

AN ABSTRACT OF THE THESIS OF

Li Xu for the degree of Master of Science in Microbiology presented on February 23, 1990

Title: Epitope Mapping and Characterization of the Glycoprotein of Infectious Hematopoietic Necrosis Virus

Redacted for Privacy

Abstract approved by: _____

Dr. Jo-Ann Ching Leong

A characterization of the antigenic determinants (epitopes) of the glycoprotein (G) of infectious hematopoietic necrosis virus (IHNV) was made with different regions of the G gene expressed in Escherichia coli. A cDNA copy of the G gene was divided into four fragments after Taq I digestion and these fragments were subcloned into the pATH vectors which put expression of each G gene fragment under the control of the trpE promoter. The resulting plasmids encoded trpE-G fusion proteins containing different regions of the viral glycoprotein. The three plasmids, pXL2, pXL3, and pXL7, were found to encode fusion proteins that were detected with anti-IHNV sera in Western immunoblots. A comparison of reactivities of the fusion proteins encoded by these plasmids was made with a number of anti-G specific monoclonal antibodies (Mabs) by Western immunoblot and radioimmunoassay. The non-neutralizing monoclonal antibody, 136J, was found to react with the trpE-G fusion protein encoded by pXL3 and the fusion proteins encoded by plasmids, p52G and p618G, which were identified in previous studies (Gilmore et al., 1988).

Another non-neutralizing Mab, 2F, was able to bind to the pXL3 fusion protein and the neutralizing Mab, RB/B5, recognized the pXL7 fusion protein. Competitive radioimmune studies with a synthetic peptide derived from the amino acid sequence encoded by pXL3 was found to inhibit the binding of a neutralizing Mab, 127B, to purified IHN virus.

Epitope Mapping and Characterization
of the Glycoprotein of Infectious
Hematopoietic Necrosis Virus

by

Li Xu

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed February 23, 1990

Commencement June 1990

APPROVED:

Redacted for Privacy

Professor of Microbiology in charge of major

Redacted for Privacy

Head of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented February 23, 1990

Typed by Li Xu for herself

ACKNOWLEDGEMENTS

It is my pleasure to thank Dr. Jo-Ann Leong, my major professor, for her kindness, supervision and for providing me with the great opportunity to study in the heart of the valley.

I want to say thanks from the bottom of my heart to the fellows in the Virology laboratory: Dan Mourich, Dr. Mark Engelking, Lisa Oberg, Carla Mason, Jim Wirkkula, Leslie Tengelson, Lawrence Songjian Chen, Eric Anderson and Rebecca Day, for many, many, many helps!

I would like to thank Dr. Sandro Ristow at Washington State University and Dr. John Fryer at Oregon State University for providing monoclonal antibodies.

I do appreciate Dr. John Fryer, Dr. Wilbert Gamble and Dr. Larry Moore for being on my committee.

A special gratefulness is to my parents, sisters, brothers and their families for their encouragement.

My final thanks goes to my sweetie, Huanchun Ye, for his help and mental support.

TABLE OF CONTENTS

REVIEW OF LITERATURE	1
Rhabdovirus	1
Infectious Hemotopoietic Necrosis Virus (IHNV)	4
Glycoprotein	4
Fish Vaccine	9
MATERIALS AND METHODS	10
Antisera and Monoclonal Antibodies	10
Neutralization Assays	10
Construction of Chimeric Plasmids	11
Colony Immunoblot Detection	12
Determination of the Sizes of the Inserts	13
DNA Sequence Analysis	13
Western Immunoblot	15
Competitive Radioimmunoassay (RIA)	16
RESULTS	18
Virus Neutralization Tests	18
Construction of Fusion Protein Expression Plasmids	18
Expressions of the Fusion Proteins	19
Characterization of G Subunits by Monoclonal Antibodies (Mabs)	20
Competitive Radioimmunoassay (RIA)	20
DISCUSSION	22
BIBLIOGRAPHY	64

LIST OF FIGURES

<u>FIGURE</u>		<u>PAGE</u>
1.	Construction of trpE-G Fusion Protein Expression Plasmids	30
2.	Determination of the Sizes of the Inserts Contained in the Recombinant Plasmids by Taq I Cleavage	32
3.	Determination of the Sizes of the Inserts Contained in the Recombinant Plasmids by Pst I Cleavage	34
4.	Plasmid Sequencing Strategy for pXL2, pXL3 and pXL7 Plasmids	36
5.	The Sequence of the IHNV-G Portions Contained in pXL7 Plasmid	38
6.	Analysis of trpE-G Fusion Proteins	40
7.	Immunoblot Characterization of G Subunits by Monoclonal Antibodies	43
8.	Physical Map of the IHNV G Gene and the Locations of the Cysteine Residue and the Glycosylation on the G Proteins	48
9.	The Location of the Synthetic Peptide of 19 Amino Acids on the IHNV Glycoprotein	50
10.	¹²⁵ I-labeled Monoclonal Antibody Competitive Binding Assay with the Synthetic Peptide Positioning on the Amino Acids Sequence 426-444 of the Original IHNV Glycoprotein	51
11.	Competition Percentage for Each ¹²⁵ I-labeled Monoclonal Antibody	58

LIST OF FIGURES (continued)

<u>FIGURE</u>		<u>PAGE</u>
12.	The Position of the Synthetic Peptide and the Inserts Contained in pXL2, pXL3 and pXL7 Plasmids on the Hydrophathy Plot of the Glycoprotein	59
13.	The Epitope Mapping of the IHNV Glycoprotein	61
14.	Comparision of the Epitope Locations of the Glycoproteins of IHNV, VSV, and Rabies Virus	62

LIST OF TABLES

<u>TABLE</u>		<u>PAGE</u>
1A.	Neutralization Assay of Monoclonal Antibodies to IHNV Cedar River	28
1B.	Neutralization Assay of Monoclonal antibodies to IHNV Cedar River	29
2.	A Summary of the Immunoblot Characterization of G Subunits by Monoclonal Antibodies	47
3.	A Summary of the Predicted T Cell Epitope Segments for IHNV Glycoprotein	63

EPITOPE MAPPING AND CHARACTERIZATION OF THE GLYCOPROTEIN OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

REVIEW OF LITERATURE

Rhabdovirus

Rhabdoviruses are known to infect a wide variety of hosts including vertebrates, arthropods, and plants. These viruses are identified by their bullet-shaped appearance and have dimensions of approximately 70 X 180 nm. They possess an unsegmented single-stranded RNA genome of negative sense and a helical nucleocapsid surrounded by a lipoprotein envelope. Like the virions of all negative-stranded RNA viruses, the rhabdoviruses also contain RNA-dependent RNA polymerase activity. This enzymatic activity is required for the transcription and replication of the viral RNA.

Animal rhabdoviruses are defined into two genera: Vesiculovirus (Vesicular Stomatitis virus Group) and Lyssavirus (Rabies Virus Group) (Matthews, 1982) as well as an unclassified group which includes the fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV). The Vesiculovirus and Lyssavirus are distinguished by their viral protein patterns (Matthews, 1982). They have in common a polymerase (L), a glycoprotein (G) and a nucleocapside protein (N). The Vesiculoviruses have an NS phosphoprotein and one matrix protein, whereas the Lyssaviruses exhibit a protein pattern with two matrix proteins M1 and M2 (Matthews, 1982; Coslett et al., 1980).

The most well-documented studies of rhabdoviral structure and replication have been carried out with VSV. The linear, single-stranded

genomic RNA of VSV was found to be tightly bound by monomers of the N protein [49 kilodalton (kd)]. In addition, the two minor proteins - L (241 kd) and phosphoprotein NS (29 kd) were found to form the transcribing ribonucleoprotein particle (RNP) together with the RNA-N complex (Baltimore et al., 1970; Bishop, 1977; Moyer and Banerjee, 1975). Emerson and Yu (1976) have indicated that both NS and L proteins were required for VSV RNA synthesis by reconstruction experiments *in vitro*. In addition, it was found that the anti-L and anti-NS antibodies inhibited the transcription of VSV *in vitro* (Harmon and Summers, 1982). Further studies suggested that the L protein of VSV (New Jersey) was the transcriptase (Ongradi et al., 1985) and the NS protein played a role in the RNA chain elongation step (De and Banerjee, 1984). One feature of the NS protein was that it was highly negatively charged and phosphorylated (Gill and Banerjee, 1985; Sokol et al., 1974). It was observed that partial dephosphorylation of the NS protein by phosphatase decreased the amount of the transcription products (Kingsbury, et al., 1981). Recently, the phosphorylation of the serine positions 236 and 242 in the NS protein was found to regulate the formation of the transcription complex which contained the NS protein, L protein and the N-RNA template (Chattopadhyay and Banerjee, 1987). The phosphorylation of the NS protein was found to be catalyzed by purified L protein *in vitro* (Sanchez et al., 1985).

The viral M protein which is located on the inside of the viral envelope has two functions. First, it serves as an endogenous inhibitor of viral transcription (Carroll and Wagner, 1979; Clinton et al., 1978; Wilson and Lenard, 1981) and second, it plays an important role in the assembly of virus particles which bud from the plasma membrane (Schnitzer and Lodish,

1979; Weiss and Bennett, 1980). It was demonstrated that the VSV M protein was phosphorylated in VSV-infected cells (Imblum and Wagner, 1974; Moyer and Summers, 1974). The function of the M protein phosphorylation remains unknown.

The glycoprotein (G) has been found on the virion surface as protruding spikes. VSV has been estimated to contain an average of 1,205 molecules of G protein per virion (Thomas, et al., 1985). Dubovi and Wagner (1977) treated VSV with cross-linking agents and found the formation of G-G dimers and trimers. Thus, they suggested that the glycoprotein spike of VSV consisted of more than one G protein. However, there is also evidence for the presence of G monomer in virion. Monomeric G protein is present in preparation of the G protein obtained by extraction with octyl β -D-glucoside from purified virions (Crimmins et al., 1983). When purified rabies virus was treated with Triton X-100 and the viral glycoprotein was purified by isoelectric focusing, a polymeric structure was detected (Dietzschold et al., 1978). It has been demonstrated for both VSV and rabies virus that the neutralizing anti-rhabdovirus antibodies are directed against the G protein (Kelley et al., 1972; Cox et al., 1977). The locations of the antigenic determinants on both VSV and rabies virus glycoproteins will be discussed later in this report.

The physical organization of VSV genome has been delineated by UV inactivation studies and the gene order is 3' N-NS-M-G-L 5' (Ball and White, 1976; Abraham and Banerjer, 1976). The order of rabies genome has been shown to be 3' N-M1-M2-G-L 5' by UV inactivation studies (Flamand and Delagneau, 1978).

Infectious Hematopoietic Necrosis Virus (IHNV)

Infectious hematopoietic necrosis virus is a rhabdoviral pathogen which causes an acute, systemic disease in salmonid fish in North America, Europe, Japan and Taiwan (Pilcher and Fryer, 1978; Wolf, 1988). Under the electron microscope, the virion morphology is that of an enveloped, bullet-shaped particle with an average diameter of 90 nm and an average length of 160 nm (Amend and Chambers, 1970). The IHNV genome is a single-stranded and unsegmented RNA of negative sense. It is approximately 11,000 nucleotides long and has a sedimentation value of 38-40S (McCain et al., 1974; Hill et al., 1975). Leong and coworkers have reported that IHNV RNA composes 4.8% of the molecular weight of the virion (Leong et al., 1983). The IHNV virion protein consists of a polymerase L (150 kd), a surface glycoprotein G (66 kd), a nucleocapsid protein N (40-44 kd) and two matrix proteins (22.5 kd and 17 kd respectively) (McAllister and Wagner, 1975; Hill et al., 1975; Kurath and Leong, 1985). Five structural proteins and one non-virion protein (12 kd) (Kurath and Leong, 1985) are encoded by the IHNV genome. The gene order of 3' N-M1-M2-G-NV-L 5' has been determined by R-loop mapping (Kurath et al., 1985). The composition IHNV virion is very similar to that of rabies virus in having the two matrix proteins.

Glycoprotein

The glycoprotein (G) of rhabdoviruses, anchored in the membrane and exposed on the surface of the viruses, acts as the virus receptor ligand for cells (Bishop et al., 1975). The VSV G protein also mediates virus penetration and uncoating (Matlin et al., 1982). Previous studies have

demonstrated that the glycoproteins of both VSV and rabies virus can induce a neutralizing antibody response and protective immunity (Kelley et al., 1972; Cox et al., 1977; Wiktor et al., 1984). By using monoclonal antibodies, nine distinct antigenic sites have been defined for VSV [New Jersey strain (NJ)]. Four of these epitopes for VSV (NJ) were recognized by neutralizing monoclonal antibodies (Bricker et al., 1987). For the Indiana strain (Ind) of VSV, eleven distinct epitopes have been identified and four of them were found to be neutralizing epitopes (Volk et al., 1982). Seven of the nine epitopes identified for VSV (NJ) have been mapped to the region of the viral glycoprotein bounded by amino acids 193-289. Included among these seven epitopes were those recognized by the four neutralizing monoclonal antibodies (Keil and Wagner, 1989). Four epitopes including two neutralizing epitopes have been mapped for VSV (Ind) G protein. These were located in the cysteine-rich region of amino acids 80-183 near the N-terminus, and another seven epitopes, which also included two neutralizing epitopes, were mapped to a region near the C-terminus between amino acids 286 and 428 (Keil and Wagner, 1989).

The antigenic structure of the rabies virus glycoprotein has also been investigated. Three CNBr cleavage fragment of the rabies virus G protein, Cr1 (amino acids 1-44), Cr3 (amino acids 292-323) and Cr4 (amino acids 103-178) were found to induce rabies virus neutralizing antibodies, although the titers of the induced antibodies were lower than that of the antibody induced by the native G protein (Dietzschold et al., 1982). Using monoclonal antibodies and mutant viruses, it was shown that there were 12 unique epitopes on the G protein of the rabies CVS strain and 17 epitopes on the G protein of the rabies ERA strain (Lafon et al., 1983; Wiktor and

Koprowski, 1980). The neutralizing monoclonal antibodies were used to select the viral mutants that were resistant to neutralization from both ERA and CVS strains of rabies virus. Nucleotide sequence analyses of the G genes of these viral mutants revealed single nucleotide changes at positions 1062, 1070, 1072 and 1133. This correlated with the amino acids at positions 333 (Arg →Ile), 336 (Asn →Asp), 336 (Asn →Lys), and 357 (Gly → Arg), respectively. It is only with change in the Arg-333 that the viral phenotype changes from pathogenic to nonpathogenic (Dietzschold et al., 1983; Seif et al., 1985; Wunner et al., 1985).

In the research described here, I have sought to make a similar characterization of the epitopes of the IHNV glycoprotein. The IHNV glycoprotein gene has been cloned and sequenced (Koener et al., 1987). It is a gene of 1,609 nucleotides and encodes a protein of 508 amino acids which is similar in size to that of VSV (517 amino for VSV-NJ and 511 amino acids for VSV-Indiana) and rabies virus (524 amino acids). A comparison of the IHNV G protein with that of VSV and rabies indicated a homology of only 20%. More importantly, the structural domains such as transmembrane region, the signal peptide, cysteine positions, and location of glycosylation sites were conserved (Koener et al., 1987). This similarity would suggest that neutralizing and binding epitopes for the IHNV G protein would be located in regions that corresponded to those found for VSV and rabies.

Engelking and Leong (1989) have prepared monospecific antisera in rabbits to four purified IHNV structural proteins - N, M1, M2 and G. They found that the glycoprotein was the only viral protein which was capable of inducing neutralizing antibodies and the antisera prepared to N, M1 and M2 did not possess neutralizing or neutralization enhancing activity. Moreover,

they found that the purified G protein could block the neutralizing activity of antiserum prepared to the whole virus. The G protein has also been found to induce protective immunity in kokanee salmon (*Oncorhynchus nerka*) and rainbow trout (*Salmo gairdneri*) fry by injection and immersion vaccination. However, it was less immunogenic in fish than the glycoprotein of rabies virus in mice because 50 times more protein was required to induce comparable immunity in fish (Engelking and Leong, 1989). A significant finding that was made in this study was that the glycoprotein from a single type of IHNV could induce protective immunity in fish against challenge with many different electrophoretic types of IHNV. This was an encouraging finding because it indicated that different types of IHNV share common neutralization epitope(s).

These studies also showed that polyvalent rabbit antiserum prepared to virus that had been deglycosylated enzymatically contained virus neutralizing activity and reacted with glycosylated G protein in Western immunoblots. This finding indicated that there were neutralizing epitopes on the IHNV G protein that did not require carbohydrate side chains (Engelking and Leong, 1989).

Several investigators have demonstrated that the expression of the rabies glycoprotein in bacteria results in a denatured protein which was unable to produce a protective immune response (Malek et al., 1984; Yelverton et al., 1983). Thus, only a part of the IHNV G gene containing one or a few epitopes, instead of the intact G gene, was used to construct recombinant plasmids for bacterial expression (Gilmore et al., 1988). The IHNV G gene was digested into a number of small fragments with *Sau3A I* and these fragments were randomly inserted into *trpE* expression plasmids.

Two recombinant plasmids, p52G and p618G, were identified by screening transformed colonies with anti-IHNV and anti-IHNV G protein antisera. DNA sequence analyses of these plasmids indicated that each contained the same Sau3A I fragment of 329 bp ligated in frame to the *trpE* protein. They differed in which additional Sau3A I fragment had been ligated to the 3' end of the 329 bp fragment. The size of the fusion proteins expressed by p52G and p618G were 49 kd and 48 kd, respectively. The 329 bp fragment encoded a region of the IHNV G protein near its center which contained a highly hydrophilic peptide sequence. Bacterial lysates containing the p52G or p618G fusion proteins were effective in inducing protective immunity against IHNV (Gilmore et al., 1988). This "vaccine" was also immunoprotective against many different types of IHNV (Engelking and Leong, 1989). Thus, a nonglycosylated, highly hydrophilic region of the IHNV G protein could be expressed in an antigenically recognizable form in bacteria and this peptide region was also immunoprotective for a number of different IHNV types (Hsu et al., 1986)

All of these findings indicated that the characterization of binding and neutralization epitope(s) by examination of different regions of the G protein expressed in bacteria was possible. Although a region of approximately 100 amino acids had proven to be effective in vaccine trials, there still remained questions concerning the number of immunoprotective regions in the G protein and the identification of those regions of the G protein which were recognized by fish. These questions led us to develop a number of expression clones containing different regions of the G protein. This thesis describes the construction of these clones and the preliminary characterization of the antigenic structure of their expressed fusion

proteins.

Fish Vaccine

Infectious hematopoietic necrosis is a severe epizootic disease in fry and juvenile fish with a rapid onset of mortality. It has resulted in large economic losses to the salmon and trout industry in the Pacific Northwest. Presently, there is no chemotherapeutic method available for prevention or control this disease. Avoidance has been the only available way to prevent the disease. But avoidance is not always possible. The fish do produce a protective serum antibody upon immunization with killed IHNV and this antibody is able to neutralize IHNV in vitro (Amend and Smith, 1974). Thus, development of vaccine to IHNV is a potential way to control the IHN disease.

An ideal fish vaccine must be safe, effective, economical and easy to administer. The aquaculture industry is looking to advances in recombinant DNA technology to provide a new way for developing such vaccines. Engelking and Leong (1989) have indicated that the IHNV glycoprotein is the only viral antigen to elicit protective immunity in fish and it also has the ability to induce cross-protective immunity for other virus isolates. Therefore, a subunit vaccine composed of the intact viral glycoprotein or a portion of it should be an effective vaccine.

The work described in this report analyzes different regions of the viral glycoprotein for antigenic reactivity in vitro. It is part of a study to characterize the important epitopes of the IHNV glycoprotein.

MATERIALS AND METHODS

Antisera and Monoclonal Antibodies

The anti-IHNV (RB1 strain) rabbit serum was provided by Dr. Mark Engeling (Oregon State University). The monoclonal antibodies to IHNV glycoprotein were developed by Dr. Sandra Ristow (Washington State University). The monoclonal antibodies 127B, 131A, 135L, 136J and 151K were prepared against the Clear Springs Trout Company IHNV isolate, O39-82-SR. The monoclonal antibodies, 6S, was prepared against the Coleman (National Fish Hatchery, California) isolate. Monoclonal antibodies 15A, 15B, 2F and 2P were prepared to a Cedar River (Washington) IHNV isolate. The monoclonal antibodies, 127B and 131A, were neutralizing antibodies. Another neutralizing monoclonal antibody, RB/B5, was obtained from Dr. J. L. Fryer (Oregon State University). It was prepared to an IHNV strain isolated from steelhead trout at Round Butte, Oregon, and was found to neutralize ten different IHNV isolates (Winton et al., 1988).

Neutralization Assays

The virus-neutralizing abilities of Mabs 2F, 2P, 15A and 15B made to IHNV CD₂ (Cedar River, WA) were tested by neutralization assays. Briefly, a series of 10-fold IHNV (CD₂) dilutions were mixed with a constant amount of Mab to give a 1:5 Mab-virus mixture or the undiluted virus was directly mixed with Mab to give a 1:5 Mab-virus mixture. The mixtures were rocked on a Neutator (Clay Adams) at 15°C for three hours. IHNV (CD₂) mixed with RPMI-5% fetal calf serum at the 1:5 ratio served as virus controls. The

0.1 ml virus dilution-Mab mixtures from dilutions 10^{-3} to 10^{-7} were plated onto the 96-well plates with 0.1 ml CHSE 214 cells. For the direct mixtures of virus and Mab, 10-fold dilutions from 10^{-3} to 10^{-8} were made before plating onto 96-well plates. The plates were allowed to be incubated at 15°C for one week. The cytopathic effects (CPE) for each well of the plates were observed under microscope after the cultures were stained with 1% crystal violet in formaldehyde/acetic acid. The TCID_{50} and neutralization index were calculated (Rovozzo and Burke, 1973).

Construction of Chimeric Plasmids

The recombinant plasmid, pG8, contains the entire 1.6 kb coding sequence of the IHNV glycoprotein (G) gene (Koener et al., 1987). A DNA fragment containing the G gene was purified by gel electrophoresis in low melting temperature agarose (Sea Plaque, FMC Products) after Pst I restriction endonuclease digestion of pG8. The purified G gene fragment was then digested by the endonuclease Taq I [Bethesda Research Laboratory (BRL)]. This yielded four fragments of 245, 609, 552 and 203 bps (Figure 1). Both the 609 bp and 552 bp fragments and a mixture containing all four fragments were ligated to the plasmid vector, pATH 3 (provided by Dr. T. J. Koerner and Dr. A. Tzagoloff, Columbia University). The pATH 3 vector had been previously cleaved by Cla I and treated with calf intestinal alkaline phosphatase (Promega). Specific ligations were performed using T4 DNA ligase (BRL). The ligation mixtures were transformed into E. coli DH5 alpha competent cells and the transformed cells were plated on Luria-Bertani (LB) agar plates containing 120 ug/ml ampicillin. Those cells containing recombinant plasmids were identified by restriction mapping of the plasmid

DNA isolated from ampicillin resistant colonies. These constructions were designed to produce fusion proteins containing part of the trpE structural protein fused in frame to an appropriate G gene sequence were expressed under the control of the trpE promoter.

Colony Immunoblot Detection

Transformant colonies were screened for the production of specific viral peptides using anti-IHNV serum (Helfman and Hughes, 1987). The transformant colonies were inoculated onto nitrocellulose filters [Schleicher and Schuell (S&S)] from master plates, and these filters were then overlaid on the LB agar plates containing 120 ug/ml ampicillin and 15 ug/ml indole-acrylic acid for trpE promoter induction. The filters were incubated for 12 hours at 37°C to permit bacterial colony growth, then removed from the agar plate and placed in a chloroform vapor chamber for 1 hour. They were shaken gently in lysis-blocking solution (150mM NaCl; 5mM MgCl₂; 10 ug/ml DNase I; 40 ug/ml lysozyme; 3% gelatin in TBS) for 4 hours at room temperature. The filters then were washed three times with TBS (30mM Tris-HCl, pH 7.5; 0.5M NaCl) and reacted for one hour with 1:100 dilution of rabbit anti-IHNV serum which had been previously absorbed with E. coli lysate. Following three washes with TBS the filters were incubated with a 1:1000 dilution of goat anti-rabbit IgG peroxidase conjugated antibody [Boehringer Mannheim (BM)] for 1 hour after three times wash with TBS. The substrate solution [0.5 X TBS; 0.6 mg/ml 4-chloro-1-naphthol; 0.1% (V/V) hydrogen peroxide] was used to react with antibody bound to bacterial colonies containing recombinant DNA clones expressing a fragment of IHNV-G gene.

Determination of the Sizes of the Inserts

Positive recombinant colonies were randomly chosen for subsequent examination. The plasmid DNA was isolated by the alkaline lysis miniplasmid preparation method (Maniatis et al., 1982). Overnight cultures in LB media containing 120 ug/ml ampicillin were centrifuged and the cell pellets were treated with lysozyme (2mg/ml lysozyme; 20% sucrose in 0.1M Tris and 0.02M EDTA, pH 8.0) followed by lysis in an alkaline solution containing 1% SDS in 0.2N NaOH. Then, the solutions were neutralized with 1M sodium acetate (pH 4.8). After removal of most of the chromosomal DNA and bacterial proteins by differential centrifugation, the supernatant fluid was treated with ribonuclease and the plasmid DNA was concentrated by ethanol precipitation. The isolated plasmid DNAs were digested with Taq I (BRL) at 65°C and electrophoresed in a 2% agarose gel (Sea Plaque, FMC Products) (Figure 2). In addition, the isolated plasmids were cleaved with Pst I (BM) at 37°C and the mixtures were separated in a 1.5% agarose (Sea Plaque, FMC) (Figure 3). The gels were stained by ethidium bromide to visualize the fragments. The inserts containing a portion of the G gene were identified by the comparison with the fragments obtained for pATH 3 DNA after Taq I or Pst I digestion. The sizes of the DNA fragments were estimated from the mobility of the fragments in gels as compared to that of digested lambda DNA and øX 174 DNA markers (BRL).

DNA Sequence Analysis

Three clones containing different portions of the G gene were identified by restriction enzyme analyses. Plasmid DNA from each clone was sequenced by the supercoil DNA sequencing procedure (Wang et al., 1988).

Unpurified templates of the G gene clones were used to determine their exact location within the G gene. The templates were prepared by the rapid boiling method (Holmes and Quigley, 1981). Overnight cultures of each E. coli clone were grown in LB-ampicillin broth media at 37°C. The DNA templates were concentrated in a microfuge for 1 minute at room temperature and resuspended in STET buffer (8% glucose; 0.5% Triton X-100; 50mM EDTA; 10mM Tris-HCl, pH 8.0). A Freshly prepared lysozyme solution (Sigma) [10 mg/ml in TE buffer (pH 8.0)] was added to a final concentration of 75 ug/ml lysozyme. The preparations were immediately placed in boiling-water for 40-50 seconds at which time they were centrifuged in a microfuge for 12 minutes at room temperature to remove genomic DNA and cell debris. The plasmid DNAs were recovered from the supernatant by isopropanol precipitation.

The template DNAs were sequenced using the Sequenase kit with ³⁵S-dATP as the labelled nucleotide (United States Biochemical Corporation) as follows: A 1:5 dilution of the template DNA solution was made and the DNA solution was then denatured in 2M NaOH and 2mM EDTA for 5 minutes at room temperature. The DNA solution was subsequently neutralized with 0.9M sodium acetate (pH 5.0) and precipitated with 95% ethanol. The sequencing procedures called for the annealing of the appropriate primers to the template DNA at 80°C for 2 minutes in Sequenase buffer (40mM Tris-HCl, pH 7.5; 20mM MgCl₂; 50mM NaCl); subsequently, the annealing mixture was cooled slowly to room temperature. The binding sites of the primers used were located in the 5'-polylinker and in the region after the 3'-polylinker of pATH 3 vectors, respectively. The synthesis of nucleotide chains was carried out in 0.1M dithiothreitol, labeling nucleotide mixture,

alpha-³⁵S dATP and Sequenase for 5 minutes at room temperature. The labeling reaction mixtures were then transferred to appropriate dideoxy termination reaction mixtures and incubated for 5 minutes at 37°C. At that time the stop solution (95% formamide, 20mM EDTA, bromophenol blue and xylene cyanol) was added and the sequencing reaction mixtures were applied to a 6% polyacrylamide-8M urea gels in TBE buffer (89mM Tris base; 89mM boric acid; 2mM EDTA). The gels were electrophoresed for an appropriate period of time and then fixed with 5% acetic acid and 5% methanol for 15 minutes. The gels were dried and autoradiographed (KodaK X-AR-5 film) at room temperature overnight.

Western Immunoblot

Bacterial colonies containing the recombinant plasmids under examination were grown in LB broth media containing 120 ug/ml ampicillin and 20 ug/ml indoleacrylic acid for 18 hours. The cells were harvested by centrifugation and lysed by high-speed agitation in a minibeadbeater (Biospec Products) in Tris-EDTA buffer (10mM Tris, pH 8.0; 1mM EDTA, pH 8.0). The lysates were electrophoresed on SDS-PAGE gels (Laemmli, 1970) (10% separating gel and 4.5% stacking gel). The gels were transferred to nitrocellulose filters (Towbin et al., 1979). The Western blots were then incubated in a solution of TBS containing 3% gelatin for 1 hour. The filters were incubated with 1:100 dilution of anti-IHNV serum or monoclonal antibodies at room temperature. After three washes with TBS, the filters were reacted with 1:1000 dilution of goat anti-rabbit IgG peroxidase-conjugated antibody for the detection of specific binding by rabbit anti-IHNV polyclonal serum or goat anti-mouse IgG or IgM-peroxidase conjugated

antibody for the detection of specific binding by mouse anti-G monoclonal antibodies. Hydrogen peroxide-containing substrate solution [0.5 X TBS; 0.6 mg/ml 4-chloro-naphthol; 0.1% (V/V) hydrogen peroxide] was used in color development. The fusion proteins were detected as purple bands.

Competitive Radioimmunoassay (RIA)

The fusion proteins were further characterized by competitive radio-immune procedures. A peptide of 19 amino acids was synthesized to match the amino acid sequence derived from the cDNA sequence of the IHNV G gene from 1325-1380 bp. This peptide was used in competitive radioimmunoassays to determine whether it would specifically inhibit the binding of radiolabeled monoclonal antibodies to purified virus. Tissue culture fluids containing the monoclonal antibodies were obtained from Dr. S. Ristow and concentrated by ammonium sulfate precipitation as described by Good and co-workers (1980). The concentrated monoclonal antibodies were labeled with ^{125}I iodine by the chloramine-T method (Lefkovits and Pernis, 1985). The 100 μl concentrated monoclonal antibodies were incubated with 5 μl Na^{125}I (100 mCi/ml in 0.01M NaOH), phosphate buffer saline (PBS), pH 7.4, 5×10^{-5} M KI and 2 mg/ml chloramine-T for 5 minutes at room temperature. The free, non-bound radioactive iodine was removed by passing the labeling mixture through a Sepharose G-25 pre-packed column (Pharmacia). The labeled monoclonal antibody was eluted with PBS and 15 fractions were collected for each antibody mixture. To each fraction tube, 25% bovine serum albumin (BSA) was added to a final concentration of 5 % to prevent radiation damage to the iodinated product. A 5 μl sample from each fraction was counted with a Beckman Gamma 5500 counter to determine which

fractions contained the labeled immunoglobulin. The peak fractions were pooled.

The saturating concentration for each labeled antibody for a standard quantity of IHNV was determined as follows: a series of increasing quantities of labeled antibody was incubated in Immulon I breakaway wells which had been coated with 0.5 ug of purified IHNV (RB1 or O39-82) in 100 ul of bicarbonate buffer [1.96 gram (g) of Na_2CO_3 and 2.93g of NaHCO_3 in one liter distilled water, pH 9.6]. The wells were incubated at room temperature for 2.5-3 hours. After this time, the wells were rinsed with PBS for 5 times and then the radioactivity bound to each well was measured in a Beckman Gamma 5500. Wells which were coated with 0.5 ug BSA instead of IHNV served as controls for non-specific binding of the radiolabeled antibody.

For the competitive assay with the synthetic peptide, virus saturating amounts of each iodinated monoclonal antibody were mixed with a series of peptide dilutions and incubated for 1 hour at room temperature. The mixture was then added to wells coated either with RB1 or O39-82-SR IHNV. Monoclonal antibodies 127B, 135L, 136J and 6S were previously shown to bind to RB1 IHNV; monoclonal antibodies 131A and 151K were previously shown to bind to O39-82-SR IHNV (S. Ristow, personal communication). After a 3-hour incubation at room temperature, the mixture in each well was removed and the well was washed with PBS. The wells were counted in a Beckman Gamma 5500 counter to determine the amount of labeled antibody which was still able to bind to the virus.

RESULTS

Virus Neutralization Tests

The neutralization abilities of Mabs 2F, 2P, 15A and 15B on IHNV (CD₂) were determined by the virus neutralization indices as described by Rovozzo and Burke (1973). An index of at least 1.7 was considered significant. The monolayer in each well was examined microscopically for cytopathology. The neutralization indices (N.I.) were calculated from the TCID₅₀ values by this equation:

$$\text{N.I.} = - \log (\text{TCID}_{50} \text{ of virus control} - \text{TCID}_{50} \text{ of tested Mab})$$

The neutralizing indices of Mabs 2F, 2P, 15A and 15B were found to be much lower than 1.7. Therefore, it was concluded that these four Mabs were non-neutralizing antibodies (Table 1A and 1B).

Construction of Fusion Protein Expression Plasmids

The characterization of the antigenic domains of the IHNV G protein was made by analyzing the immunogenic reactivity of the different cloned regions of the G protein. Portions of the G gene were subcloned into trpE expression vectors to create trpE-G protein fusions (Figure 1). The fusion proteins were expressed in E. coli after indoleacrylic acid induction and lysates of induced proteins were analyzed by immunoblot or radioimmunoassay.

A cDNA clone of the G gene, pG8, was found to contain three Taq I cleavage sites at positions base pair 245, 854 and 1406, respectively (Figure 4). Taq I digestion of purified G gene resulted in four fragments of 245 bp, 609 bp, 552 bp and 203 bp and these fragment sizes were

confirmed by agarose gel analysis (Figure 2 and Figure 3). Constructions of recombinant plasmids containing these fragments were made and eighty-eight transformant colonies were screened for G protein expression by colony immunoblot with anti-IHNV sera. Three plasmids were identified for subsequent analysis, pXL2, pXL3 and pXL7. Restriction analysis of the purified plasmid DNAs indicated that pXL2 contained a G gene fragment of 609 bp and pXL3 contained a fragment of 552 bp. Both of these constructions were made with purified G gene fragments. The pXL7 construction was made with a mixture of G gene fragments and an analysis of the G gene inserts in this plasmid indicated that the two smaller G gene fragments of 245 bp and 203 bp had been ligated at the Pst I sites and then inserted into the Cla I site of the pATH 3 as shown in Figures 4 and 5. Each plasmid construction was verified by direct DNA sequence analysis of the purified plasmid DNA (Wang et al., 1988).

The plasmid sequencing strategy for pXL2, pXL3 and pXL7 is shown in Figure 4. For pXL7, the D fragment at the C-terminal end of the G gene was ligated in frame to the trpE gene of pATH 3. Sequence analysis indicated that the A fragment of 245 bp had been ligated to the D fragment at the Pst I site in the same orientation as the G gene (Figure 5).

Expressions of the Fusion Proteins

The expressed fusion proteins encoded by pXL2, pXL3 and pXL7 were detected both by Coomassie Brilliant Blue staining of the protein distributed by SDS-PAGE gel (Figure 6A) and by Western blot with anti-IHNV serum (Figure 6B). The trpE protein of the pATH vector is approximately 37 kd. The protein bands indicated by arrows in the pXL2 and pXL3 lanes of

Figure 6 have estimated molecular weights 59.3 and 57.3, respectively. These molecular weight estimations coincide with that calculated for the trpE protein and the fused G protein segment for each plasmid. For pXL7, there is a termination codon (TAA) at position base pair 1574 (Figure 5) which resulted in the addition of only 55 amino acids of the G protein to the C terminal region of the trpE protein. Thus, the calculated molecular weight of the fusion protein encoded by pXL7 was 43. Although no specific band of this size was detected in the Coomassie brilliant blue stained gel, an immunologically reactive band of this size was detected by immunoblot assay (Figure 6B).

Characterization of G Subunits by Monoclonal Antibodies (Mabs)

An attempt was made to determine the location of linear antigenic determinants of the IHNV G protein by reaction with Mabs in Western immunoblots. Proteins synthesized in bacteria containing the pXL2, pXL3 or pXL7 plasmids were resolved by SDS-PAGE and transferred to nitrocellulose. These Western blots were treated with ten different Mabs. The non-neutralizing monoclonal antibody 136J bound to pXL3, p52G and p618G (Figure 7A) and non-neutralizing Mab 2F reacted with pXL3 (Figure 7B). The pXL7 subunit was detected by neutralizing Mab RB/B5 (Figure 7C). Table 2 summarizes the binding activity of each Mab and the results of Western immunoblot assays with the 10 Mabs.

Competitive Radioimmunoassay (RIA)

Mabs 127B, 131A, 135L, 136J, 151K and 6S were assayed for their ability to bind to a synthetic peptide of 19 amino acids derived from the G

gene sequence present in pXL3, p52G and p618G recombinant plasmids (Figure 9). Saturating concentrations of ^{125}I -labeled antibody to IHNV-coated wells were determined for each Mab. Then, increasing concentrations of the synthetic peptide was added to determine whether the binding of the labeled Mab would be specifically affected by the synthetic peptide. Only Mab 127B was competitively inhibited by the peptide (Figure 10A and Figure 11). The peptide did not abolish the bindings of Mabs 131A, 135L, 136J, 151K and 6S to viruses (Figure 10B, 10C, 10D, 10E and 10F). These data suggest that the synthetic peptide of 19 amino acid derived from pXL3, p52G and p618G contains a neutralizing epitope, 127B.

DISCUSSION

The IHNV glycoprotein has been identified as the virion protein which elicits neutralizing antibody in rabbits and induces protective immunity in fish to homologous and heterologous strains of IHNV (Engelking and Leong 1989). These findings suggested that genetic engineering might be used to develop an economically feasible IHNV vaccine for fish. Thus, a clone of the IHNV glycoprotein gene was made (Kurath and Leong, 1985; Kurath et al., 1985; Koener et al., 1987) and expression of a portion of this gene in bacteria resulted in a prototype IHNV subunit vaccine (Gilmore et al., 1988). Only 350 bases of IHNV sequence was expressed in this initial vaccine construction because there were 16 cysteine residues (Figure 8B) in the glycoprotein gene. Previous work with the rabies glycoprotein had shown that when the entire gene was expressed in bacteria, a denatured protein was produced, presumably because appropriate folding mechanisms for disulfide bond formation in protein were absent in *E. coli*. The IHNV vaccine clone contained a region of the gene which encoded only one cysteine residue.

Despite the efficacy of the vaccine in laboratory trials, it seemed useful to determine whether other regions of the IHNV glycoprotein gene would be expressed in an antigenically recognizable form in bacteria and thereby, provide increased protection in fish. The recombinant plasmids pXL2, pXL3, and pXL7 were constructed so that all regions of the glycoprotein gene were expressed in bacteria as trpE-G fusion proteins. All of these recombinant plasmids produced fusion proteins that were also analyzed in Western immunoblots with anti-IHNV sera and specific monoclonal antibodies. These

results were compared with the proteins produced by p52G and p618G, the plasmids identified in the original vaccine construction. The results of this comparison is shown in Table 2.

The pXL2 plasmid encoded a trpE-G fusion protein containing the 201 amino acids of the glycoprotein near the N-terminus. This fusion protein was recognized by anti-IHNV serum but not by anti-G monoclonal antibodies. The pXL7 plasmid contains two regions of the G gene, A and D as shown in Figure 5. Although there are 510 nucleotides of G gene inserted into pXL7, the D fragment from the C terminal region of the glycoprotein is the only sequence expressed as part of the trpE-fusion protein. There is a TAA termination codon at the end of the D fragment (position 1547 in the original glycoprotein gene) and there is no appropriate recognition site for initiation of translation (Shine-Dalgarno sequence) at the 5' end of the downstream A fragment. Therefore, a peptide of only 55 amino acids was encoded by pXL7. This 55 amino acid peptide reacted with both anti-IHNV serum and the neutralizing monoclonal antibody RB/B5. The result indicates that there is a neutralizing epitope within this C-terminal region of 55 amino acids.

Although the plasmids, pXL3, p52G and p618G, have different size DNA inserts, they contain common G gene sequences from bases 1052 to 1379. The plasmid, pXL3, contains an additional 198 nucleotides of G gene sequence at the 5' end. These plasmids express trpE-G fusion proteins of 57.3 kd, 49 kd and 48 kd, respectively. All of these proteins react with polyclonal anti-IHNV sera and the non-neutralizing monoclonal antibody 136J in Western immunoblots. This result indicates that the common 109 amino acid sequence (336-444) encoded by these three plasmids contains a

linear epitope. In addition, the neutralizing monoclonal antibody, 127B, was able to bind to an epitope on the viral glycoprotein in radioimmune assays. The binding of 127B to IHNV or any of the fusion proteins did not occur in Western immunoblots (Table 2). This suggested that the epitope recognized by 127B was not strictly a linear array of amino acids, but rather a sequence that required a certain secondary conformation.

The binding of labeled 127B to purified virus could be effectively blocked in radioimmunoassays with a synthetic peptide derived from the amino acid sequence present in pXL3, p52G, and p618G (Figures 10A and 11). This 19 amino acid peptide, NH₃-lys-ser-val-pro-his-pro-ser-ile-leu-ala-phe-tyr-asn-glu-thr-asp-leu-ser-gly-COOH, corresponded to amino acid residues 426 to 444 of the IHNV glycoprotein (Koener et al., 1987) and contained proline residues at positions 429 and 431. Analysis of the peptide by the rules of Chou and Fasman (1974) indicated that there was a possible β turn in the molecule which would lend a secondary conformation to the peptide. This peptide was shown to be slightly hydrophilic by computer analysis using Kyte and Doolittle hydrophathy scale with a window of 10 consecutive amino acid residues (Koener et al., 1987) (Figure 12).

The monoclonal antibody, 2F, did recognize an epitope which was found only in pXL3. The recognition was made in Western immunoblot and indicated that there was a distinct linear epitope located in the unique 66 amino acids at N terminus of the pXL3 encoded G protein sequence. Mab 2F was generated against the Cedar River strain of IHNV and Mabs 136J and 127B were prepared to the O39-82-SR strain. Since pXL3, a clone containing G gene sequences from the Round Butte strain of IHNV, reacted with all three Mabs, the three viral strains must contain common epitopes

defined by 2F, 136J and 127B (Figure 13).

Although there is a very low level of homology between the glycoprotein sequences of IHNV, VSV and rabies virus, the structural domains on these three glycoproteins have been conserved (Koener et al., 1987). It should be possible to determine whether there is also a conservation in the antigenic structure of the rhabdovirus glycoproteins. The seven epitopes of VSV (Ind) which included two neutralizing epitopes were mapped to the amino acid sequence 286 to 428 (Figure 13). For rabies virus (ERA) glycoprotein, the amino acids arg-333, asn-336 and gly-357 were found to be important for its antigenic structure. Interestingly, the three epitopes of the IHNV glycoprotein which have been identified are localized in the region of amino acids 270 to 444 (Figure 14). This region corresponds to those of the other two rhabdoviruses. This finding suggests that the glycoproteins of IHNV, VSV and rabies virus are related in their three-dimensional structure.

T Lymphocytes (T cells) are involved in both cellular and humoral immune systems. Previous studies have indicated that there are epitopes on some antigen proteins, such as insulin, influenza hemagglutinin A, myoglobin, foot-and-mouth disease virus VP1, hepatitis B surface antigen and rabies virus glycoprotein, which are able to activate T cell division (Roitt et al., 1989). Studies with the rabies virus glycoprotein have shown that the T cell epitope(s) differ from the neutralizing epitopes (Wunner et al., 1985). Because this work was initiated to develop an effective IHNV vaccine for fish and inclusion of appropriate IHNV T cell antigens would be a logical addition to vaccine, an analysis of glycoprotein for T cell antigens was made. Current models for T cell function implicate that T cells in the recognition of the processed antigens which are presented on the surface of

antigen-presenting cells (APC) by the major histocompatibility complex (MHC) molecules (Schwartz, 1985). In recent years, the structural properties of T cell antigens have been examined. One of the models for the requisite structure of a T cell antigen involves an amphipathic helix, which has a hydrophobic domain on one side of the helix and a hydrophilic domain on the other. It is postulated that the hydrophobic side of processed antigen segments would bind to MHC molecules on the surface of APCs and the hydrophilic side would be recognized by the T cells receptors (DeLisi and Berzofsky, 1985). A computer algorithm for amphipathic helix model called TCELL has been created (Margalit et al., 1987). By this program, 18 out of the 23 known T cell antigenic sites (77%) were found to overlap amphipathic helix regions (Margalit et al., 1987). This result suggested that amphipathic helix-containing regions of antigens are potential T cell binding sites.

There are a number of other factors which should be considered in the evaluation of amphipathic helices as T cell antigen. The location of lysine as the ultimate or penultimate C-terminal residue in helical antigenic sites has been reported to be strongly correlated with T cell antigen structures by Spouge and co-workers (1987). Proline was not usually found in helices except near the N-terminus and N-glycosylation was thought to mask the T cell epitope (Margalit et al., 1987). An analysis of the IHNV glycoprotein was made with the TCELL computer program to determine the number and location of the possible amphipathic helix fragments. The most likely candidates for T cell antigens which were predicted by the analysis is shown in Table 3.

The glycoprotein peptide sequence with the highest probability as a T cell antigen (peptide number 1 in Table 3) was located in a region of the

glycoprotein (amino acids 77-110) encoded by pXL2. This region did not react with any of the monoclonal antibodies that were available although it did react with polyclonal rabbit anti-IHNV serum. The peptides 8 and 9 which were identified as possible T cell antigens were also found in the trpE-fusion proteins expressed by the plasmids pXL3, p52G, and p618G. Its presence in this region may account for the "immunodominance" of this region in the development of G protein reactive monoclonal antibodies.

We are unable at this time to determine whether fish lymphocytes react in a manner similar to the mammalian (mouse) T cell in the recognition of amphipathic helical structure. If fish do respond in an immunologically similar fashion, then it is possible that these T cell antigenic regions may help amplify the immune response to IHNV G protein in fish. We may be able to examine this question by creating synthetic peptides containing the amino acids sequences identified in Table 3 and using these peptides as adjuvants in vaccination. They would be used with synthetic peptides from the antigenic region identified by neutralizing monoclonal antibodies.

Table 1A. Neutralization Assay of Monoclonal Antibodies to IHNV Cedar River

	Infected Wells (CPE% > 50%)					TCID ₅₀ /0.08ml	Neutralizing Index
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷		
IHNV	ND ^b	12/12	3/12	0/12	0/12	10 ^{-4.67}	—
2F	ND	11/11	6/12	0/11	0/11	10 ⁻⁵	-0.33
2P	ND	8/12	5/12	1/12	0/12	10 ^{-4.65}	0.02
IHNV	12/12	11/12	2/12	1/12	0/12	10 ^{-4.6}	—
15A	12/12	11/12	6/12	0/12	0/12	10 ^{-5.0}	-0.4
15B	12/12	11/12	4/12	1/12	0/12	10 ^{-4.76}	-0.16

^a IHNV Cedar River strain (WA) designated CD₂ (Hsu et al., 1986).

^b Not done

Table 1B. Neutralization Assay of Monoclonal Antibodies to IHNV Cedar River

	Infected Wells (CPE% > 50%)						TCID ₅₀ /0.08ml	Neutralizing Index
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸		
IHNV	ND ^b	12/12	7/12	0/10	0/11	0/8	10 ^{-5.14}	—
2F	ND	11/12	2/12	0/10	0/10	0/10	10 ^{-4.56}	0.575
IHNV	12/12	7/12	1/12	0/12	0/12	0/12	10 ^{-4.21}	—
15A	12/12	10/12	4/12	0/12	0/12	0/12	10 ^{-4.64}	-0.43
15B	12/12	11/12	7/12	0/12	0/12	0/12	10 ^{-5.07}	-0.86

^aNot done

Figure 1. **Construction of trpE-G fusion protein expression plasmids.** The 1.6 kb cDNA of IHNV G gene isolated from the pG8 was digested with Taq I. The portions of G gene were subcloned into pATH 3 vectors pre-cleaved by Cla I as described in Materials and Methods.

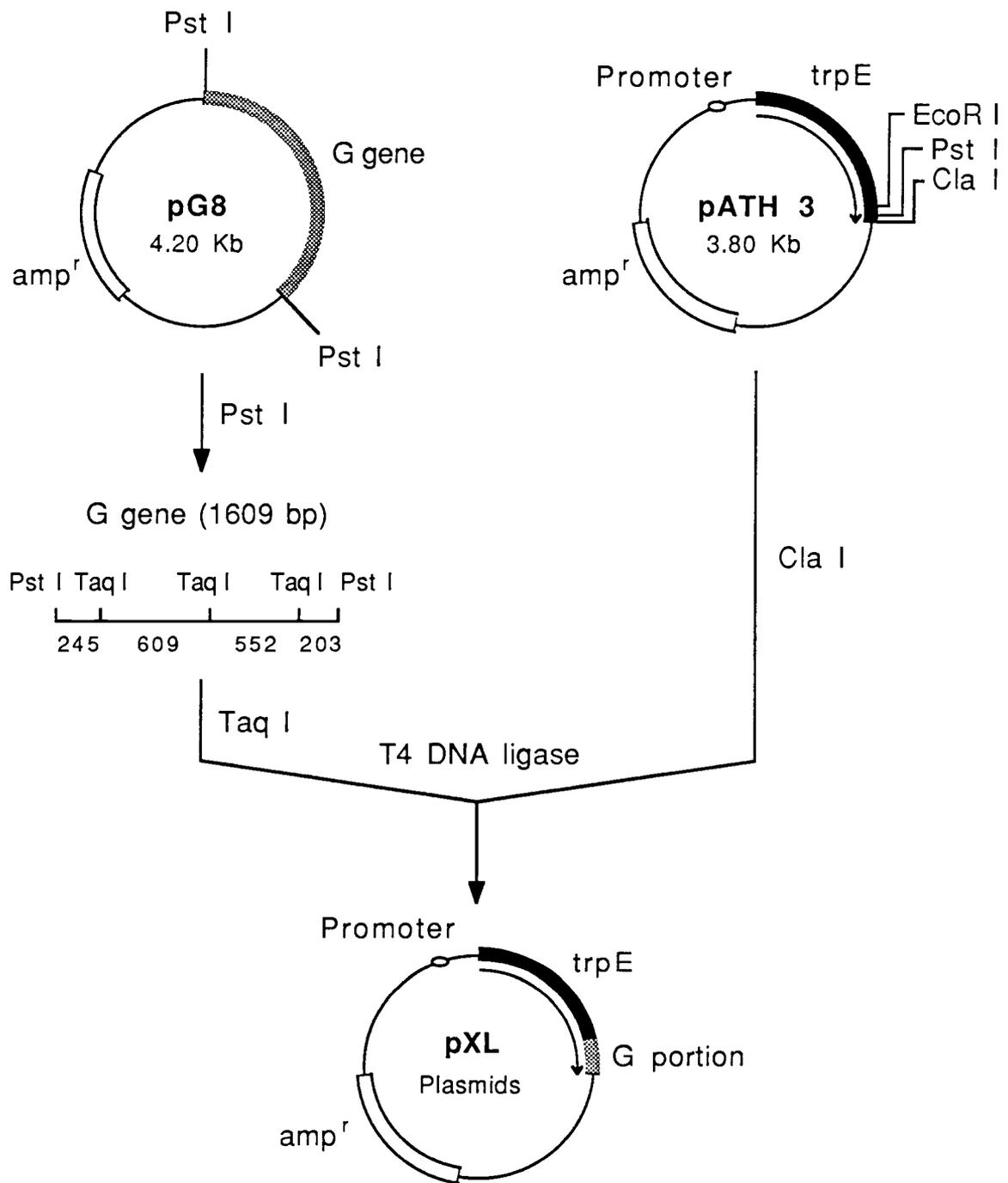


Figure 1.

Figure 2. **Determination of the sizes of the inserts contained in the recombinant plasmids by Taq I cleavage.** The plasmid isolated from immunopositive colonies pATH 3, pXL2 and pXL7 plasmids were digested with Taq I and the DNA fragments were separated by electrophoresis in a 2% agarose gel. The insert sizes in pXL2 and pXL7 were estimated from the gel in comparison with pATH 3 vector. Marker lane contains the Hind III-digested lambda phage DNA and Hae III-digested phiX 174 phage DNA.

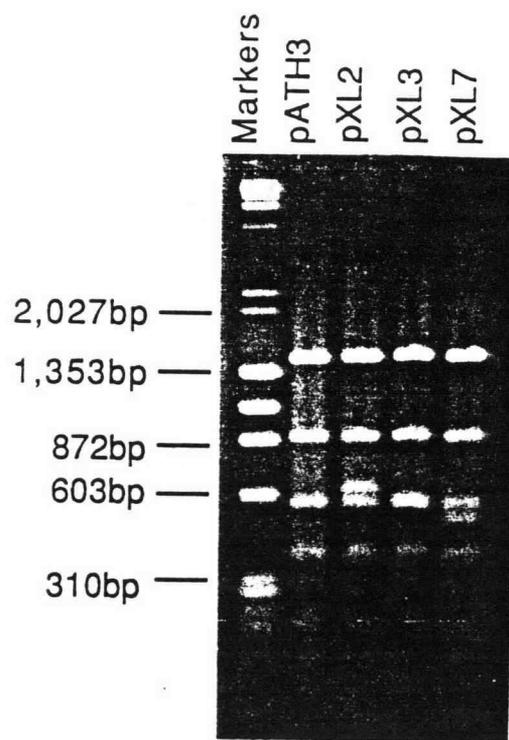


Figure 2.

Figure 3. Determination of the sizes of the inserts contained in the the recombinant plasmids by Pst I cleavage.

The isolated pXL2, pXL3 and pXL7 plasmids were digested with Pst I and electro phoresed in a 1.5% agarose gel. The insert sizes in pXL2, pXL3 and pXL7 plasmids were estimated from the gel using Cla I-digested pATH 3 vector as control. Marker lane contains the Hind III-digested lamda and hae III-digested øX174 phage DNAs.

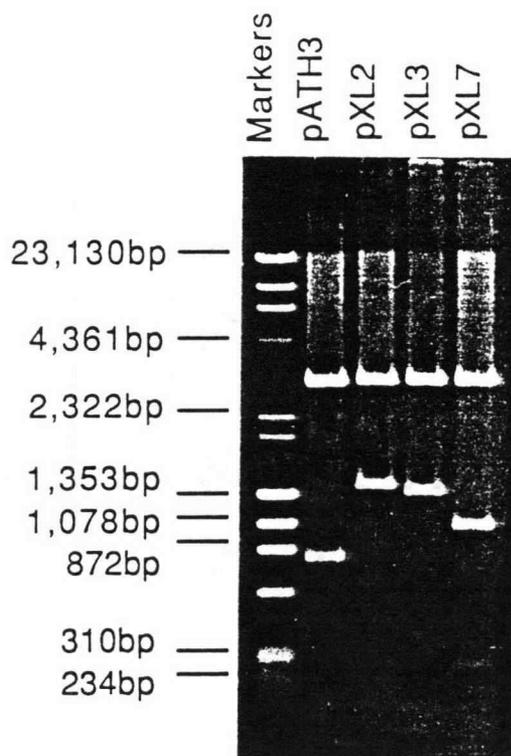


Figure 3.

Figure 4. **Plasmid sequencing strategy for pXL2, pXL3 and pXL7 clones.** Arrows indicate the direction of sequencing and the sequenced region.

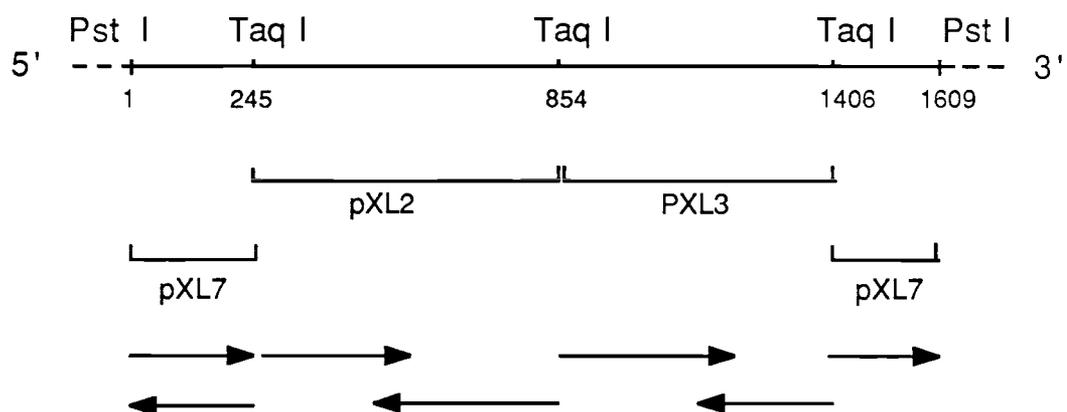


Figure 4.

Figure 5. **The sequence of IHNV-G portions contained in pXL7 plasmid.**

Figure 6. **Analysis of trpE-G fusion proteins.** Bacterial lysates were resolved on 10% SDS-PAGE gels. (A) Proteins on one gel was stained with 0.1% Commassie Brilliant Blue and destained in a solution containing 30% methol and 10% acetic acid. Arrows indicate the positions of fusion proteins expressed by pXL2 and pXL3 plasmids. (B) Proteins on one gel were transferred to nitrocellulose, and analyzed with anti-IHNV serum. The trpE-G fusion proteins are shown. The major band expressed by pXL2, pXL3, pXL7, p52G and p618G migrated at a position of 59.3 kd, 57.3 kd, 43 kd, 49 kd and 48 kd, respectively.

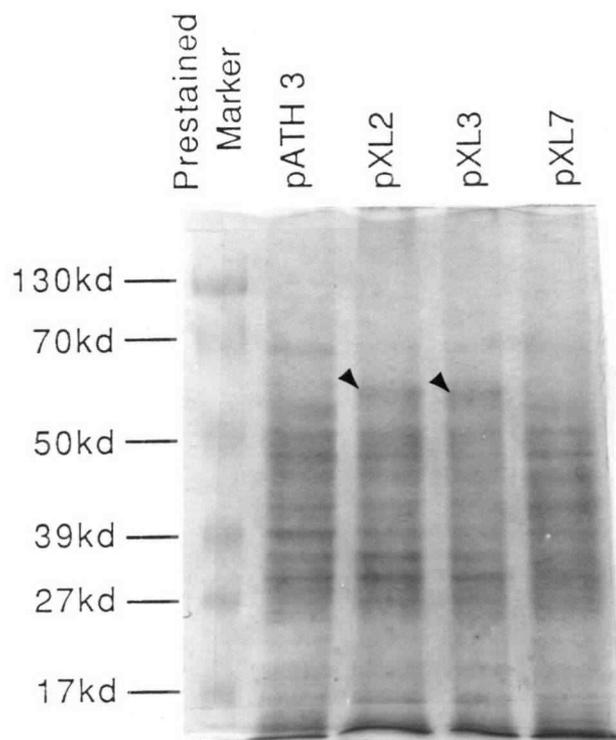


Figure 6A.

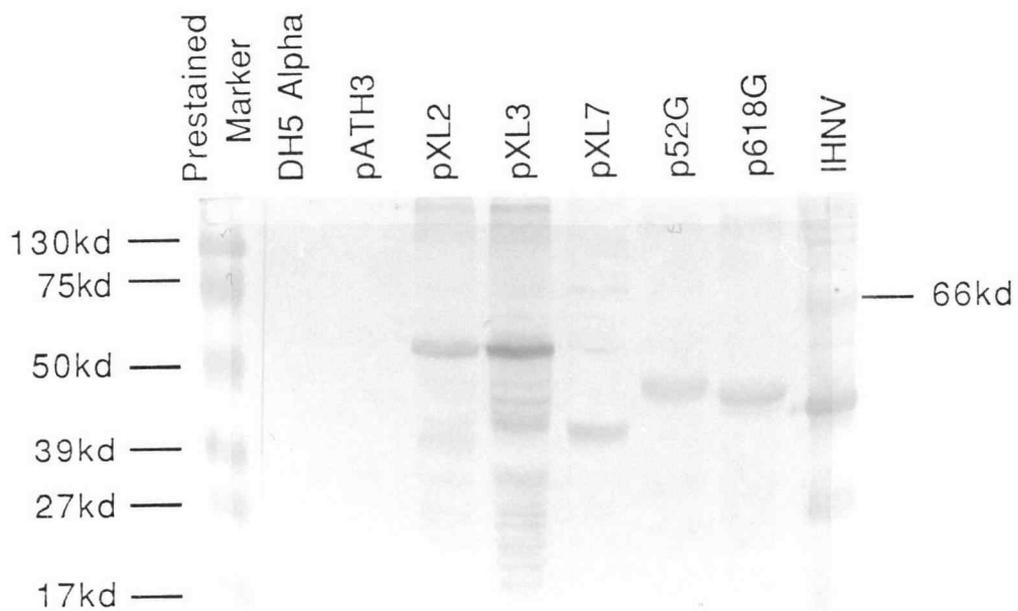


Figure 6B.

Figure 7. **Immunoblot characterization of G subunits by monoclonal antibodies.** Bacterial lysates were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose. (A) Immunoblot with 136J. The pXL3, p52G and p618G fusion proteins are shown to be recognized by 136J. (B) Immunoblot with 2F. The 57.3 kd pXL3 fusion protein is indicated by a arrow. (C) Immuno blot with RB/B5. The reactive major band is the pXL7 fusion protein.

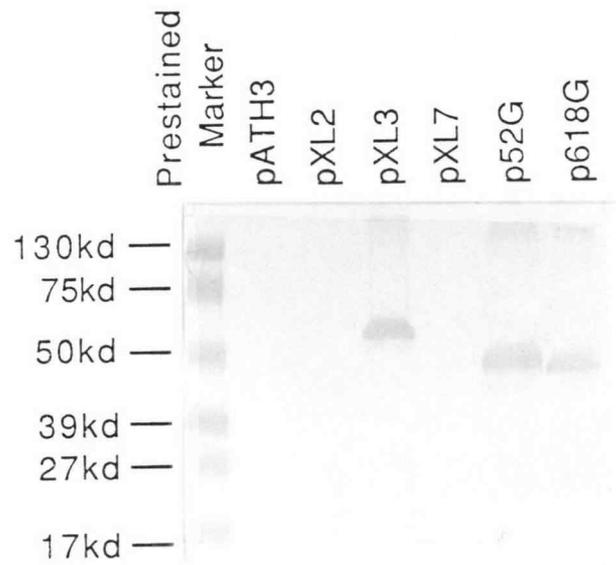


Figure 7A.

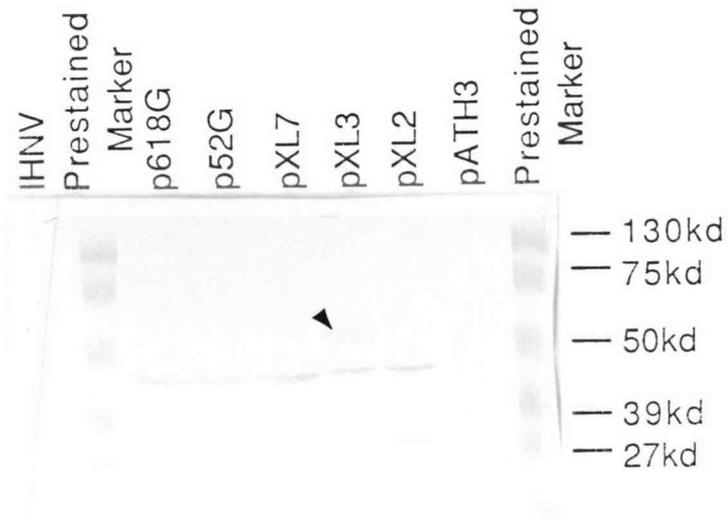


Figure 7B.

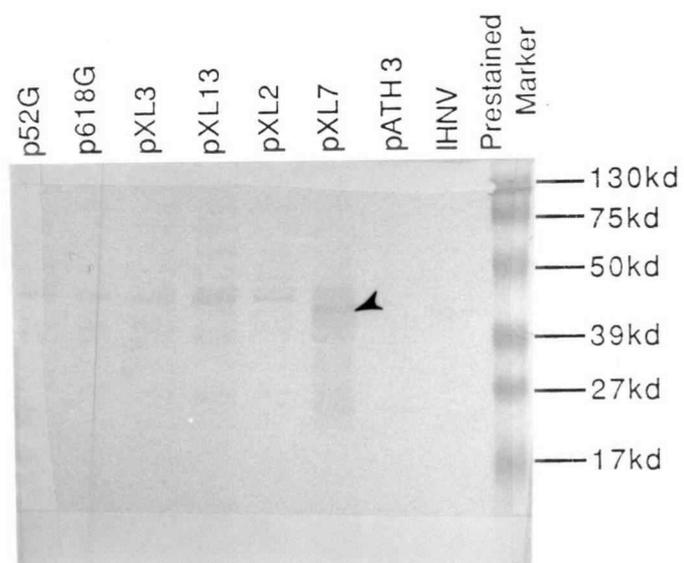


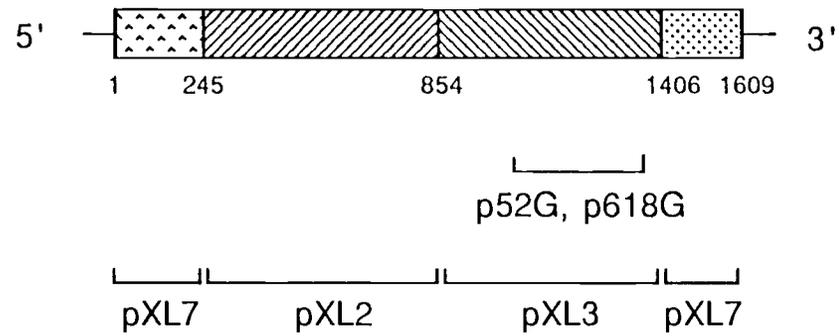
Figure 7C.

Table 2. A summary of the immunoblot characterization of G subunits by monoclonal antibodies

Mabs to IHNV	Ig type	Neutralizing ability	Western Blot Assay				
			pXL2	pXL3	pXL7	p52G	p618G
Hagerman							
127B	M	+	-	-	-	-	-
131A	M	+	-	-	-	-	-
135L	G	-	-	-	-	-	-
136J	G	-	-	+	-	+	+
151K	G	-	-	-	-	-	-
Round Butte							
RB/B5	M	+	-	-	+	-	-
Cedar River							
15A	M	-	-	-	-	-	-
15B	M	-	-	-	-	-	-
2F	M	-	-	+	-	-	-
2P	M	-	-	-	-	-	-

Figure 8. Physical map of the IHNV G gene and the locations of the cysteine residue and the glycosylation on G Proteins

A. The physical map of the IHNV G gene



B. Cysteine residues (c) and glycosylation sites (g) on G protein

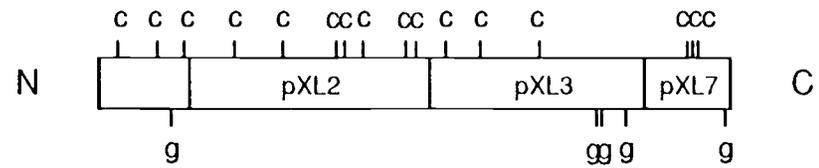


Figure 8.

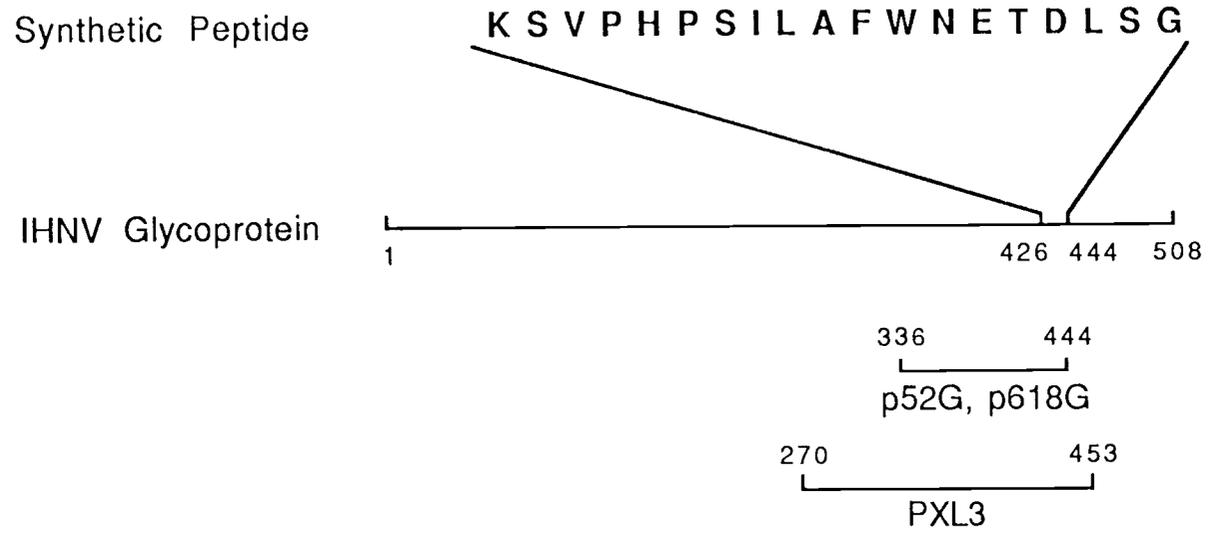


Figure 9. The location of the synthetic peptide of 19 amino acids on the IHNV glycoprotein.

Figure 10. **¹²⁵I-labeled monoclonal antibody competitive binding assay with the synthetic peptide positioning on the amino acid sequence 426-444 of the original IHNV glycoprotein.** The counts per minute were plotted against the concentration of the peptide.

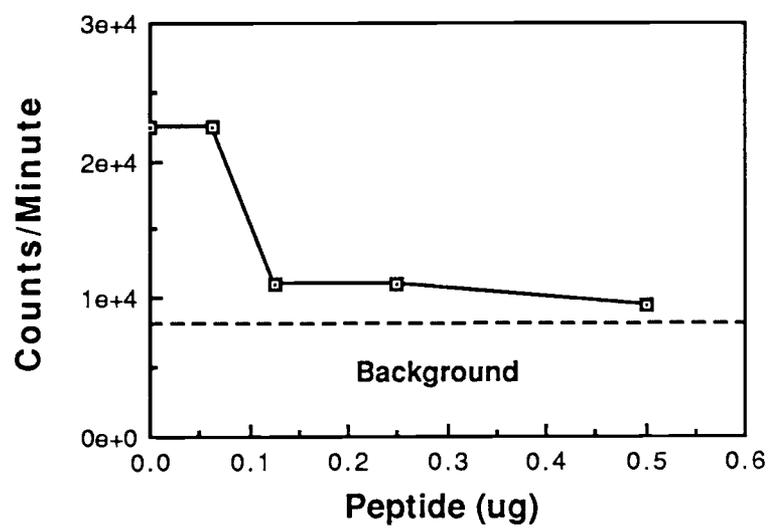


Figure 10A. 127B vs Peptide

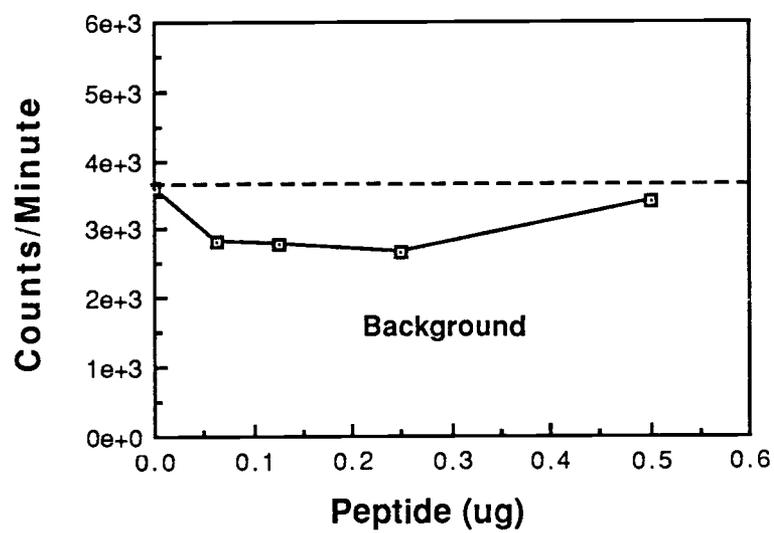


Figure 10B. 131A vs Peptide

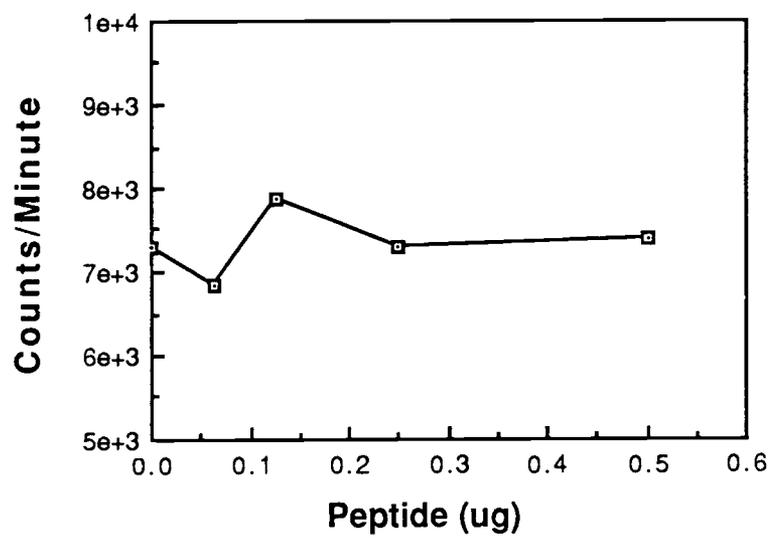


Figure 10C. 135L vs Peptide

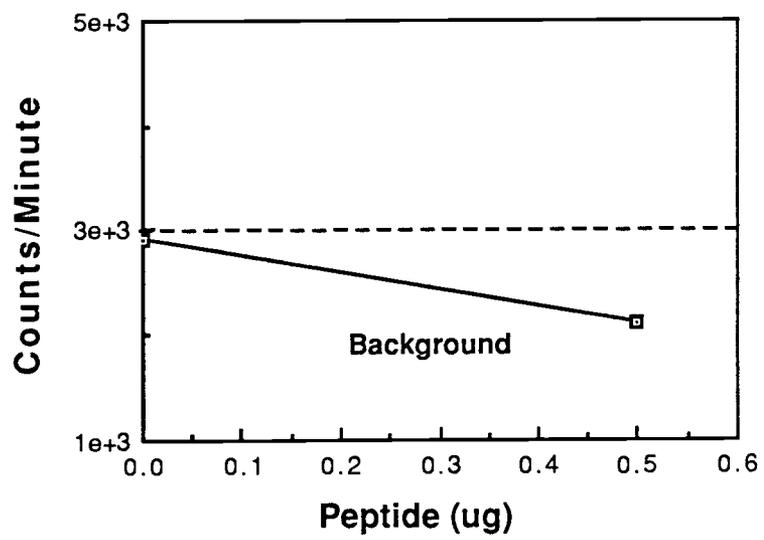


Figure 10D. ^{136}J vs Peptide

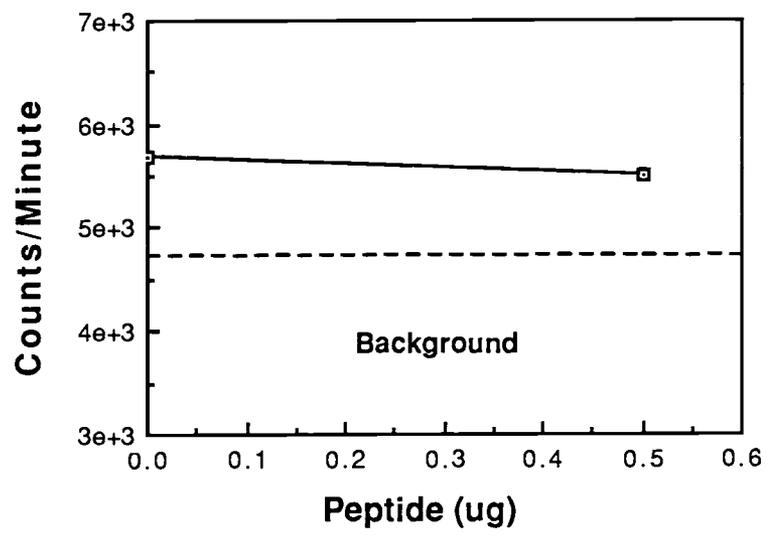


Figure 10E. 151K vs Peptide

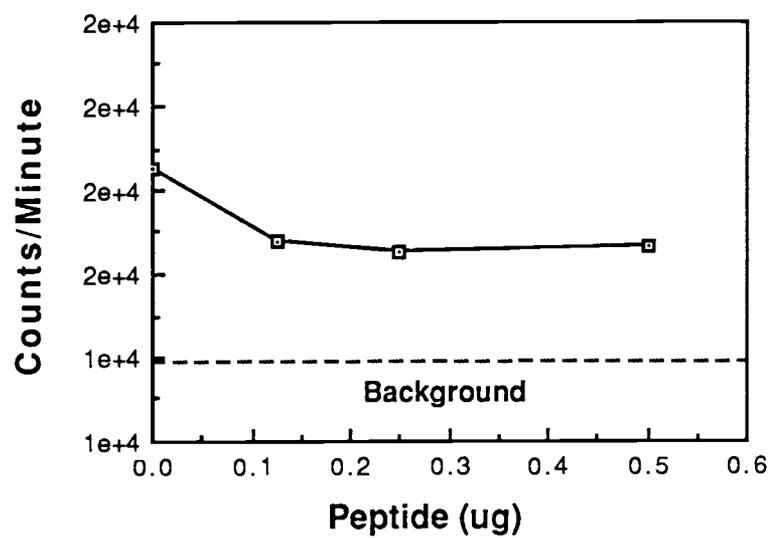


Figure 10F. 6S vs Peptide

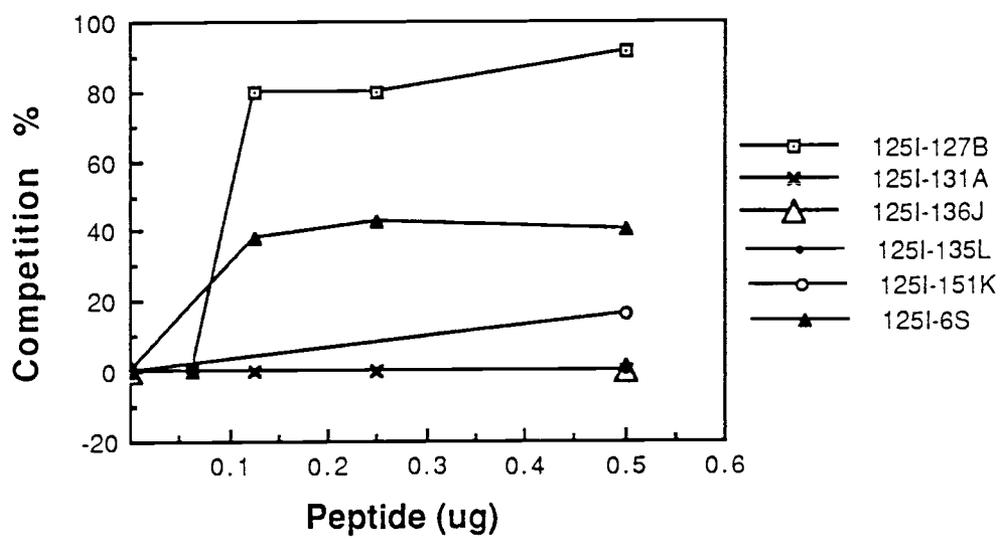


Figure 11. Competition percent for each ^{125}I -monoclonal antibody

Figure 12. **The positions of the synthetic peptide and the inserts contained in pXL2, pXL3 and pXL7 plasmids on the hydropathy plot of the IHNV glycoprotein.** The hydropathy plot was originally created by Koener and co-workers (1987) using the method of Kyte and Doolittle with a window of 10 consecutive amino acid residues. Negative values indicate hydrophilicity.

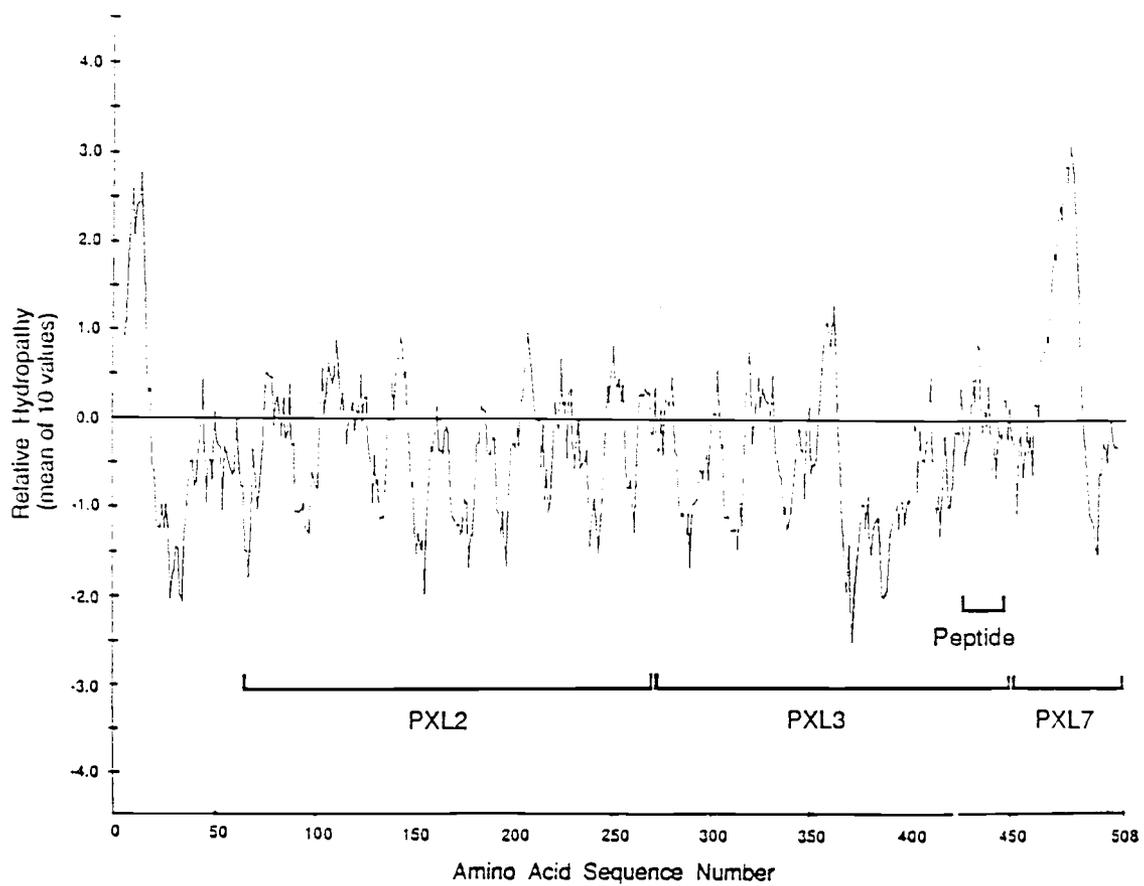


Figure 12.

IHNV glycoprotein

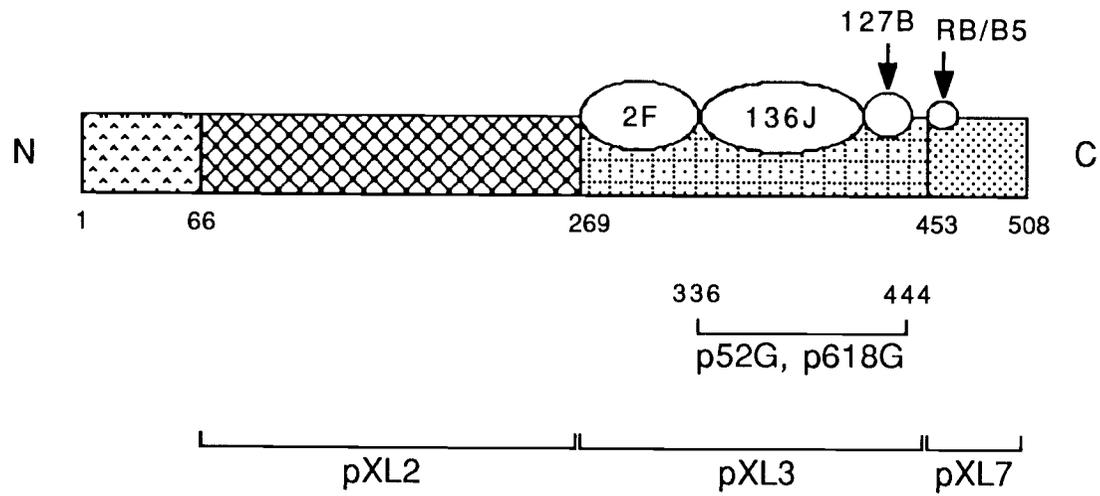


Figure 13. The epitope mapping of the IHNV glycoprotein

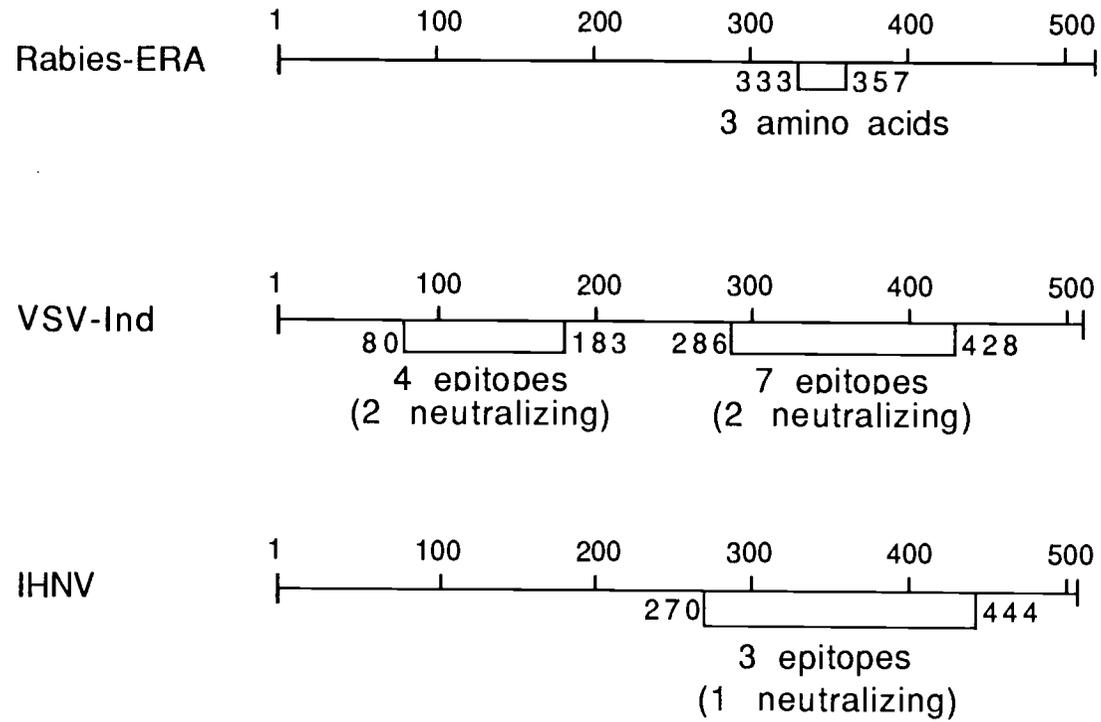


Figure 14. Comparison of the epitope locations of the glycoproteins of IHNv, VSV, and rabies virus.

Table 3. A summary of the predicted T cell epitope segments for IHNV glycoprotein

No. of segment	Mid-point of blocks ^a	Amino acid sequence	Range of angles	Amphipathic score (>8)
1	82-105(K)	77 PTSIRSLSVGNDLGD ^I HTQ GNH ^I H ^K VLYRTICST 110	80-135	53.2
2	133-138	28 STKEAGAYDTTTAAAL 43	120-135	10.7
3	188-199	183 SDFIGGKCTKSPCQTHWSNVVW 204	80-90	21.2
4	213-217(K)	208 AGIPACDSSQE ^I KAH 222	120-125	9.7
5	226-232(K)	221 AHLFVDKISNRV ^V KATS 237	80-95	16.5
6	248-256(K)	243 WGLHRACMIEFCG ^K QWIRT 261	80-135	13.5
7	261-268	256 QWIRTDLGD ^L ISVEYNS 273	85-135	13.9
8	340-346(K)	335 RSPHPGINDVYAMH ^K GGS 351	95-125	15.7
9	407-413(K)	402 TTIIPDLEKYVAQY ^K TS 418	100-110	15.1
10	447-453	442 LSGISIRKLDSFDLQSL 458	130-135	16.7

^a The data in this column gives information on the mid-point positions of the predicted 11 residue blocks. A K indicates that a lysine residue is located after the first 10 residues at the N-terminus of the segment. In example 1, the midpoint of the first block of 11 residues is located at amino acid 82 and the midpoint of the last block of 11 residues is located at amino acid 105.

BIBLIOGRAPHY

Abraham, G., A. K. Banerjee. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci.* 73:1504-1508.

Amend, D. F., and L. Smith. 1974. Pathophysiology of infectious hematopoietic necrosis diseases in rainbow trout (*Salmo gairdneri*): Early changes in blood and aspects of the immune response after injection of IHN virus. *J. Fish. Res. Board. Can.* 31:1371-1378.

Amend, D. F., and V. C. Chambers. 1970. Morphology of certain viruses of salmonid fishes. I. *In vitro* studies of some viruses causing hematopoietic necrosis. *J. Fish. Res. Board. Can.* 27:1285-1293.

Ball, L. A., and C. N. White. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci.* 73:442-446.

Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. *Proc. Natl. Acad. Sci. USA* 66:572-576.

Bishop, D. H. L. 1971. Complete transcription by the transcriptase of vesicular stomatitis virus. *J. Virol.* 7:486-490.

Bishop, D. H. L., P. Repik, J. F. Obueski, N. F. Moore, and R. R. Wagner. 1975. Restitution of infectivity to spikeless vesicular stomatitis virus by solubilized viral components. *J. Virol.* 16:75-84.

Bricker, B. J., R. M. Snyder, J. W. Fox, W. A. Volk, and R. R. Wagner. 1987. Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus (New Jersey Serotype): A method for preliminary mapping of epitopes. *Virology* 161:533-540.

Carroll, A. R., and R. R. Wagner. 1979. Role of the membrane (M) protein in endogenous inhibition of *in vitro* transcription by vesicular stomatitis virus. *J. Virol.* 29:134-142.

Chattopadhyay, D., and A. K. Banerjee. 1987. Phosphorylation within a specific domain of the phosphoprotein of vesicular stomatitis virus regulates transcription *in vitro*. *Cell* 49:407-414.

- Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* 13:222-245.
- Clinton, G. M., S. P. Little, F. S. Hagen, and A. S. Huang. 1978. The matrix (M) protein of vesicular stomatitis virus regulates transcription. *Cell* 15:1455-1462.
- Coslett, G. D., B. P. Holloway, and J. F. Obijeski. 1980. The structural proteins of rabies virus and evidence for their synthesis from separate monocistronic RNA species. *J. Gen. Virol.* 49:161-180.
- Cox, J. H., B. Dietzschold, and L. G. Schneider. 1977. Rabies virus glycoprotein II. Biological and serological characterization. *Infect. and Immunity* 16:754-759.
- Crimmins, D. L., W. B. Mehard, and S. Schlesinger. 1983. Physical properties of a soluble form of the glycoprotein of vesicular stomatitis virus at neutral and acidic pH. *Biochemistry* 22: 5790-5796.
- De, B. P., and A. K. Banerjee. 1984. Specific interactions of L and NS proteins of vesicular stomatitis virus with heterologous genome ribonucleoprotein template lead to mRNA synthesis *in vitro*. *J. Virol.* 51:628-634.
- DeLisi, C., and J. A. Berzofsky. 1985. T-cell antigenic sites tent to be amphipathic structures. *Proc. Natl. Acad. Sci. USA* 82:7048-7052.
- Dietzschold, B., J. H. Cox, L. G. Schneider, T. J. Wiktor and H. Koprowski. 1978. Isolation and purification of a polymeric form of the glycoprotein of rabies virus. *J. Gen. virol.* 40:131-139.
- Dietzschold, B., T. J. Wiktor, R. Macfarlan, and A. Varrichio. 1982. Antigenic structure of rabies virus glycoprotein: Ordering and immunologic characterization of the large CNBr cleavage fragments. *J. Virol.* 44:595-602.
- Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proc. Natl. Acad. Sci. (USA)* 80:70-74.

- Dubovi, E. J., And R. R. Wagner. 1977. Spatial relationships of the proteins of vesicular stomatitis virus: Induction of reversible oligomers by cleavable protein cross-linkers and oxidation. *J. Virol.* 12:500-509.
- Emerson, S. U., and Y.-H. Yu. 1975. Both NS and L proteins are required for *in vitro* RNA synthesis by vesicular stomatitis virus. *J. Virol.* 15:1348-1356.
- Engelking, H. M., and J. C. Leong. 1989. The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. *Virus Research* 13:213-230.
- Flamand, A., and J. F. Delagneau. 1978. Transcriptional mapping of rabies virus *in vivo* . *J. Virol.* 28:518-523.
- Gill, D. S., and A. K. Banerjee. 1985. Vesicular stomatitis virus NS protein: Structural similarity without extensive sequence homology. *J. Virol.* 55:60-66.
- Gilmore, R. D., H. M. Engelking, D. S. Manning, and J. C. Leong. 1988. Expression in *Escherichia coli* of an epitope of the glycoprotein of infectious hematopoietic necrosis virus protects against viral challenge. *Bio/Technology* 6:295-300.
- Good, A. H., L. Wofsy, J. Kimura, and C. Henry. 1980. Purification of immunoglobulins and their fragments. *In* : B. B. Mishel and S. M. Shiigi (Eds.). *Selected methods in cellular immunology*. W. H. Freeman and Company, San Francisco. pp.278-280.
- Harmon, S. A., and D. F. Summers. 1982. Characterization of monospecific antisera against all five vesicular stomatitis virus specific proteins: Anti-L and anti-NS inhibit transcription *in vitro* . *Virology* 120:195-204.
- Helfman, D. M., and S. H. Hughes. 1987. Use of antibodies to screen cDNA expression libraries prepared in plasmid vectors. *In* : Berger, S. L. and R. Kimmel (Eds.). *Guide to molecular cloning techniques: Method in enzymology vol.152*. Academic Press, Inc., Orlando. pp 451-457.

- Hill, B. J., B. O. Underwood, C. J. Smale, and F. Brown. 1975. Physico-chemical and serological characterization of five rhabdoviruses infecting fish. *J. Gen. Virol.* 27:369-378.
- Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacteria plasmids. *Anal. Biochem.* 114:193-197.
- Hsu, Y.-L., H. M. Engelking, and J. C. Leong. 1986. Occurrence of different types of infectious hematopoietic necrosis virus in fish. *Applied and Environmental Microbiology* 52:1353-1361.
- Imblum, R. L., and R. R. Wagner. 1974. Protein kinase and phosphoproteins of vesicular stomatitis virus. *J. Virol.* 13:113-124.
- Keil, W., and R. R. Wagner. 1989. Epitope mapping by deletion mutants and chimeras of two vesicular stomatitis virus glycoprotein genes expressed by a vaccinia virus vector. *Virology* 170:392-407.
- Kelly, J. M., S. U. Emerson, and R. R. Wagner. 1972. The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibodies. *J. Virol.* 10:1231-1235.
- Kingsbury, D. W., C.-H. Hsu, and E. M. Morgan. 1981. A role for NS protein phosphorylation in vesicular stomatitis virus transcription. *IN : The Replication of Negative Stand Viruses.* D. H. Bishop and R. W. Compans (Eds.), Elsevier/North-Holland, New York. p.821.
- Koener, J. F., C. W. Passavant, G. Kurath, and J. C. Leong. 1987. Nucleotide sequence of a cDNA clone carrying the glycoprotein gene of infectious hematopoietic necrosis virus, a fish rhabdovirus. *J. Virol.* 61:1342-1349.
- Kurath, G., and J. C. Leong. 1985. Characterization of infectious hematopoietic necrosis virus mRNA species reveals a nonvirion rhabdovirus protein. *J. Virol.* 53:462-468.
- Kurath, G., K. G. Ahern, G. D. Pearson, and J. C. Leong. 1985. Molecular cloning of the six mRNA species of infectious hematopoietic necrosis virus, a fish rhabdovirus, and gene order determination by R-loop mapping. *J. Virol.* 53:469-476.

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

Lafon, M., T. J. Wiktor, and R. I. Macfarlan. 1983. Antigenic sites on the rabies virus glycoprotein: Analysis with monoclonal antibodies. *J. Gen. Virol.* 64:843-851.

Lefkovits, I., and B. Pernis. 1985. Immunological methods, volume III. Academic Press, Inc., Orlando, Florida. pp286-287.

Leong, C. L., Y. L. Hsu, and H. M. Engelking. 1983. Synthesis of the structural proteins of infectious hematopoietic virus. *In* : Bacterial and viral disease of fish. J. H. Crosa (Ed.), Washington Sea Grant Program, Univ. of Washington, Seattle. pp61-71.

Malek, L., G. Soostmayer, R. T. Garvin, and E. James. 1984. The rabies glycoprotein gene is expressed in *E. coli* as a denatures polypeptide. *In* : R. H. Chanock and Lerner, R. A. (Eds.). Modern approaches to vaccines. Cold Spring Harbor Laboratory, New York. pp203-208.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. p.368.

Margalit, H., J. L. Spouge, J. L. Cornette, K. B. Cease, C. DeLisi, and J. A. Berzofsky. 1987. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. *J. Immuno.* 138:2213-2229.

Matlin, K., A. Reggio, K. Simons, and A. Helenius. 1982. Pathway of vesicular stomatitis virus entry leading to infection. *J. Mol. Biol.* 156:609-631.

Matthews, R. E. F. 1982. Classification and nomenclature of viruses, Rhabdoviridae. *Intervirology* 17:109-111.

McAllister, P. E., and R. R. Wagner. 1975. Structural proteins of two salmonid rhabdoviruses. *J. Virol.* 15:733-738.

Moyer, S., and A. K. Banerjee. 1975. Messenger RNA species synthesized *in vitro* by the virion-associated RNA polymerase of vesicular stomatitis virus. *Cell* 4:37-43.

Moyer, S. A., and D. F. Summers. 1974. Phosphorylation of VSV *in vivo* and *in vitro*. *J. Virol.* 13:455-465.

Ongradi, J., C. Cunningham, and J. F. Szilagyi. 1985. The role of polypeptides L and NS in the transcription process of vesicular stomatitis virus New Jersey using the temperature-sensitive mutant tsE1. *J. Gen. Virol.* 66:1011-1023.

Pilcher, K. S. and J. L. Fryer. 1978. Viral diseases of fish: a review through 1978, Part 1. *CRC Crit. Reviews in Microbiology* 7:287-364.

Roitt, I., J. Brostoff, and D. Male. 1989. *Immunology*. Mosby, New York. P.7.9.

Rovozzo, G., and C. N. Burke. 1973. A manual of basic virological techniques. Prentice-Hall, Inc., Englewood Cliffs, N. J.. pp 87-98.

Sanchez, A., B. P. De, and A. K. Banerjee. 1985. *In vitro* phosphorylation of the NS protein by the L protein of vesicular stomatitis virus. *J. Gen. Virol.* 66:1025-1036.

Schnitzer, T. J., and H. F. Lodish. 1979. Noninfectious vesicular stomatitis virus particles deficient in the viral nucleocapsid. *J. Virol.* 29:443-447.

Schwartz, R. H.. 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu. Rev. Immunol.* 3:237-261.

Seif, I., P. Coulon, P. E. Rollin, and A. Flamand. 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies mutations affecting antigenic site III of the Glycoprotein. *J. Virol.* 53:926-934.

Sokol, F. H. K. Clark, T. J. Wiktor, M. L. McFalls, D. H. L. Bishop, and J. F. Obijeski. 1974. Structural phosphoproteins associated with ten rhabdoviruses. *J. Gen. Virol.* 24:433-445.

Spouge, J. L., H. R. Guy, J. L. Cornette, H. Margalit, K. Cease, J. A. Berzofsky, and C. DeLisi. 1987. Strong conformational propensities enhance T-cell antigenicity. *J. Immunol.* 138:204-212.

Thomas, D., W. W. Newcomb, J. C. Brown, J. S. Wall, J. f. Hainfield, B. L. Trus, and S. C. Alasdair. 1985. Mass and molecular composition of vesicular stomatitis virus: A scanning transmission electron microscopy analysis. *J. Virol.* 54:598-607.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.

Volk, W. A., R. M. Snyder, D. C. Benjamin, and R. R. Wagner. 1982. Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus: comparative neutralizing activity. *J. Virol.* 42:220-227.

Wang, L-M., D. K. Weber, T. Johnson and A. Y. Sakaguchi. 1988. Supercoil sequencing using unpurified templates produced by rapid boiling. *BioTechniques* 6:839-843.

Weiss, R. A., and R. L. R. Bennett. 1980. Assembly of membrane glycoprotein studied by phenotypic mixing between mutants and vesicular stomatitis virus and retrovirus. *Virology* 100:252-274.

Wiktor, T. J., and H. Koprowski. 1980. Antigenic variants of rabies virus. *J. exp. med.* 152:99-112.

Wiktor, T. J., R. I. Macfarlan, K. J. Reagan, B. Dietzschold, P. J. Curtis, W. H. Wunner, M.-P. Kieny, R. Lathe, J.-P. Lecocq, M. Mackett, B. Moss, and H. Koprowski. 1984. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. *Proc. Natl. Acad. Sci. USA* 81:7194-7198.

Wilson, T., and J. Lenard. 1981. Interaction of wild-type and mutant M protein of vesicular stomatitis virus with nucleocapsids *in vitro*. *Biochemistry* 20:1349-1354.

Winton, J. R., C. K. Arakawa, C. N. Lannan, J. L. Fryer. 1988. Neutralizing monoclonal antibodies recognize antigenic variants

among isolates of infectious hematopoietic necrosis virus. Dis. Aquat. Org. 4:199-204.

Wolf, K. 1988. Fish viruses and fish viral diseases. Cornell University Press, New York. p83.

Wunner, W. H., B. Dietzschold, R. I. Macfarlan, C. L. Smith, E. Golub, and T. J. Wiktor. 1985. Localization of immuogenic domains on the rabies virus glycoprotein. Ann. Inst. Pasteur/Virol. 136E:353-362.

Yelverton, E., S. Norton, J. F. Obijeski, D. V. Goeddel. 1983. Rabies virus glycoprotein analogs: biosynthesis in *Escherichia coli*. Science 219:614-620.