation of an intact 42S band after agarose gel electrophoresis in the presence of methyl mercuric hydroxide (Alfa, Danvers, MA), an RNA denaturant (6). One liter of infected cell tissue culture fluid yielded 10–15 ug of genomic RNA.

Purification of RNA from virus infected cells. Monolayers of CHSE-214 cells were infected with IHNV at a multiplicity of infection of 1 in the presence of actinomycin D (Calbiochem) and allowed to incubate at 16^{o}C for about 24-28 h at which time cytopathic effects (CPE) were visible in approximately 25% of the cells. The cells were gently scraped from the walls of the tissue culture flask with a sterile rubber policeman and the cell pellet collected by mild centrifugation. The cell pellet was dispersed by homogenization, and the RNA purified by the guanidinium/cesium chloride method of Glisan et al (22) and Ullrich et al (61). Polyadenylated RNA species (mRNAs) were isolated by passage of the RNA preparation through an oligo-(dT) cellulose (Sigma, St. Louis, MO) column and the subsequent elution of the poly-A tailed RNAs as described by Maniatis et al (45).

Synthesis of genomic cDNA clones. cDNA copies of genomic fragments were generated using the following conditions. In a 50 ul reaction, 1 ug of 42S genomic RNA was denatured by the addition of 10 mM CH_3HgOH at room temperature for 5 min. Then 50 uCi of ^{32}P dTTP (S.A. 3200 Ci/mmole) (New England Nuclear, Boston, MA), 2 ul placental RNAse inhibitor (Promega, Madison, WI), and 2 ul 700 mM B-mercaptoethanol

were added and the mixture was allowed to incubate for 5 min at room temperature. The first strand cDNA reaction was carried out in the presence of 50 mM Tris (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 1 mM dGTP, 1 mM dCTP, 1 mM dATP, 0.1 mM dTTP, 500 ng calf thymus primer (Sigma), and 40 U of avian myeloblastosis virus (AMV) reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) at 42°C for 2 h. The RNA:DNA hybrid strands were extracted with phenol/chloroform, precipitated with ethanol, washed, and dried by standard procedures. Second strand cDNA synthesis was performed in a 100 ul reaction containing 100 mM HEPES (pH 6.9), 4 mM $MgCl_2$, 15 mM B-mercaptoethanol, 70 mM KCl, 0.1 mM dATP, 0.1 mM dTTP, 0.1 mM dGTP, 10 uCi ^{32}P dCTP, 60 U *E. coli* DNA polymerase I (BRL), 1 U RNAse H (BRL), 0.02 U T_4 DNA ligase (BRL) at 12°C for 1 h, and then at room temperature for 1 h. The double stranded cDNA was tailed with approximately 20 dCTP residues and was annealed to similarly dGTP-tailed pUC8 vector linearized with PstI (BRL). The resultant recombinant plasmids were transformed in *E. coli* strain SC181, and plated onto agar containing 100 ug/ml ampicillin.

Colony blot hybridization. Transformant colonies were screened for the presence of viral inserts by colony blot hybridizations. The colonies were transferred to nitrocellulose filters overlaid on agar plates containing ampicillin by the replica plating method of Grunstein and Hogness (24) or by direct transfer with sterile toothpicks. The replica plates were incubated overnight at 37°C to allow the cells to grow. The cells were lysed by treatment with 10% SDS followed by 0.5 M NaOH. The filters were then washed, dried, and placed at 80°C to allow the exposed DNA to bake onto the nitrocellulose. The filters were then

hybridized to a ^{32}P end-labeled genomic probe according to standard methods (45). The probe was made by end-labeling the 3' terminus of the IHNV genome with ^{32}P cytidine 3', 5'-bis (phosphate) using RNA ligase (BRL). When hybridization of the labeled probe to the filters was complete (16 h), recombinant colonies were detected on Kodak X-AR film exposed to the filters.

Recombinant plasmid screening. Colonies containing recombinant plasmids detected by colony blot hybridization were transferred to an agar plate containing 100 ug/ml ampicillin and allowed to grow overnight at 37°C. The colonies were transferred by sterile toothpicks and shaken vigorously in a lysing solution of 0.5% SDS, 50 mM NaOH, 5 mM EDTA, and 2% ficoll and incubated at 68°C for 30-60 min. The samples were loaded onto a 1% agarose gel and subjected to electrophoresis using a Tris-acetate buffering system (45). After electrophoresis, plasmid DNA could be observed under ultra-violet light after prior staining with ethidium bromide.

Plasmid preparations. Large scale plasmid preparations were performed by the method of Holmes and Quigley (27) and purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (45). Mini-preparations of plasmid DNA were made using the alkaline lysis of Birnboim and Doly (10). Large scale plasmid preparations yielded approximately 300-500 ug DNA, and the smaller scale preparations commonly yielded approximately 5-10 ug of plasmid DNA. Both procedures resulted in DNA that was pure enough for restriction enzyme digestions and for subcloning into different vector systems.

Identification of inserts by Northern blot analysis. RNA isolated from IHNV infected CHSE-214 cells was separated electrophoretically under denaturing conditions in agarose gels using methyl mercuric hydroxide (6). The distributed RNA was subsequently transferred electrophoretically to nylon hybridization transfer membranes (Pall, Glen Cove, NY) using an Electroblot apparatus (E-C Corp., St. Petersburg, FL). Following RNA transfer, the membranes were incubated at 80^{o} to fix the RNA. The membranes were subjected to hybridization to labeled probes derived from the recombinant plasmids according to standard procedures (45). Radioactively labeled probes were made by nick translation of the plasmids containing inserts of viral origin according to the method of Rigby et al. (50).

Southern blot analysis. The inserts of the recombinant plasmids were excised from the plasmid vector by digestion with the restriction enzyme PstI and separated by agarose gel electrophoresis as described above. The DNA was transferred to nitrocellulose filters by the method of Southern (55). Hybridization to radioactively labeled probes was performed as described above.

DNA sequencing. Nucleotide sequence determination was performed by the Sanger dideoxy chain termination method (54) after subcloning the DNA fragments into the appropriate M13 sequencing vector (47).

RESULTS

Detection and analysis of recombinant colonies. In this cloning experiment, there were a total of 472 transformant colonies screened for viral inserts of which 80 contained inserts in the range of 200 to 1800 base pairs (bp). The colonies containing viral inserts were detected by hybridization to radioactively labeled IHNV genome RNA as shown in Figure 1. The intact genomic RNA was used as a probe because it contains all the genetic information of the virus and thus will identify any clone of viral origin. The plasmids of the "positive" colonies were then analyzed by agarose gel electrophoresis to determine their relative size. Figure 2 shows the recombinant plasmids in relation to the parental plasmid, pUC8. The mobility of each plasmid gives an estimate of the size of the insert each carries when compared to the standard pUC8. Based on size analysis, plasmids with the largest inserts were chosen for subsequent analysis.

Genomic mapping of recombinants. Plasmid preparations of various clones were made and insert sizes were more accurately determined after excision of the insert from the vector with PstI and subsequent gel electrophoresis analysis. Southern blot analysis was performed using IHNV mRNA clones of known genomic location as probes (33). Alternatively, the genomic clones were nick translated for use as probes in Northern blot analyses, in which the gene location of each

Figure I.1. Detection of colonies containing recombinant plasmids by colony blot DNA hybridization. Representative colony blots are shown on the autoradiogram. Colonies with plasmids containing inserts of viral origin are seen as dark spots following hybridization to radioactively labeled IHNV genome probe.

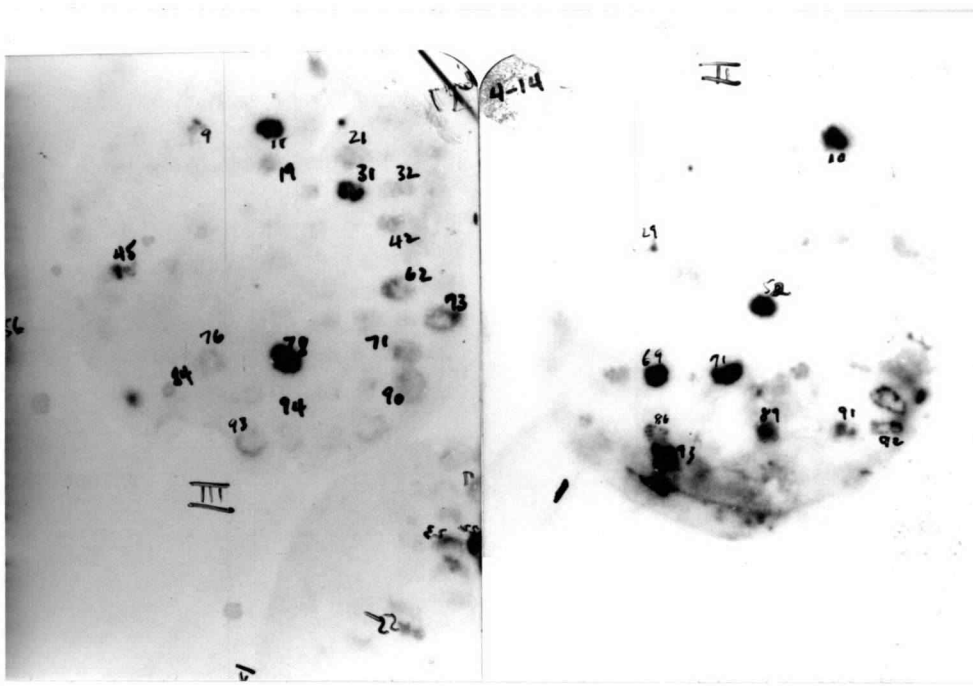
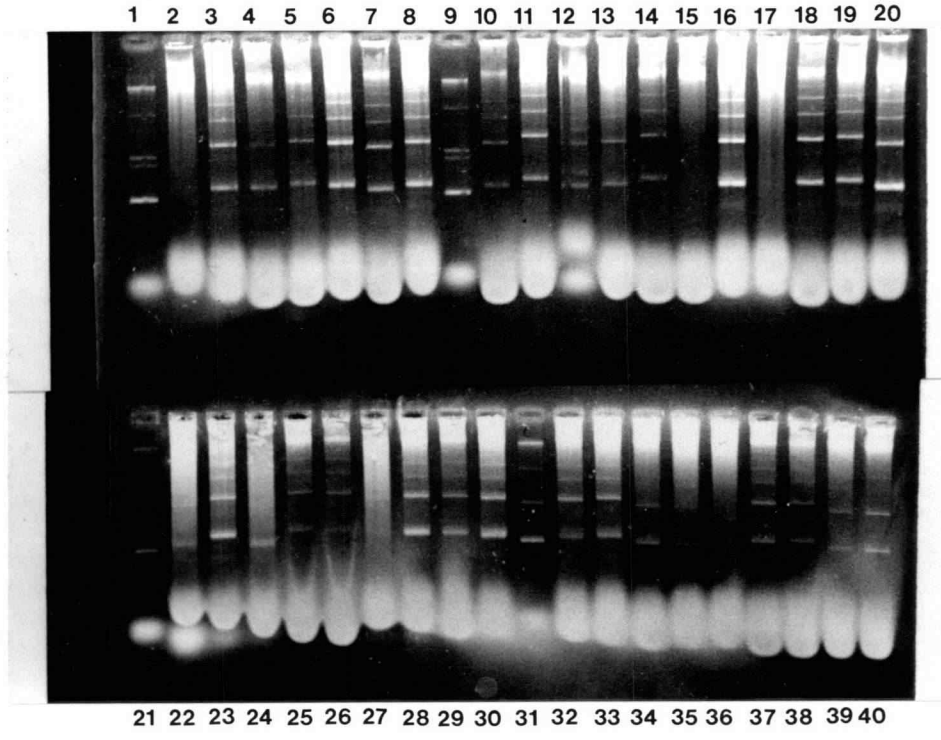


Figure I.2. Estimation of insert size by plasmid purification and gel electrophoresis. Plasmids from individual recombinant colonies are shown in relationship to the parental plasmid, pUC8. Bands in each lane represent different forms of plasmid supercoiling. Note the variation between colonies in migration of plasmid DNA indicating size differences of the insert. Lanes 1,9,21, and 31 - pUC8; lanes 2-8, 10-20, 22-30, and 32-40 - recombinants.



clone was identified by its hybridization to a specific mRNA. A battery of nucleocapsid (N) gene clones were identified in this manner (Fig. 3e). Figure 3a shows an agarose gel with the PstI cut inserts of plasmids 470, 163, 522 and 292 with insert sizes of approximately 750, 615, 800 and 750 bp respectively. Figure 3b shows hybridization of these plasmids to a probe derived from a mRNA clone that maps to the 5'end of the gene, pN144 (33). Note that there is no hybridization between p470 and pN144. However, p470 does hybridize to a mRNA probe corresponding to the 3' of the gene, pN419, which does not hybridize to p163 (Fig. 3c). Southern blots in Figure 3d show the hybridization between p522 (probe), and plasmids 470 and 163, thus mapping the locations of these clones to their genomic position (Fig. 3e). In addition, the genomic location of plasmids 470 and 163 was further verified by their hybridization to the N mRNA in Northern blots of RNA purified from IHNV infected CHSE-214 cells (data not shown).

The genomic position of other clones mapped in similar fashion as described above are shown in Figure 4 indicating the method of random oligomer priming generated clones along the entirety of the genome. Of special interest is p337, a clone with an insert of approximately 900 bp. p337 hybridizes with a probe derived from the 3'end of the G mRNA (33) (Fig. 5a). Figure 5b shows a Northern blot of polyadenylated RNA purified from IHNV-infected CHSE-214 cells using p337 as a probe. p337 hybridizes to both the G mRNA and the NV mRNA indicating that its location on the genome spans the junction between the G and NV genes.

DNA sequence analysis. The nucleotide sequence of the N gene was determined using the genomic and mRNA clones described. A partial

Figure I.3. Mapping of genomic clones to the N gene by Southern blotting. a) agarose gel electrophoresis indicating the insert size of clones following digestion with PstI. Lane 1, 123 bp ladder marker DNA; lane 2, p470; lane 3, p163; lane 4, p522; lane 5, p292. b) Southern blot of gel in (a). The probe is pN144, a mRNA clone from the 5' end of the gene. c) Southern blot of p470 and p163 using pN419 as the probe. Lane 11, p470; lane 12, p163; lane 13, same as lane 12 only using pN144 as a probe (noted as a positive reference). d) Southern blot of p470 and p163 as in (c) using a p522 probe. e) Map of the N gene showing locations of the mRNA probes, pN419 and pN144, and the genomic clones. The N gene is measured in increments of 100 bases.

Figure I.3.

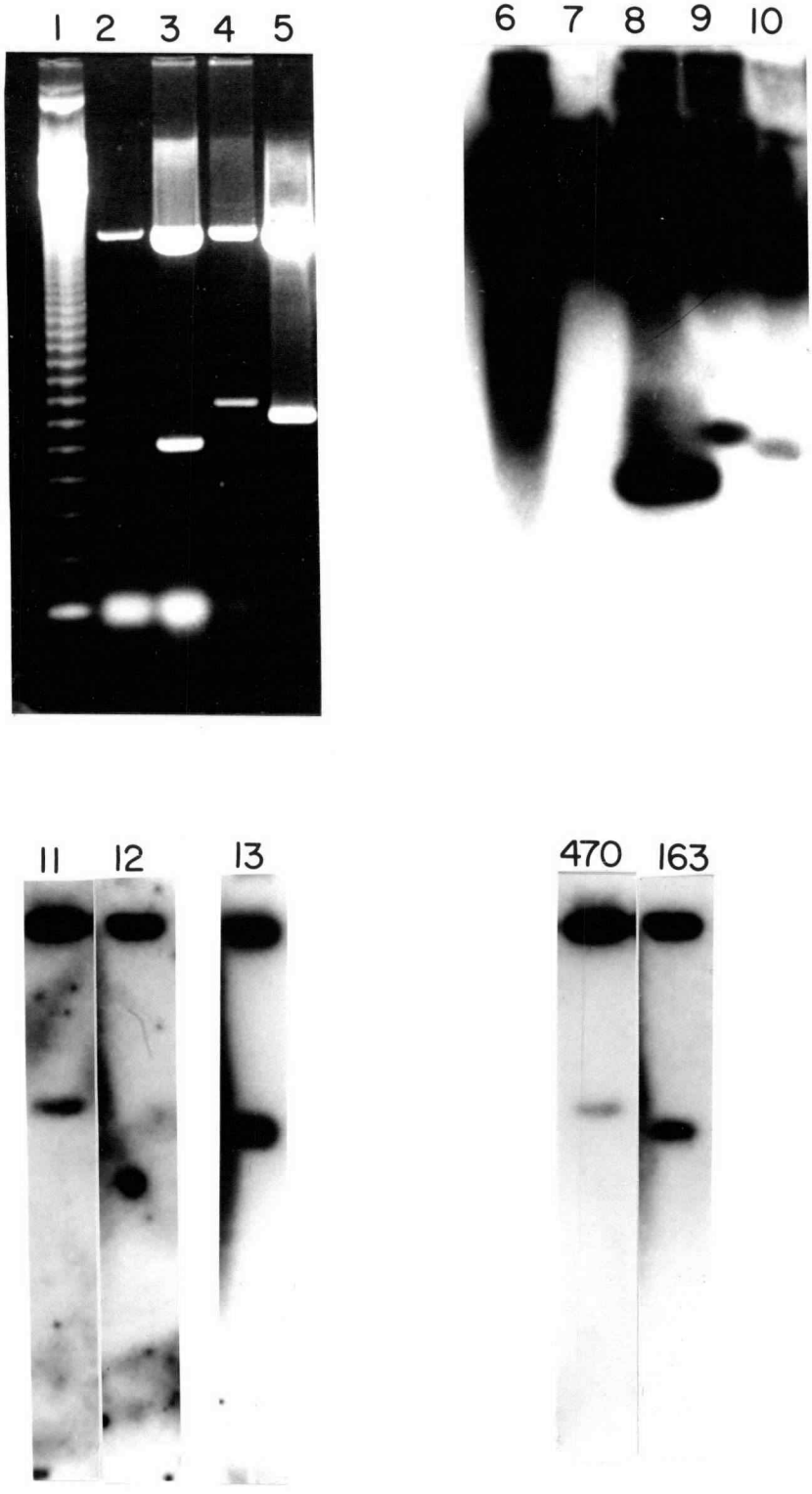


Figure I.3. (continued)

e

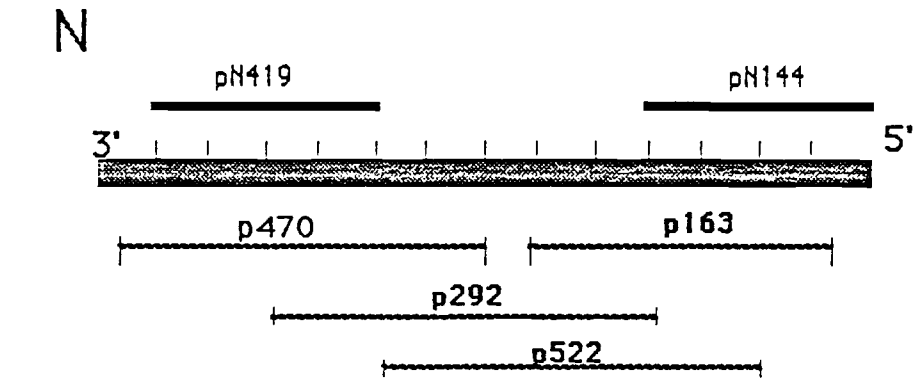


Figure I.4. IHNV genome map showing genomic clones mapped to their respective locations using mRNA probes. N,M1,M2,G,NV, and L indicate gene position. Lines above the genome represent mRNA probes. Lines below the genome represent genomic clones. The genome is measured in increments of 2 kilobases.

Figure I.4.

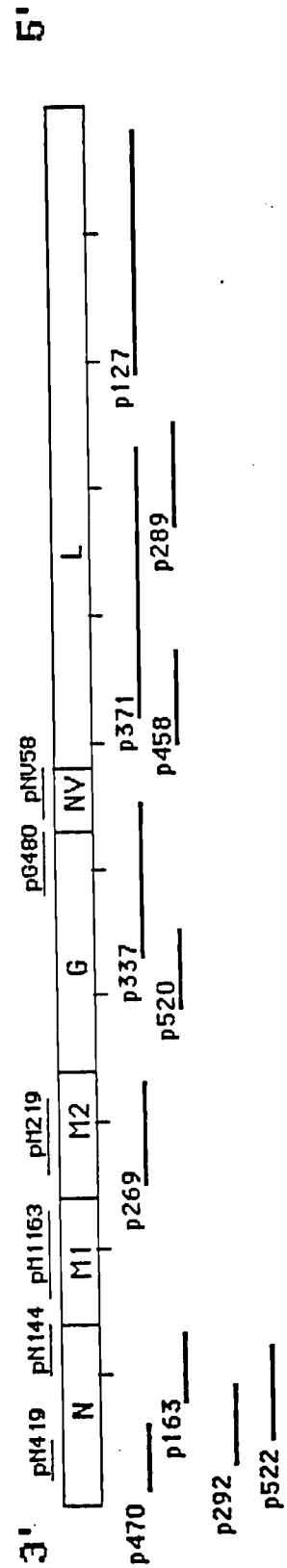
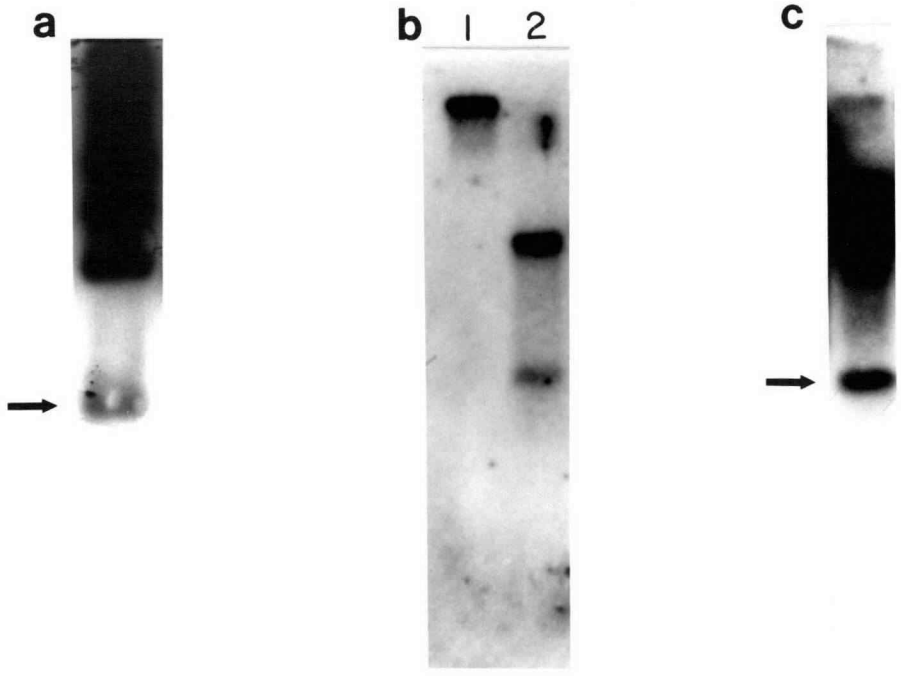

5'
3'
N
M1
M2
G
NV
L
pN419
pN144
pM1163
pM219
pG480
pNV58
p470
p163
p292
p522
p269
p520
p337
p458
p371
p289
p127

Figure I.5. Mapping of genomic clone, p337, to the G-NV intergenic region by Northern and Southern blotting. a) Southern blot of p337 hybridizing to pG480 probe. pG480 maps to the 5'end of the G gene (Fig.I.4). b) Northern blot using a p337 probe. Lane 1, 42S IHNV genome RNA; lane 2, poly-A+ RNA purified from IHNV-infected cells. c) Southern blot of p337 hybridizing to a pNV58 probe.



restriction map was determined by digesting the N gene clones with an array of available restriction enzymes so that a sequencing strategy could be formulated to subclone fragments of the clones into the appropriate M13 sequencing vector. The sequencing strategy is shown in Figure 6.

The nucleotide sequence of the G-NV intergenic region was determined by sequencing p337 according to the sequencing strategy shown in Figure 7. Features of each region are discussed below.

Figure I.6. The genomic and mRNA clones used for the dideoxy sequencing of the IHNV N protein gene. Above is a partial restriction map of a cDNA copy of the N gene marked in kilobases. The end of the gene is shown by the location of the poly-A tails. The segments with no arrows represent the size of clones pN2216 and p470, and the segments with arrows indicate the extent and direction of nucleotide sequencing. No genomic clones were available to sequence past the poly-A tails of p144.

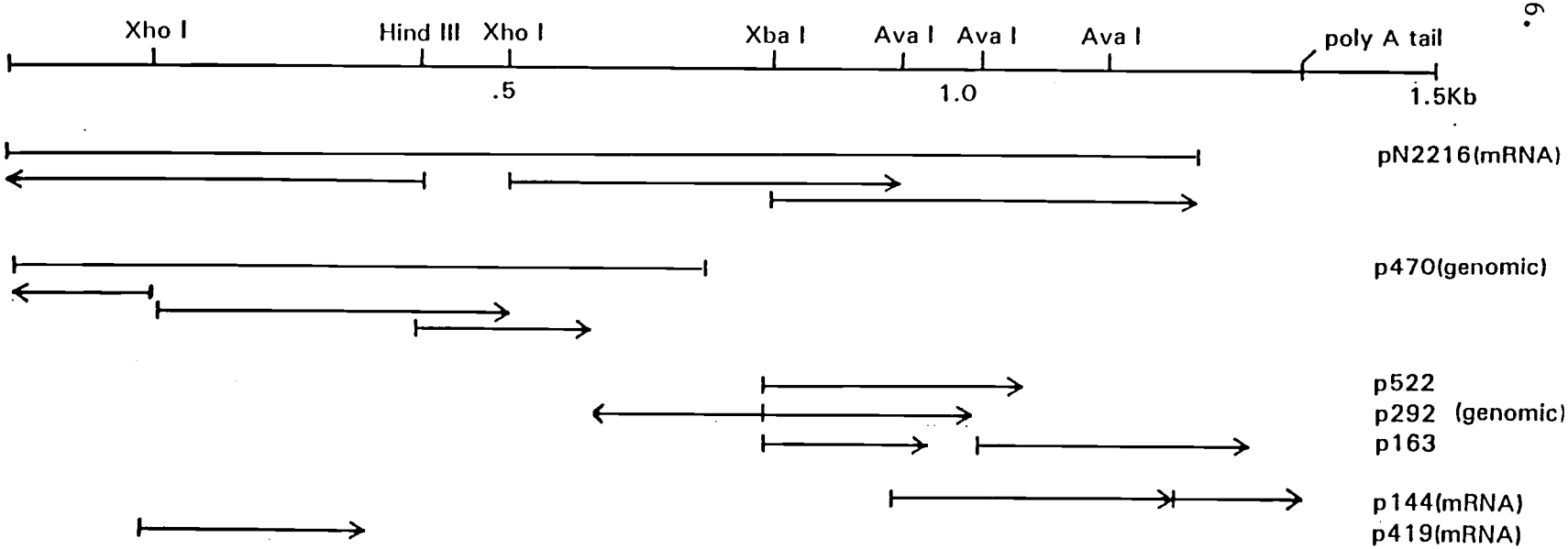
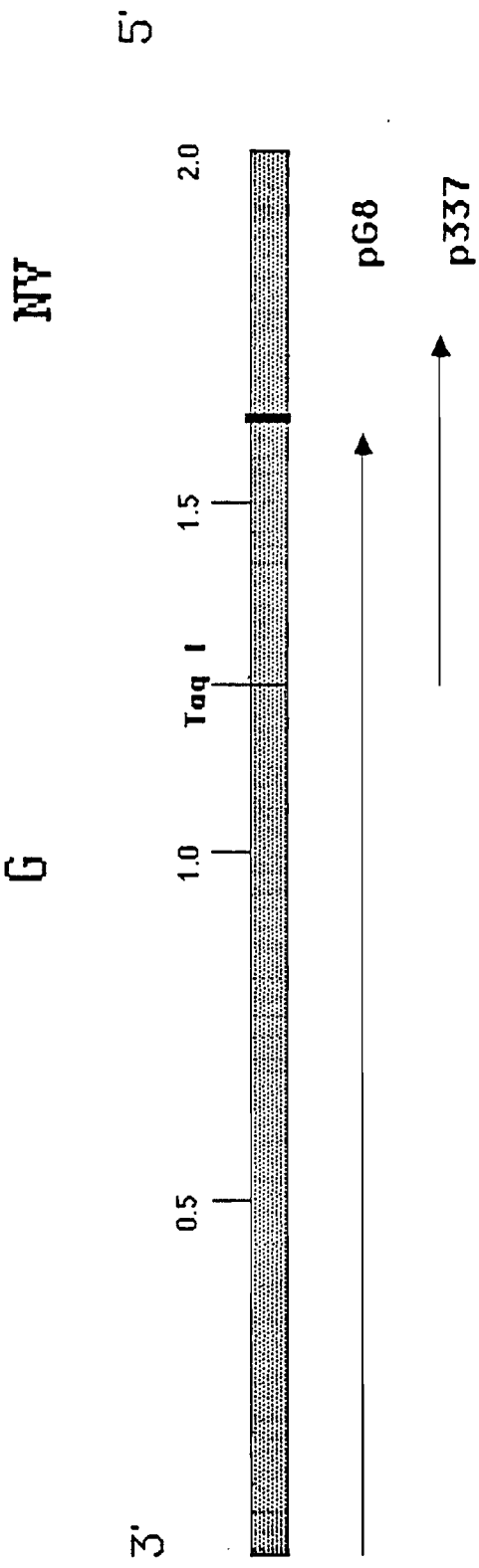

Xho I
Hind III
Xho I
Xba I
Ava I
Ava I
Ava I
poly A tail
.5
1.0
1.5Kb
pN2216(mRNA)
p470(genomic)
p522
p292 (genomic)
p163
p144(mRNA)
p419(mRNA)

Figure I.6.

Figure I.7. The direction of dideoxy sequencing of p337. The arrow represents the direction and extent of nucleotide sequencing. The 5' sequence of the G gene was confirmed by the sequence reported by Koener et al (31) and is represented by the segment pG8.

Figure I.7.



DISCUSSION

N gene sequence. The nucleotide sequence of the IHNV N gene is shown in Figure 8. An open reading frame beginning with an ATG codon at position 107 and terminating at position 1346 is shown with the predicted amino acid sequence. This frame potentially encodes a protein of 413 amino acids with a calculated molecular weight of approximately 45,600, slightly larger than the 40,500 daltons estimated by the migration of the IHNV N protein on SDS polyacrylamide gels (28). No other reading frame has a potential for encoding more than 75 amino acid residues.

Using the alignment program of Wilbur and Lipman (62) under these parameters: K-tuple size=2, window=40, gap penalty=2, comparisons between the IHNV N protein gene and that reported for vesicular stomatitis virus (VSV) (9,20), rabies virus (57), and sonchus yellow net virus (SYNV, a plant rhabdovirus) (Zuidema et al, manuscript submitted), showed no significant homology at the nucleotide level. These findings were not unexpected because there is no hybridization between IHNV N gene probes and VSV N mRNA on Northern blots (data not shown). Amino acid sequence comparisons using the same parameters also showed no significant homologies among viruses.

Using the Kyte and Doolittle program (35), hydropathy plots of the deduced amino acid sequences of the N protein for VSV (Indiana), rabies virus, and SYNV are compared to that of IHNV (Fig. 9). The IHNV N protein resembles the other rhabdoviruses in that it has a hydrophilic

Figure I.8. The nucleotide sequence of the IHNV N gene is shown in the mRNA (+) sense along with the deduced amino acid sequence of the continuous open reading frame beginning with the Met codon at position 107 and terminating at position 1346. The underlined bases at positions 75-78 and 1349-1352 indicate regions of homology with other rhabdoviruses in the non-coding regions. The dotted bases at position 35-39 indicate bases in common with rhabdoviral leader RNA. The adenine residues at the 3' end of the sequence indicate the poly-A tails of the mRNA.

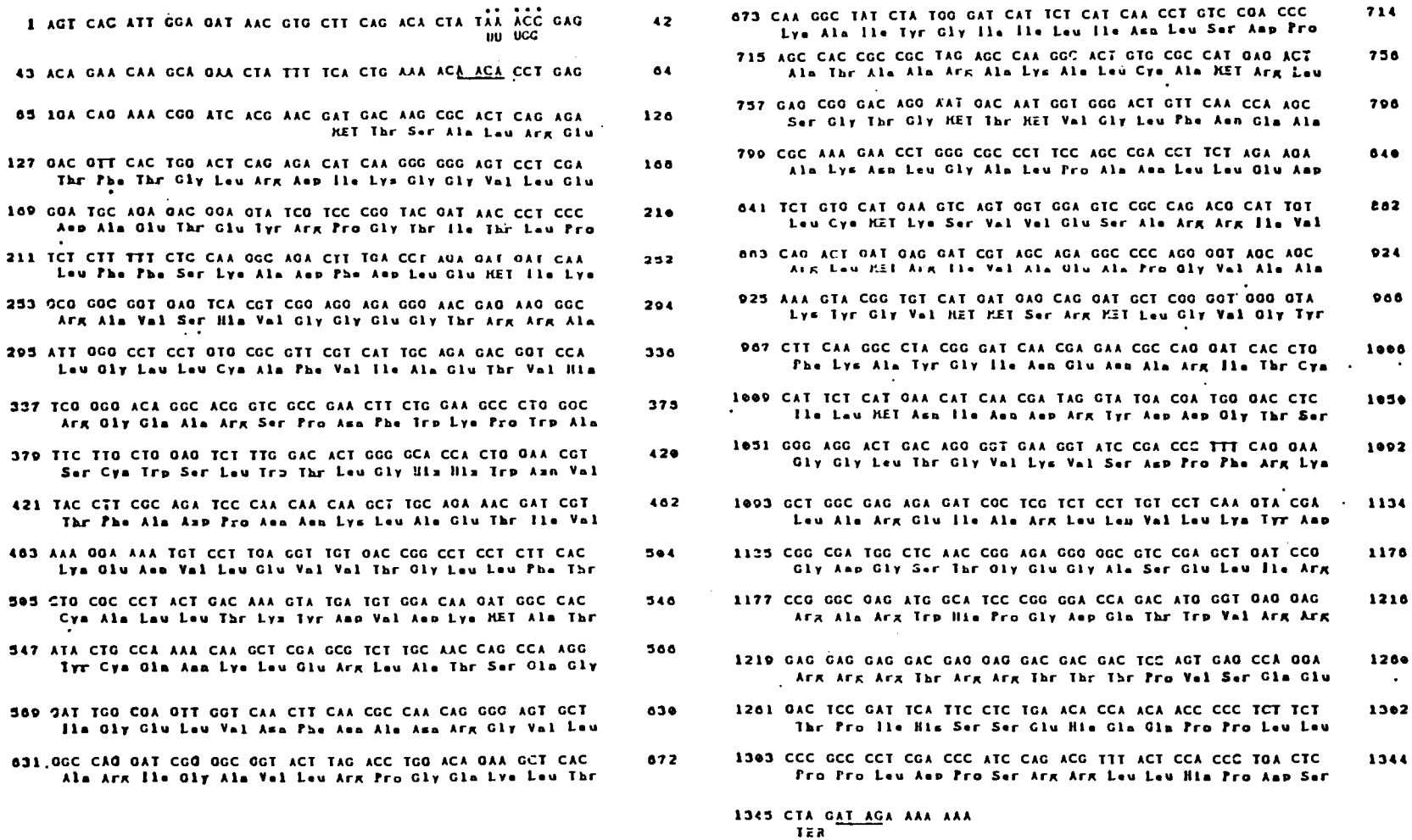
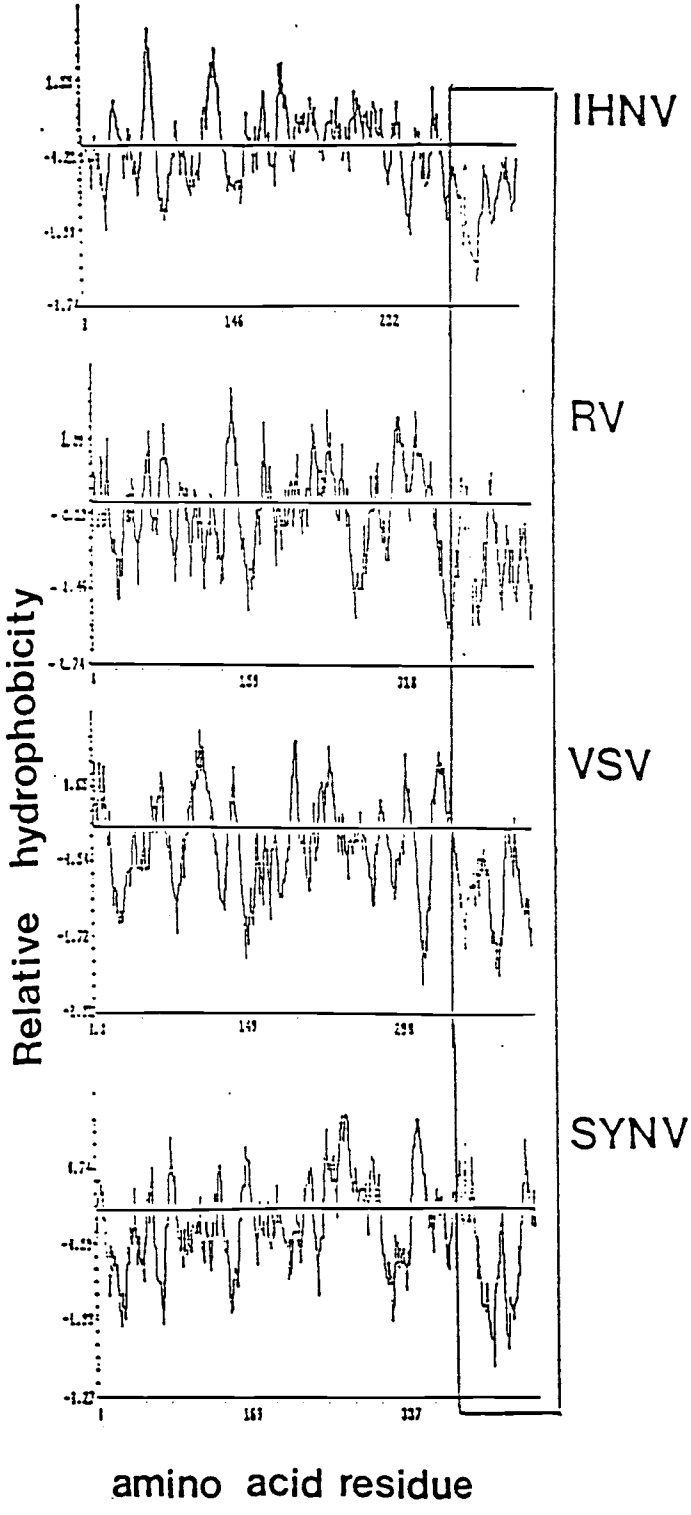

1 AGT CAC ATT GGA GAT AAC GTG CTT CAG ACA CTA TAA ACC GAG 42
UU UCG
43 ACA GAA CAA GCA GAA CTA TTT TCA CTG AAA ACA ACA CCT GAG 84
85 TGA CAG AAA CGG ATC ACG AAC GAT GAC AAG CGC ACT CAG AGA 126
MET Thr Ser Ala Leu Arg Glu
127 GAC GTT CAC TGG ACT CAG AGA CAT CAA GGG GGG AGT CCT CGA 168
Thr Phe Thr Gly Leu Arg Asp Ile Lys Gly Gly Val Leu Glu
169 GGA TGC AGA GAC GGA GTA TCG TCC CGG TAC GAT AAC CCT CCC 210
Asp Ala Glu Thr Glu Tyr Arg Pro Gly Thr Ile Thr Leu Pro
211 TCT CTT TTT CTC CAA GGC AGA CTT TGA CCT AUA GAT GAT CAA 252
Leu Phe Phe Ser Lys Ala Asp Phe Asp Leu Glu MET Ile Lys
253 GCG GGC GGT GAG TCA CGT CGG AGG AGA GGG AAC GAG AAG GGC 294
Arg Ala Val Ser His Val Gly Gly Glu Gly Thr Arg Arg Ala
295 ATT GGG CCT CCT GTG CGC GTT CGT CAT TGC AGA GAC GGT CCA 336
Leu Gly Leu Leu Cys Ala Phe Val Ile Ala Glu Thr Val His
337 TCG GGG ACA GGC ACG GTC GCC GAA CTT CTG GAA GCC CTG GGC 378
Arg Gly Gln Ala Arg Ser Pro Asn Phe Trp Lys Pro Trp Ala
379 TTC TTG CTG GAG TCT TTG GAC ACT GGG GCA CCA CTG GAA CGT 420
Ser Cys Trp Ser Leu Trp Thr Leu Gly His His Trp Asn Val
421 TAC CTT CGC AGA TCC CAA CAA CAA GCT TGC AGA AAC GAT CGT 462
Thr Phe Ala Asp Pro Asn Asn Lys Leu Ala Glu Thr Ile Val
463 AAA GGA AAA TGT CCT TGA GGT TGT GAC CGG CCT CCT CTT CAC 504
Lys Glu Asn Val Leu Glu Val Val Thr Gly Leu Leu Phe Thr
505 CTG CGC CCT ACT GAC AAA GTA TGA TGT GGA CAA GAT GGC CAC 546
Cys Ala Leu Leu Thr Lys Tyr Asp Val Asp Lys MET Ala Thr
547 ATA CTG CCA AAA CAA GCT CGA GCG TCT TGC AAC CAG CCA AGG 588
Tyr Cys Gln Asn Lys Leu Glu Arg Leu Ala Thr Ser Gln Gly
589 GAT TGG CGA GTT GGT CAA CTT CAA CGC CAA CAG GGG AGT GCT 630
Ile Gly Glu Leu Val Asn Phe Asn Ala Asn Arg Gly Val Leu
631 GGC CAG GAT CGG GGC GGT ACT TAG ACC TGG ACA GAA GCT CAC 672
Ala Arg Ile Gly Ala Val Leu Arg Pro Gly Gln Lys Leu Thr
673 CAA GGC TAT CTA TGG GAT CAT TCT CAT CAA CCT GTC CGA CCC 714
Lys Ala Ile Tyr Gly Ile Ile Leu Ile Asn Leu Ser Asp Pro
715 AGC CAC CGC CGC TAG AGC CAA GGC ACT GTG CGC CAT GAG ACT 756
Ala Thr Ala Ala Arg Ala Lys Ala Leu Cys Ala MET Arg Leu
757 GAG CGG GAC AGG AAT GAC AAT GGT GGG ACT GTT CAA CCA AGC 798
Ser Gly Thr Gly MET Thr MET Val Gly Leu Phe Asn Gln Ala
799 CGC AAA GAA CCT GGG CGC CCT TCC AGC CGA CCT TCT AGA AGA 840
Ala Lys Asn Leu Gly Ala Leu Pro Ala Asn Leu Leu Glu Asp
841 TCT GTG CAT GAA GTC AGT GGT GGA GTC CGC CAG ACG CAT TGT 882
Leu Cys MET Lys Ser Val Val Glu Ser Ala Arg Arg Ile Val
883 CAG ACT GAT GAG GAT CGT AGC AGA GGC CCC AGG GGT AGC AGC 924
Arg Leu MET Arg Ile Val Ala Glu Ala Pro Gly Val Ala Ala
925 AAA GTA CGG TGT CAT GAT GAG CAG GAT GCT CGG GGT GGG GTA 966
Lys Tyr Gly Val MET MET Ser Arg MET Leu Gly Val Gly Tyr
967 CTT CAA GGC CTA CGG GAT CAA CGA GAA CGC CAG GAT CAC CTG 1008
Phe Lys Ala Tyr Gly Ile Asn Glu Asn Ala Arg Ile Thr Cys
1009 CAT TCT CAT GAA CAT CAA CGA TAG GTA TGA CGA TGG GAC CTC 1050
Ile Leu MET Asn Ile Asn Asp Arg Tyr Asp Asp Gly Thr Ser
1051 GGG AGG ACT GAC AGG GGT GAA GGT ATC CGA CCC TTT CAG GAA 1092
Gly Gly Leu Thr Gly Val Lys Val Ser Asp Pro Phe Arg Lys
1093 GCT GGC GAG AGA GAT CGC TCG TCT CCT TGT CCT CAA GTA CGA 1134
Leu Ala Arg Glu Ile Ala Arg Leu Leu Val Leu Lys Tyr Asp
1135 CGG CGA TGG CTC AAC CGG AGA GGG GGC GTC CGA GCT GAT CCG 1176
Gly Asp Gly Ser Thr Gly Glu Gly Ala Ser Glu Leu Ile Arg
1177 CCG GGC GAG ATG GCA TCC CGG GGA CCA GAC ATG GGT GAG GAG 1218
Arg Ala Arg Trp His Pro Gly Asp Gln Thr Trp Val Arg Arg
1219 GAG GAG GAG GAC GAG GAG GAC GAC GAC TCC AGT GAG CCA GGA 1260
Arg Arg Arg Thr Arg Arg Thr Thr Thr Pro Val Ser Glu Glu
1261 GAC TCC GAT TCA TTC CTC TGA ACA CCA ACA ACC CCC TCT TCT 1302
Thr Pro Ile His Ser Ser Glu His Gln Gln Pro Pro Leu Leu
1303 CCC GCC CCT CGA CCC ATC CAG ACG TTT ACT CCA CCC TGA CTC 1344
Pro Pro Leu Asp Pro Ser Arg Arg Leu Leu His Pro Asp Ser
1345 CTA GAT AGA AAA AAA
TER

Figure I.8.

Figure I.9. Hydropathy plots of the deduced amino acid sequence of IHNV, rabies virus PV strain (RV), vesicular stomatitus virus Indiana strain (VSV), and sonchus yellow net virus (SYNV) using the Kyte and Doolittle program with a window size of 10. Boxed is the hydrophilic COOH terminus in common with all four viruses.

Figure I.9.


IHNV
RV
VSV
SYNV
Relative hydrophobicity
amino acid residue

COOH terminus, but no other obvious similarities using this type of analysis were defined. However, one would predict some structural similarity because the N protein in VSV is known to complex with the genomic RNA (8), and this function is most likely conserved among rhabdoviruses.

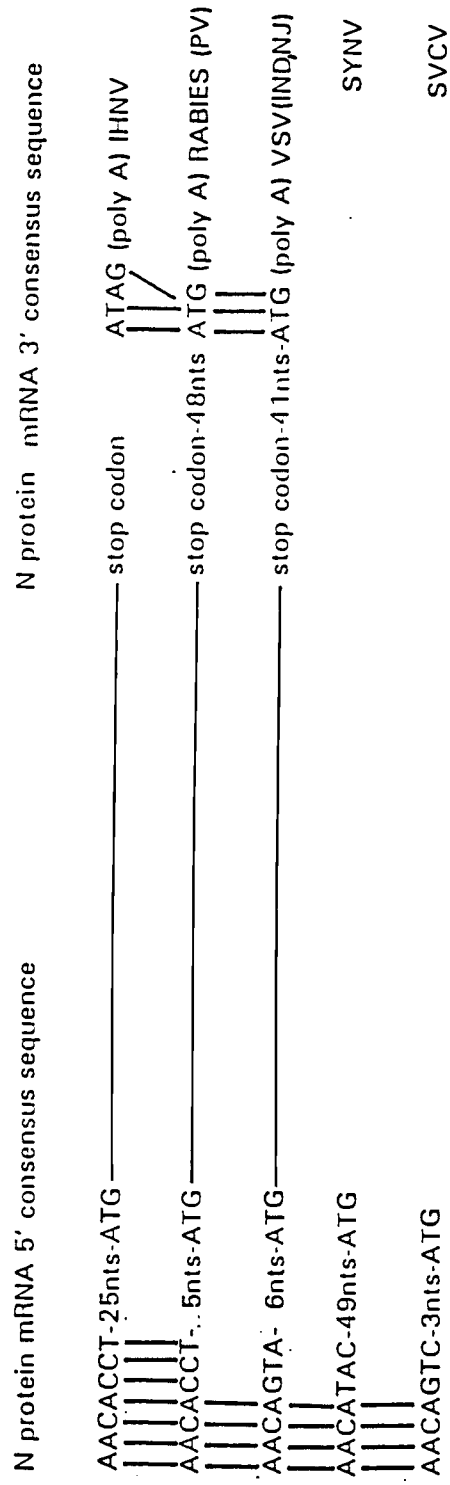
Even with no extensive nucleotide or amino acid homology between the N gene of IHNV and that of VSV, SYNV, and rabies virus, the non-protein coding regions do share consensus sequences. Examination of the 5'non-coding region of the IHNV N mRNA reveals the consensus sequence 5' AACA 3' beginning 32 bases upstream from the ATG translation start codon at position 75. This is identical with that regarded as the mRNA initiation site in rabies virus (34), VSV (44), SYNV (66), and spring viremia of carp virus (SVCV, another fish rhabdovirus) (52). This putative IHNV N mRNA initiation site has even more sequence homology with the PV strain of rabies virus, matching a septanucleotide 5' AACACCT 3' (58) (Fig. 10).

At the 3' end, downstream from the protein coding region, what must represent the polyadenylation signal, is the sequence 5' ATAG 3', which is similar to that of rabies virus (58) and VSV (51) (Fig. 10). The 3' non-coding region of the IHNV NmRNA is short, consisting of only four nucleotides following the termination codon (Fig. 8).

An unusual finding from the nucleotide sequencing of the NmRNA clone was the presence of a non-coding region of approximately 100 bases before the first ATG start site. With the exception of the SYNV N gene which is 56 bases (Zuidema, submitted manuscript), other rhabdovirus mRNA sequences have only an approximate 10-30 base non-coding region before the translation start (58). This observation

Figure I.10. Consensus sequences of the 5' and 3' untranslated regions of the N gene are indicated (when known) for IHNV, rabies virus PV strain, VSV strains Indiana and New Jersey, SYNV, and spring viremia of carp virus.

Figure I.10.

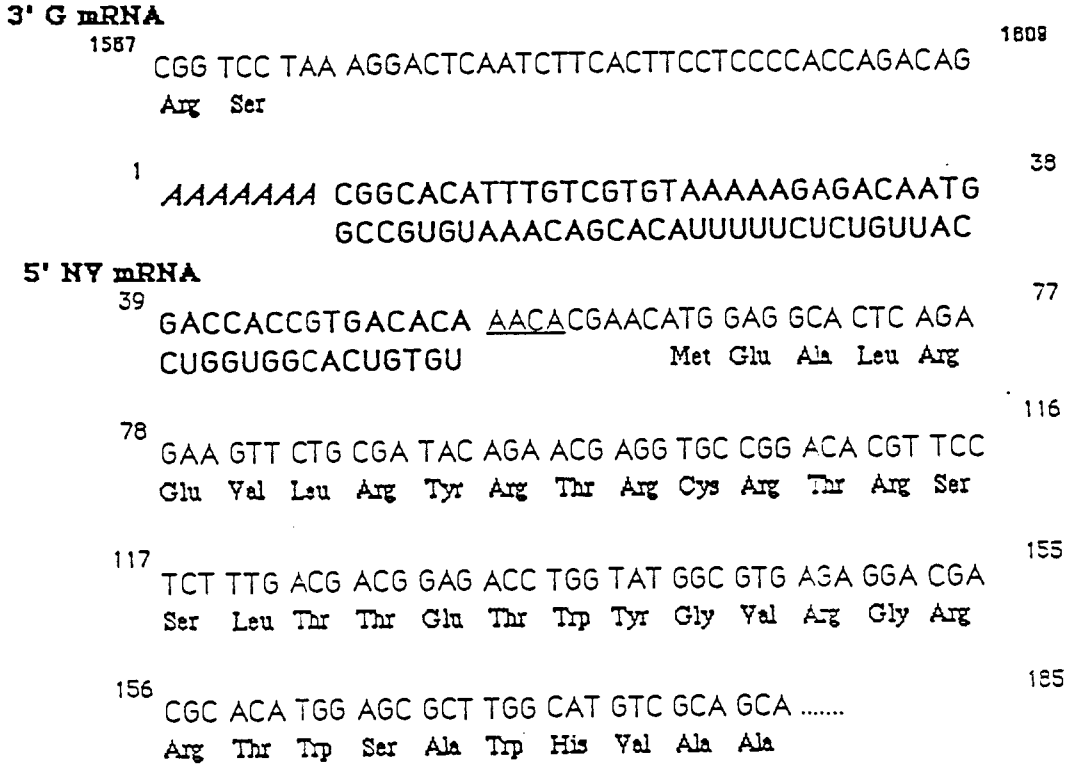

N protein mRNA 5' consensus sequence
N protein mRNA 3' consensus sequence
AACACCT-25nts-ATG
stop codon
ATAG (poly A) IHNV
AACACCT-..5nts-ATG
stop codon-48nts
ATG (poly A) RABIES (PV)
AACAGTA- 6nts-ATG
stop codon-41nts-ATG (poly A) VSV(IND,NJ)
AACATAC-49nts-ATG
SYNV
AACAGTC-3nts-ATG
SVCV

raises interesting speculation concerning the molecular biology of the IHN virus. Leader RNAs are transcribed from the terminal 3' end of the genome, immediately upstream of the N gene, and are thought to serve an important role in the transcription and replication of rhabdoviruses (8). Leader RNAs typically range in size from 47 to 50 bases and have been detected in VSV (16), rabies virus (34), spring viremia of carp virus (52), and SYNV (with a leader of 147 bases) (66). All leader RNAs described are transcribed apart from the mRNAs, and no precursor forms have been detected in which a leader RNA is attached to a mRNA, although there is evidence of polycistronic mRNAs in VSV (46). The 5' non-coding region of the IHNV N mRNA shares the pentanucleotide 3' UUUGG 5' (genome sense) at position 35, in common with other rhabdovirus leader RNAs (21) (Fig. 8). This homology and the long 5' non-translated region of the IHNV N mRNA suggests the possibility that the equivalent of an IHNV leader RNA sequence may be transcribed as part of the NmRNA. This model is only speculative at this point, and direct RNA sequencing of the genome must be done to determine the 3' terminal base sequence, as well as RNA leader detection experiments to clarify the existence of the putative leader RNA and the mechanism in which it is transcribed. These results will be crucial in determining if the IHNV leader RNA transcription process is different from other rhabdoviruses as seen from the nucleotide sequence of poly-A selected N mRNA.

G-NV intergenic region sequence. The nucleotide sequence of p337 is shown in Figure 11 indicating the 3' terminus of the G gene mRNA, the intergenic region between the G and NV genes, and the predicted 5'mRNA

Figure I.11. The nucleotide sequence of the G-NV intergenic region is shown in the mRNA (+) sense. The region between the 3' end of the G mRNA and the putative 5' end of the NV mRNA is shown in the (+) and (-) sense in boldface. Italicized sequences indicate the polyadenylation residues. The underlined sequences indicate the consensus mRNA initiation site. A partial 5' NV coding sequence with the predicted amino acid sequence is shown.

Figure I.11.


3' G mRNA
1567
1608
CGG TCC TAA AGGACTCAATCTTCACTTCCTCCCCACCAGACAG
Arg Ser
1
38
AAAAAAA CGGCACATTTGTCGTGTAAAAAGAGACAATG
GCCGUGUAAACAGCACAUUUUUCUCUGUUAC
5' NY mRNA
39
77
GACCACCGTGACACA AACACGAACATG GAG GCA CTC AGA
CUGGUGGCACUGTGU Met Glu Ala Leu Arg
78
116
GAA GTT CTG CGA TAC AGA ACG AGG TGC CGG ACA CGT TCC
Glu Val Leu Arg Tyr Arg Thr Arg Cys Arg Thr Arg Ser
117
155
TCT TTG ACG ACG GAG ACC TGG TAT GGC GTG AGA GGA CGA
Ser Leu Thr Thr Glu Thr Trp Tyr Gly Val Arg Gly Arg
156
185
CGC ACA TGG AGC GCT TGG CAT GTC GCA GCA .....
Arg Thr Trp Ser Ala Trp His Val Ala Ala

start of the NV gene. The 5' end of the G gene (mRNA sense) is characterized by a 34 nucleotide non-coding region after the TAA stop codon, which is immediately followed by a stretch of seven adenine residues, presumably constituting the initiation of the poly-A tail of the mRNA. This oligo(A) run represents a consensus sequence that is invariably found at the termini of genes encoding each protein of VSV (51) and rabies virus (58). An investigation of the 3' mRNA termini of viruses of the paramyxovirus group, also with negative strand RNA genomes, reveals a similar run of from 4 to 7 adenine residues; A_5 for Sendai virus (25) and human parainfluenza virus type 3 (56), A_{4-7} for human respiratory syncytial virus (15), and A_6 for Newcastle disease virus (14). Since there is no known analogous gene junctions to the IHNV G-NV region in other negative strand RNA viruses, our comparisons of this region are limited to the more general category of 3'mRNA polyadenylation signals. It is apparent, however, that each gene studied in these viruses possesses a genomic stretch of uracil residues that signal the polyadenylation of each transcribed mRNA, and the G gene of IHNV appears to be no different.

Further study of the G-NV region reveals the sequence 5' AACA 3' at position 54, indicating the putative cap site and mRNA initiation site of the NV gene. 5' AACA 3' is regarded as the consensus sequence for mRNA initiation in the 5' non-coding region in VSV and rabies virus as noted previously. Considering this as the NV mRNA start, an ATG translation start codon is found four nucleotides downstream beginning a potential open reading frame encoding the NV protein.

Defining an intergenic region as the sequences between the 3' end of one mRNA and the 5' end of the following one, the G-NV intergenic

region consists of 46 bases (Fig. 11). There is a possibility of a second polyadenylation signal of five adenine residues 17 bases downstream of the A_7 stretch, which would shorten the intergenic region to 24 bases. In either case, this intergenic region of IHNV is substantially longer than any of the intergenic regions reported for VSV or rabies virus. The intergenic sequences for each of the VSV genes consists of the dinucleotide AG (except for the NS and M genes where it is AC) (51). In rabies virus there is more variation. The N-M1, M1-M2, M2-G, and G-L intergenic regions are 2,5,5, and 423 nucleotides long, respectively (58). This long non-coding region between the G and L genes has been hypothesized to represent a remnant gene, similar to the NV gene in IHNV (58). In the Paramyxoviridae, Sendai virus and human parainfluenza virus type 3 intergenic sequences consist of the trinucleotide GAA (25,56) but human respiratory syncytial virus intergenic regions vary from one to 52 nucleotides long (15). Thus, there is a precedent for variations in the intergenic regions within virus families. Extensive comparisons between IHNV intergenic regions and those of other negative strand RNA viruses will have to be made pending the sequence determination of the other gene junctions in IHNV.

In conclusion, these results have enabled us to define some sequences potentially involved in the regulatory processes of IHNV gene transcription and to compare those regions with those known in other unsegmented negative strand RNA viruses. Further insight has been provided into the wide divergence of rhabdoviral N genes as well as sequence conservation in non-translated regions presumed to be involved in transcriptional initiation and termination. Subsequent sequence

determination of the remainder of the IHNV genome should provide additional information for comparisons to other like viruses and should aid in defining its evolutionary relationship with mammalian and plant rhabdoviruses.

EXPRESSION IN *ESCHERICHIA COLI* OF AN EPITOPE OF THE GLYCOPROTEIN OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS WHICH PROTECTS AGAINST VIRAL CHALLENGE

ABSTRACT

Plasmid vectors were constructed which expressed an antigenic determinant of the glycoprotein gene of infectious hematopoietic necrosis virus (IHNV) as a fusion protein with the trpE protein of Escherichia coli. Insertion of Sau3AI fragments from the IHNV glycoprotein gene into trpE expression plasmids led to a fusion protein containing a hydrophilic segment of 104 amino acids from the middle portion of the viral glycoprotein. After induction with indoleacrylic acid, fusion proteins accumulated stably in the E.coli cells and accounted for approximately 10% of the total protein in the cell. Immunization trials in fish with the crude bacterial lysate containing the fusion protein have indicated that the trpE-glycoprotein fusion protein produced in bacteria induced protective immunity.

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus endemic to the North American northwest and causes a fatal disease in salmon and trout (2). IHNV is characterized by its bullet-shaped morphology, enveloped nucleocapsid and glycoprotein projections (3); its RNA genome being of negative polarity and approximately 11,000 bases encoding for the five virion associated proteins, N (nucleocapsid), M1 and M2 (matrix proteins), G (glycoprotein), and L (polymerase) (26,33). In addition, IHNV transcribes a sixth mRNA for a non-virion associated protein, NV, of unknown function; a feature not described for other studied rhabdoviruses (32).

Recent studies in our lab have recently shown that the glycoprotein is the only antigen responsible for producing neutralizing antibody against the virus *in vitro* (Engelking and Leong, manuscript submitted). Further experiments have shown that virion-purified glycoprotein is capable of producing a protective response in salmonid fishes to a lethal virus challenge (Engelking and Leong, manuscript submitted). Currently, there is no available vaccine to control outbreaks of IHNV, which results in severe economic damage to the aquaculture industry. Development of a subunit vaccine produced by the expression in *E. coli* of a viral epitope immunoreactive with IHNV neutralizing antisera was undertaken and the results are described here.

MATERIALS AND METHODS

Cells and virus. The IHNV isolates (Elk River, Dworshak, and Round Butte strains) used in these studies were obtained from W. Groberg (Oregon Department of Fish and Wildlife). The virus used for challenge studies was prepared by infecting rainbow trout fry and reisolating the virus from fish dying of the virus infection. Subsequently, the virus was grown for two passages in chinook salmon embryo cells, CHSE-214 (18).

Construction of recombinant plasmids. The construction of a recombinant plasmid containing the trp promoter and gene fused to a fragment of the IHNV glycoprotein gene is shown in Figure 1. Plasmid pG8 contains the entire coding sequences for the IHNV glycoprotein (31). The 1.6 kb glycoprotein gene insert of pG8 was excised by cleavage with PstI and purified by gel electrophoresis in low melting temperature agarose. The purified fragment, digested with restriction endonuclease Sau3AI, yielded 9 fragments ranging in size from 40 to 329 basepairs (bp). These fragments were ligated to pATH2 and pATH3 vectors previously cleaved with BamHI in a T_4 DNA ligase reaction (45) and the entire mixture was used to transform E. coli strain MC1061 (13). Transformants containing recombinants were grown on LB plates containing 120 ug/ml ampicillin. The pATH vectors were the generous gift of T.J. Koerner and A. Tzagaloff (Columbia University).

Immunologic detection of viral peptide protein expression. Transfor-

mants were analyzed for viral peptide production by immunologic detection. The transformant colonies were transferred to nitrocellulose by replica plating (24) and the nitrocellulose was overlayed on minimal media M9 containing 1% casamino acids, 10 ug/ml indoleacrylic acid, and 120 ug/ml ampicillin (45). The cells were allowed to grow overnight at 37°C. The cells were then lysed by exposure to chloroform vapor for 2 h and subsequently soaked in lysis/blocking solution (20 mM Tris-HCl, pH 8.0; 0.5 M NaCl; 3% gelatin; 0.1 ug/ml DNAse I; 0.4 ug/ml lysozyme) for 3-4 h. After rinsing with TBS (20 mM Tris-HCl, pH 8.0; 0.5 M NaCl), the filters were treated with rabbit anti-IHNV sera (diluted 1:500) for 2 h. After a rinse with TBS, the filters were then treated with a solution of horseradish peroxidase conjugated goat anti-rabbit immunoglobulin sera (diluted 1:1000, Sigma, St. Louis, MO) for 1 h. The positive colonies were detected by the addition of the substrate (3 mg/ml 1-chloro-4-napthol in methanol diluted 1:5 in 0.5X TBS plus 0.3% hydrogen peroxide) as purple staining colonies. Replicates of the positive colonies were transferred to LB plates containing ampicillin.

Colony blot hybridization. Nitrocellulose filters containing transformants were overlayed on LB with ampicillin plates. The colonies were grown overnight at 37°and lysed by treatment with 10% sodium dodecyl sulfate followed with 0.5 M NaOH. The filters were washed, dried, and hybridized to ^{32}P-labeled IHNV glycoprotein gene probes by standard methods (45). The probe was made by nick translation of a purified G gene fragment (50). Recombinant colonies were detected on Kodak X-AR film exposed to hybridized filters.

Preparation of anti-IHNV sera. Three month old New Zealand white rabbits were immunized with purified IHNV, Round Butte strain Type 1 (28). A series of three injections were administered. The first injection of purified IHNV (0.5 mg in 0.5 ml emulsified with an equal volume of Freund's complete adjuvant) was inoculated intradermally into four sites around the neckline of the rabbit (0.25 ml per site). The second and third injections were given two and four weeks later, respectively. Again, four sites around the rabbit neckline received intradermal injections of 0.25 ml containing about 0.25 mg purified IHNV and an equal volume of Freund's incomplete adjuvant. Two weeks after the last injection the rabbits were exsanguinated by heart puncture and the serum prepared and stored at -70°C

Preparation of crude lysates. Procedures for preparation of bacterial lysates are modified from that of Kleid et al (29). Cells from colonies testing positive by immunologic detection were transferred to 10 ml of Luria-Bertani (LB) broth (45) containing 120 ug/ml ampicillin and grown to stationary phase overnight. These cells were then inoculated into one liter of M9 containing 1.0% casamino acids and 100 ug/ml ampicillin. The cultures were incubated at 37°C for 1 h at which time the cells were induced by the addition of 5 ug/ml indole-acrylic acid and incubated with agitation until stationary phase. The cells were harvested by centrifugation at 4000 g after which the cells were lysed and the insoluble protein fraction collected by centrifugation.

Western immunoblotting. The insoluble *E. coli* lysates from positive cultures were prepared as described above. Proteins were analyzed by SDS polyacrylamide gel electrophoresis on a 10% gel by the discontinuous gel method of Laemmli (37). The proteins distributed on the gel were electrophoretically transferred to 0.2 um nitrocellulose (59). The Western blots were developed with rabbit anti-IHNV sera and rabbit antisera prepared against purified IHNV glycoprotein.

DNA sequence analysis. Nucleotide sequence determination was done by the subcloning of the EcoRI and HindIII restriction endonuclease digested DNA fragments (Fig. 1) into the M13 sequencing vector mp19. Sequencing was performed by the Sanger dideoxy method (54).

Immunization trials in fish. Bacterial crude lysates were prepared as described and used to immunize fish by immersion. A set of approximately 100 rainbow trout (*Salmo gairdneri*) or chinook salmon (*Oncorhynchus tshawytscha*) fry were used for each control and immunized experimental group. The average weight of each fry at the time of immunization was 0.4 g.

Immunization by immersion was accomplished by bathing groups of 100 fry in 25 ml of the preparation (approximately 3 mg/ml total protein concentration) for 1 min. At that time the immersion solution volume was increased to 250 ml with water and fish were incubated in this diluted solution for an additional 2 min. These fish were then placed in aquaria of 5 gallons with a water flow rate of 0.25 gal/min in a constant water temperature of 10^{o}C. Approximately one month after immunization, each fish group was subdivided and challenged with

varying doses of live, virulent IHNV. All dead fish were assayed for the presence of infectious IHNV in chinook salmon cells (CHSE-214) as described by Engelking and Leong (18). The data was analyzed statistically by estimation of logistic regression models.

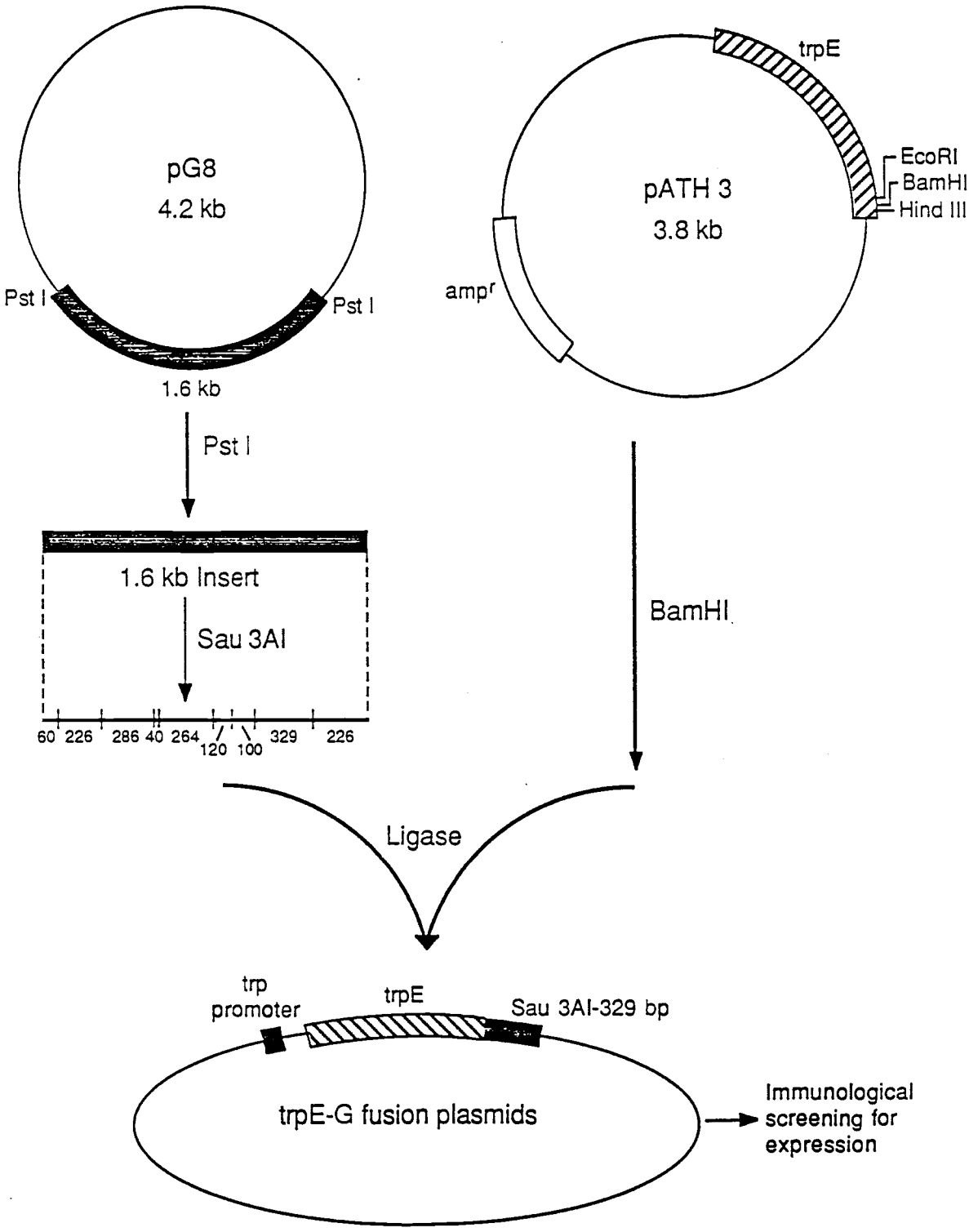
RESULTS

Construction of trpE-G expression plasmid. We have shown that purified IHNV-G protein will elicit a protective immune response in salmonid fry (Engelking and Leong, manuscript submitted). The objective of this study was the insertion of the IHNV glycoprotein gene into a suitable expression vector and the subsequent use of the expressed protein as an antiviral vaccine. Previous work with the rabies virus glycoprotein gene expressed in bacteria (65) indicated that the expressed glycoprotein was denatured in bacteria and did not produce a protective immune response. A cloning strategy was therefore employed that might circumvent this problem for the IHNV-G protein. Recombinant plasmids containing fragments of the G gene were generated rather than plasmids containing the intact G gene. Only those recombinant plasmids expressing an epitope of the G protein would be detected upon screening with anti-G protein specific sera.

Examination of the nucleotide sequence of a cDNA clone of the IHNV G mRNA indicated that Sau3AI digestion would result in nine fragments ranging in size from 40 to 329 bp. The isolated G cDNA from pG8 was digested with Sau3AI, and a mixture of these fragments was randomly ligated to the BamHI site of pATH2 and 3 expression vectors (Fig.1). The pATH vectors are so designed so that insertion of a DNA fragment at the BamHI site results in placement of the desired DNA sequence in all three reading frames.

Figure II.1. Construction of trpE-G gene fusions. The 1.6 kb cDNA cloned insert of the IHNV glycoprotein gene was fragmented and subcloned as described in Materials and Methods. The resulting plasmids used for expression analyses, p52G and p618G, were generated.

Figure II.1.


pG8
4.2 kb
Pst I
Pst I
1.6 kb
Pst I
1.6 kb Insert
Sau 3AI
60 226 286 40 264 120 100 329 226
trpE
pATH 3
3.8 kb
EcoRI
BamHI
Hind III
ampr
BamHI
Ligase
trp promoter
trpE
Sau 3AI-329 bp
trpE-G fusion plasmids
Immunological screening for expression

Transformant colonies were screened for IHNV-G epitope expression with anti-IHNV sera. Positive colonies were detected with pATH3 as the vector (Fig. 2). For subsequent analysis, twelve pATH3 positively staining colonies were selected. The presence of viral G gene information in the recombinant plasmids carried by these colonies was confirmed by colony blot DNA hybridization (data not shown).

Plasmids were isolated from individual immunopositive colonies and digested with EcoRI and HindIII. An estimate of each insert's size was determined by agarose gel electrophoresis of the digested plasmid. The results indicated that there were only two different insert sizes, approximately 600 and 1800 bp, among the twelve selected colonies (Fig. 3). The large size of these inserts indicated that two or more Sau3AI fragments were ligated together during the cloning procedure, a result confirmed by DNA sequencing of the inserts. Representatives of the two groups, p52G for the smaller insert and p618G for the larger insert, were used in the studies described below.

Expression of trpE-G fusion protein. Individual immunopositive colonies were grown in broth culture and induced with indoleacrylic acid. Bacterial lysates were prepared and the insoluble protein fraction was analyzed by SDS-PAGE. Coomassie blue staining of these gels indicated that there were two groups of immunopositive colonies. One group, represented by p618G, produced a trpE- fusion protein with an apparent molecular weight of 48,000 daltons that was easily detected in the gel (Fig. 4A). The second group, represented by p52G, produced a fusion protein that was only detected with Western Blotting (Fig. 4B). The fusion protein expressed in this group had an apparent

Figure II.2. Immunodetection of recombinant colonies expressing fusion protein. Colonies were transferred from crowded areas of the original transformant plates, where positive immunoreaction to anti-IHNV sera occurred, to gridded filters overlaid on inducing media so that transformant colonies could be more accurately rescreened for protein expression. Immunopositive colonies, seen as colored (dark) spots on the membrane, were streaked for isolation and rescreened to insure purity of individual colonies. Concentrations of purified IHNV is included as the positive control. Colonies harboring the pATH vector alone are included as negative controls (arrows).

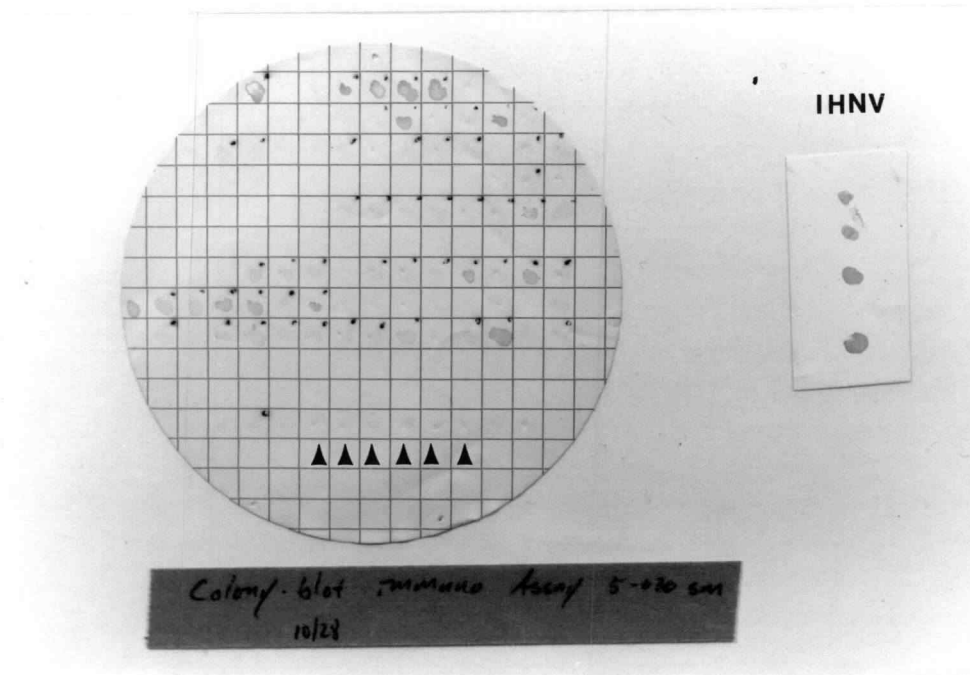


Figure II.3. Determination of the plasmid insert size of immunopositive colonies. Agarose gel electrophoresis of plasmids prepared from immunopositive colonies digested with EcoRI and HindIII showing insert sizes. Lanes a and b contain 123 bp ladder marker DNA. The series of expression clones 69-618 contain an insert of approximately 1800 bp; the series 52-513 contain an insert approximately 600 bp. Clone 615-7 contains a small insert and is expression negative.

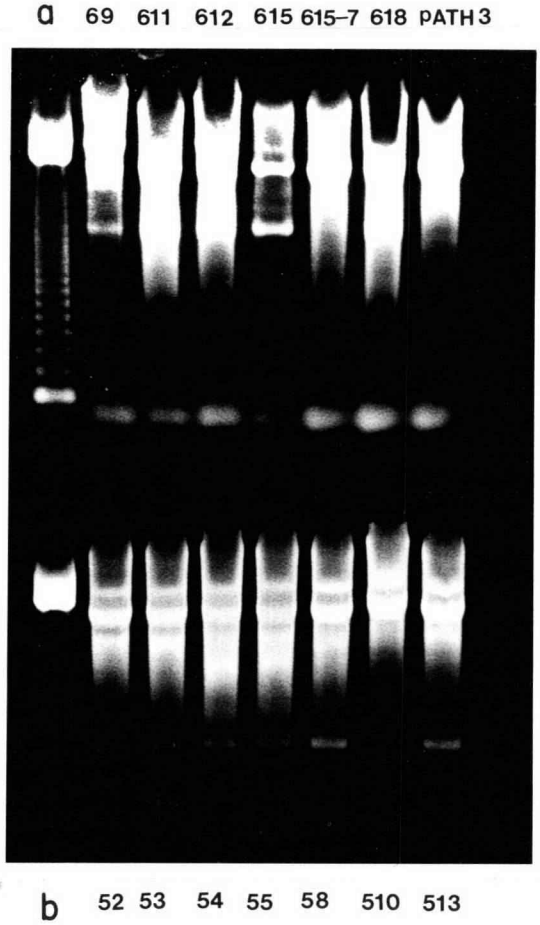
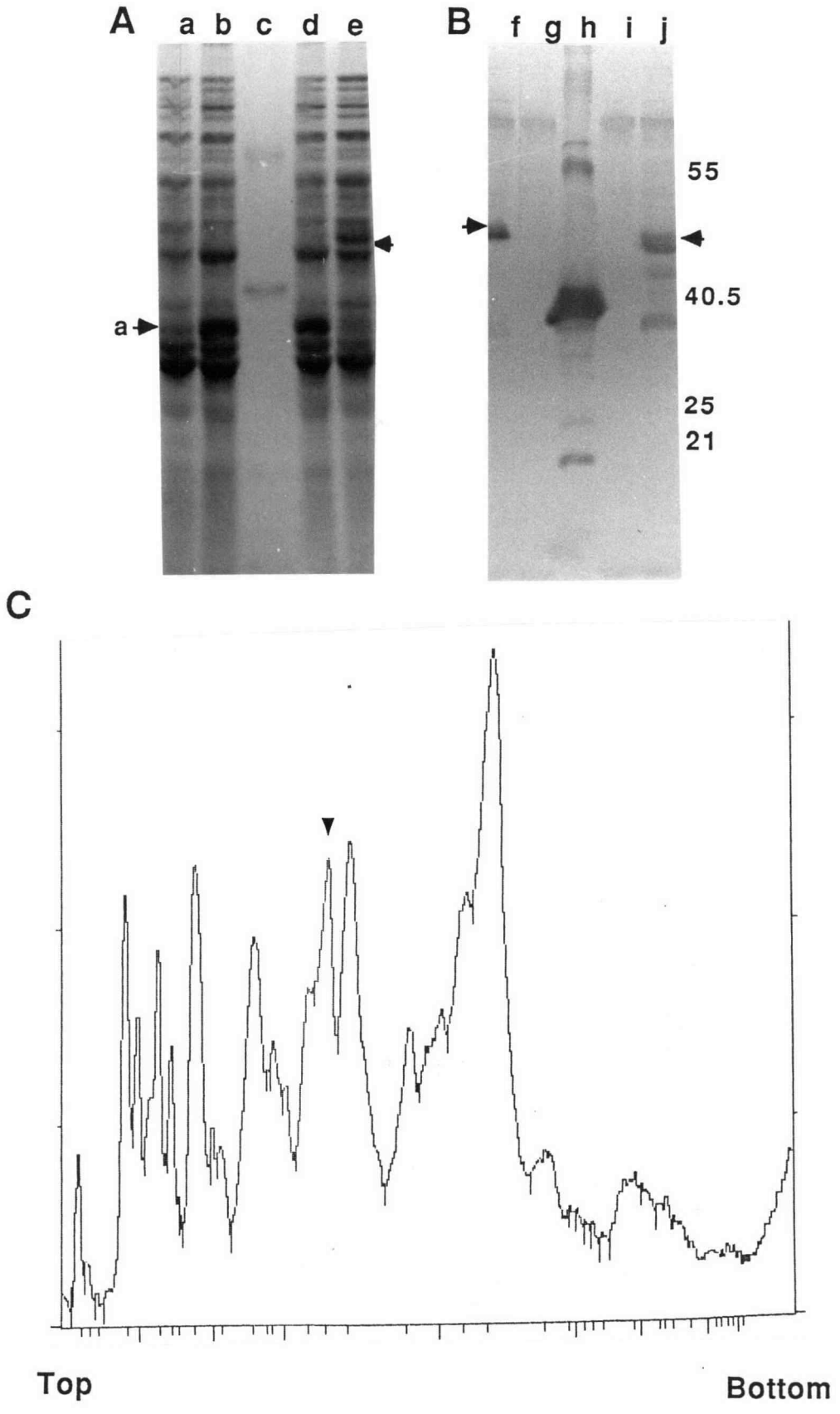


Figure II.4. Analysis of bacterial production of trpE-G fusion protein on 10% SDS-polyacrylamide gel and antibody reactivity on electrophoretic transfer blot. A) Coomassie stained gel of proteins prepared from bacteria with various plasmids. Lanes: a) insoluble proteins of cells containing plasmid 52G; e) insoluble proteins of cells containing plasmid 618G; b) and d) insoluble proteins of cells containing pATH3; c) IHNV structural proteins. Arrow a indicates the position of the trpE protein in cells containing the pATH vector alone. B) Electrophoretic blot of protein fractions in (A). Lanes a through e were analyzed with rabbit antisera produced against the IHNV virion and are shown as f through j. Lanes f and j show the trpE-G fusion protein (arrows) of plasmids 52G and 618G respectively. The major band for p618G migrated at a position of 48,000 and p52G at 49,000 daltons. Lane g and i contained the protein samples from cells containing the pATH3 vector alone. Lane h contains the IHNV structural proteins with G at 55 kd, N at 40.5 kd, M1 at 25 kd, and M2 at 21 kd. C) Laser scanning densitometric tracing of lane e of (A). Arrow indicates the fusion protein peak.

Figure II.4.

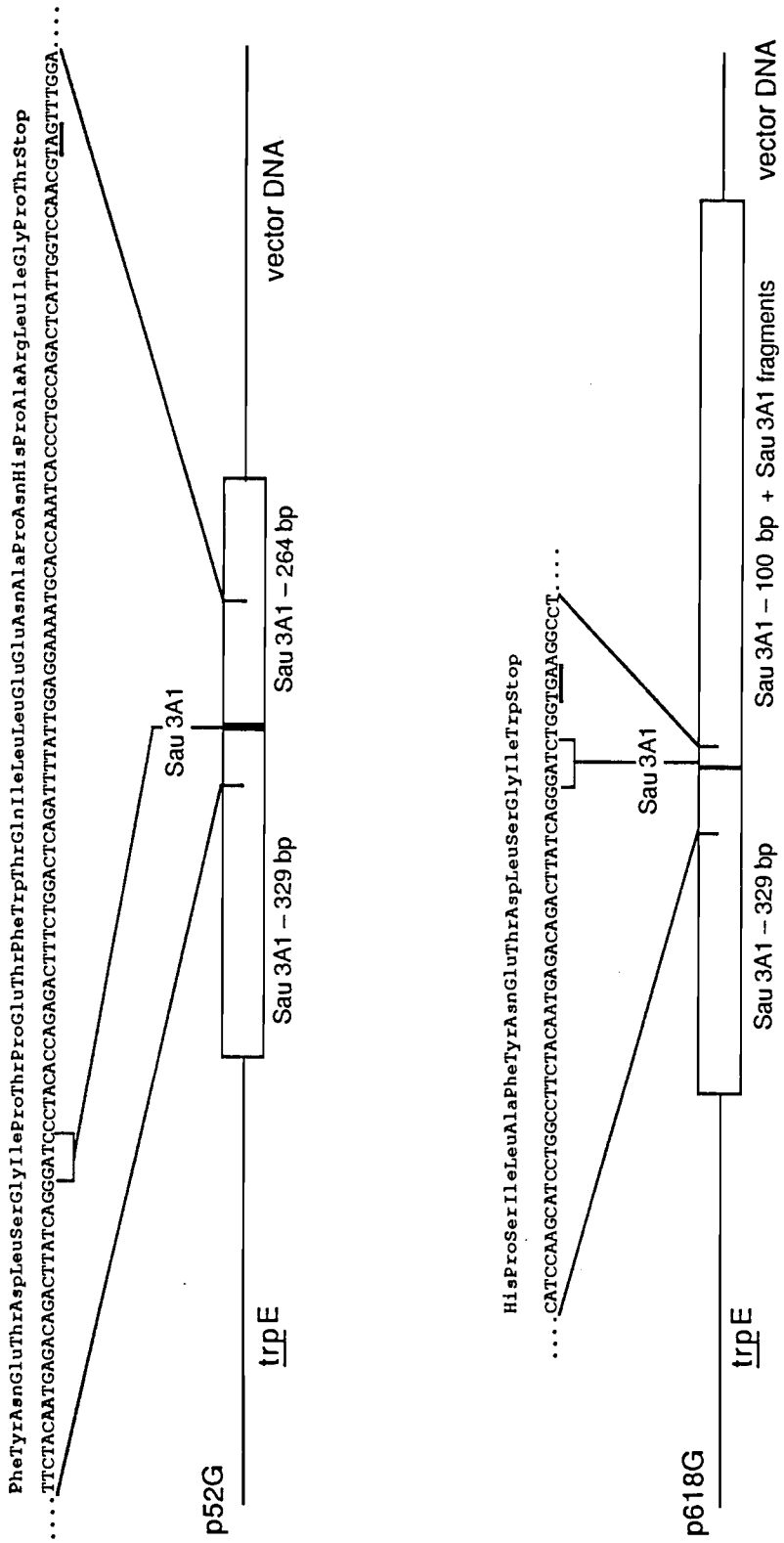


molecular weight of 49,000 daltons and seemed to be masked on Coomassie stained gels by other bacterial proteins. Some lower molecular weight minor bands were also detected in lanes f and j (Fig. 4B). One explanation for the presence of these bands is proteolytic processing by the bacteria of the larger protein into smaller polypeptides. These bands were absent from control lanes g and i (Fig. 4B). We found that extended incubation of the cells or storage of lysates at either 4^{o} or $-20^{o}C$ did not change the pattern or formation of the smaller bands. Laser densitometric scanning of the Coomassie stained gel of the bacterial lysate of p618G showed that the fusion protein made up approximately 10% of the total cell protein (Fig. 4C).

Sequence determination of the viral insert. Examination of the IHNV glycoprotein nucleotide sequence (31) indicated that only one Sau3AI fragment could be inserted in the correct reading frame in pATH3. This was a 329 bp fragment from the approximate carboxyl third of the IHNV glycoprotein gene. DNA sequence analysis verified this by demonstrating that in both p52G and p618G, the Sau3AI-329 bp fragment was inserted in frame with the structural gene for trpE (Fig. 5B). The 329 bp fragment encoded a peptide of approximately 11,000 daltons. Thus, the trpE-G fusion protein of 49,000 daltons contained 37,000 daltons of the trpE polypeptide and the additional 11,000 dalton G protein fragment. Due to the "shotgun" approach of cloning the nine Sau3AI fragments randomly into each expression vector, there was the potential for the fragments to be ligated together as well as into the vector. DNA sequence examination of these positive expression plasmids showed that extraneous Sau3AI fragments did indeed ligate tandemly. However,

Figure II.5. DNA sequence determination of p52G and p618G. Shown are the partial nucleotide sequences of plasmids 52G and 618G indicating the extra IHNV glycoprotein gene Sau3AI fragments ligated to the Sau3AI-329 fragment that is translated as described in the text. For p52G, a TAG stop codon follows in frame 84 bases from the end of the Sau3AI-329 fragment. For p618G, a TGA stop codon occurs 6 bases from the end of the Sau3AI-329 fragment. The complete nucleotide sequence of the IHNV glycoprotein gene has been described (31).

Figure II.5.


PheTyrAsnGluThrAspLeuSerGlyIleProThrProGluThrPheTrpThrGlnIleLeuLeuGluGluAsnAlaProAsnHisProAlaArgLeuIleGlyProThrStop
....TTCTACAATGAGACAGACTTATCAGGGATCCCTACACCAGAGACTTCTGGACTCAGATTTTATTGGAGGAAAATGCACCAAATCACCCTGCCAGACTCATTGGTCCAACGTAGTTTGGA....
Sau 3A1
Sau 3A1 – 329 bp
Sau 3A1 – 264 bp
vector DNA
p52G
trpE
HisProSerIleLeuAlaPheTyrAsnGluThrAspLeuSerGlyIleTrpStop
....CATCCAAGCATCCTGGCCTTCTACAATGAGACAGACTTATCAGGGATCTGGTGAAGGCCT....
Sau 3A1
Sau 3A1 – 329 bp
Sau 3A1 – 100 bp + Sau 3A1 fragments
vector DNA
p618G
trpE

as observed from the Western blot, even though these plasmids differed greatly in size, their respective fusion proteins were approximately equal in size, indicating that the extra fragments were not in frame and therefore not translated. A determination of the DNA sequence of p52G indicated that the 264 bp Sau3AI fragment (Fig. 1) was ligated adjacent to the 329 bp fragment and the termination codon TAG is found 84 bp from the end of the 329 bp fragment (Fig. 5). The 593 bpfragment provided genetic information for a peptide of 49,000 daltons (49 kd = 37 kd [trpE] + 11 kd [Sau3AI] + 1 kd [84 bp extra]). Determination of the DNA sequence of p618G indicates that the 100 bp Sau3AI fragment (Fig.1) is ligated adjacent to the 329 bp fragment and the termination codon TGA is found 6 bp from the end of the 329 bp fragment, thus allowing for the approximate 1000 dalton difference in fusion protein size (Fig. 5). DNA sequencing past the Sau3AI-100 bp fragment in p618G was not performed.

The Sau3AI-329 bp fragment is located in the carboxyl terminal third of the coding region of the IHNV glycoprotein gene (Fig.1) just before the transmembrane domain. An examination of a hydropathy plot of the deduced amino acid sequence of this region indicated that the glycoprotein region expressed in the fusion protein was very hydrophilic (31).

Immunization of fish with crude fusion protein. Immunization of rainbow trout fry with a lysate prepared from bacteria containing p52G and/or p618G was performed as described, and subsequent viral challenges provided data on the efficacy of the fusion protein as a vaccine (Table 1).

Table 1. Immunization trials using *E. coli* expressed fusion product. Comparison of immersed vaccinated rainbow trout fry to unvaccinated control fry against challenge with IHNV Round Butte type I strain.

IHNV Round Butte strain vs. rainbow trout						
	Immunized p52G			Control		
Virus dilution*	No.	SL	%	No.	SL	%
-2	25	5	20	25	23	92
-3	26	4	15	25	18	72
-4	26	0	0	25	15	60
-5	26	0	0	25	4	16

No. = Number of fish in group.

SL = Specific loss, i.e., number of fish dying from IHNV infection.

% = Percent mortality

* = Virus dilutions are shown as ten-fold dilutions of a stock virus with a titer of 7.2×10^7 $TCID_{50}$/ml

Table 1 shows the significant protection conferred on immunized fish versus unimmunized fish when challenged with the Round Butte strain of IHNV. The glycoprotein gene from which the fusion protein was derived was from this strain. Cross protection against a more virulent strain of IHNV, Dworshak, is shown in Table 2. A significant decrease in mortalities of from 50 to 70% is seen at the higher virus dilutions in this challenge experiment.

A group of fish immunized with bacterial lysate derived from E. coli that contained the pATH3 vector with no insert was used in a challenge experiment to determine if nonspecific immunity was conferred. Table 3 shows that the mortality rate of the mock immunized group was essentially the same as the non-immunized group. In addition, the immunized groups showed cross protection with another IHNV strain, Elk River, and in a different salmonid species, the chinook salmon.

Table 2. Immunization trials using *E. coli* expressed fusion product. Comparison of immersed vaccinated rainbow trout fry to unvaccinated control fry against challenge with IHNV Dworshak strain.

IHNV Dworshak strain vs. rainbow trout						
	Immunized p52G			Control		
Virus dilution*	No.	SL	%	No.	SL	%
-2	28	26	93 +	24	24	100
-3	26	20	77 +	27	25	93
-4	27	14	52	26	26	100
-5	27	3	11	26	22	85

No. = Number of fish in group.

SL = Specific loss, i.e., number of fish dying from IHNV infection.

% = Percent mortality

* = Virus dilutions are shown as ten-fold dilutions of a stock virus with a titer of 2.8×10^8 $TCID_{50}/ml$

+ = not statistically different from control

Table 3. Immunization trials using *E. coli* expressed fusion product. Comparison of immersed vaccinated chinook salmon fry to unvaccinated control fry and mock vaccinated control fry against challenge with IHNV Elk River strain.

IHNV Elk River strain vs. chinook salmon

Virus dilution*	Immunized p52G			Immunized p618G			Mock vaccinated control			Unvaccinated control		
	No.	SL	%	No.	SL	%	No.	SL	%	No.	SL	%
-2	28	2	7	29	0	0	19	10	53	25	16	64
-3	28	2	7	29	0	0	19	4	21	25	6	24
-4	28	0	0	29	3	10	18	3	17	25	3	12
-5	29	0	0	30	2	7	17	1	6	25	5	20

No. = Number of fish in group.

SL = Specific loss, i.e., number of fish dying from IHNV infection.

% = Percent mortality

* = Virus dilutions are shown as ten-fold dilutions of a stock virus with a titer of 1.9×10^8 $TCID_{50}/ml$

DISCUSSION

The expression of a portion of the IHNV glycoprotein gene in bacterial cells provides an economically feasible means of preparing antigenic material for the immunization of fish against this viral infection. The simultaneous ligation of all Sau3AI fragments into each expression vector enabled us to quickly and efficiently search for a region(s) on the gene that encoded an antigenic determinant. Portions of the IHNV glycoprotein gene were randomly attached to the trpE operon promoter and a portion of the trpE gene of E. coli to generate fusion proteins. The transformant colonies were screened for antigen production by immunologically assayed colony blots and thus, only those fusion constructions that resulted in the expression of an antigenic domain recognized by polyclonal anti-glycoprotein sera were selected.

Twelve positive colonies derived from pATH3 were analyzed and only two types of inserts were found containing 600 bp or 1800 bp. Representatives from each group were analyzed and both were found to contain the Sau3AI-329 bp fragment in frame with the trpE gene (Fig. 5). Western blot analyses of these groups showed immunoreactivity with a protein of the predicted molecular weight of a fusion product with anti-IHNV sera. These results prove that the Sau3AI-329 bp fragment encodes a domain that is recognized by the polyclonal rabbit anti-IHNV glycoprotein sera.

An examination of the deduced amino acid sequence and the hydropathy of the Sau3AI-329 bp fragment indicated that this region of the glycoprotein gene contained a sequence of very hydrophilic amino

acids from residue 361 to 369. This hydrophilic domain protrudes at a predicted beta turn in the glycoprotein chain (31) and may appear as an epitope in the native molecule.

The cloned region also contains three predicted N-linked glycosylation sites at position 400, 401, and 438 (31). We had shown previously that the rabbit anti-IHNV glycoprotein sera bound to the nonglycosylated G-protein as well as to the glycosylated G-protein (28). However, the results reported here show specifically that a small region of approximately 100 amino acids remains antigenically recognizable without glycosylation.

The finding that the trpE glycoprotein fusion protein was detected in a Western blot of the bacterial lysate prompted us to test the fusion protein in immunization trials in fish. The crude protein lysate from a 1 liter culture was used to immunize 200 fish which were approximately 1 month old and less than 0.5 g in size. Although only a few trials have been completed, a protective immunity in fish seems to be induced one month after immunization. These results indicate that fish as small as 0.5 g are capable of developing a protective immune response to IHNV. The duration of the immunity and the effect on booster immunization doses were not determined in this study. Further trials and experiments are underway to elucidate the effects of these and other parameters. Since the virus levels used in the challenge studies are considerably higher than found in natural outbreaks, these initial trials suggest that the trpE fusion protein, even in crude form, can be utilized as an effective and economical vaccine against IHNV.

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