AN ABSTRACT OF THE THESIS OF

Donald Scott Manning for the degree of Doctor of Philosophy in Microbiology presented on May 20, 1988.

Title: Deletion Mapping and Expression of the Large Genomic Segment of Infectious Pancreatic Necrosis Virus.

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Abstract approved: ____________________________

Dr. J. C. Leong

The A Segment of infectious pancreatic necrosis virus (IPNV) encodes three primary gene products, $\beta_1$, $\gamma_1$, and $\gamma_2$, in one open reading frame. The gene order is $5'\beta_1-\gamma_2-\gamma_1^3'$. The production and processing of the $\beta_1$, $\gamma_2$ and $\gamma_1$ polypeptides were studied by both in vitro translation and bacterial expression of the A segment coding region with various deletions. Virus-specific polypeptides were identified with antisera which were specific for groups of the viral proteins. The bacterial expression of these proteins for use as a subunit vaccine is also described.

In both the bacterial expression and in vitro translation systems, truncated polyproteins were produced that provided evidence of a
polyprotein precursor in the production of the $\beta_1$, $\gamma_1$ and $\gamma_2$ proteins.

Since the truncated polyproteins were produced when deletions were made to the $\gamma_2$ gene, the $\gamma_2$ protein was implicated as a virus-specific protease responsible for the polyprotein processing. The putative protease activity of the $\gamma_2$ protein was not found to act in trans and specific antisera to it did not inhibit its activity in in vitro translation reactions.

In the in vitro translation system, internal initiations of translation were demonstrated for the $\gamma_2$ and $\gamma_1$ proteins. Therefore, internal initiations of translation may be an alternative mechanism for the production of these proteins.

The A Segment gene products were expressed in bacteria as a fusion with the $\text{TrpE}$ gene and used in a preliminary trial as an immersion vaccine for rainbow trout fry. The vaccination resulted in substantial protection from subsequent viral challenge.
Deletion Mapping and Expression of the
Large Genomic Segment of
Infectious Pancreatic Necrosis Virus

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DELETION MAPPING AND EXPRESSION OF THE LARGE GENOMIC SEGMENT OF INFECTIOUS PANCREATIC NECROSIS VIRUS

INTRODUCTION

Infectious pancreatic necrosis (IPN) is a disease affecting a number of important fish species. Its etiological agent, infectious pancreatic necrosis virus (IPNV), is the most thoroughly studied member of a recently defined group of viruses, the Birnaviridae (Brown, 1986). This thesis describes the use of molecular cloning techniques to investigate birnavirus biology and to create a subunit vaccine for IPNV.

Birnaviruses, have small (70 nm) nonenveloped, icosahedral capsids, containing two segments of double-stranded genome RNA. The two RNA segments code for four primary gene products (Dobos and Rowe, 1977; see Figure 1). The larger genome RNA, Segment A, encodes three proteins, β (VP2), γ1 (VP3) and γ2 (NS) in the order 5'β-γ2-γ1 (Huang et al., 1986; see Figure 2). This order has also been confirmed for the Jasper strain of IPNV (Nagy et al., 1987) and for another birnavirus, avian infectious bursal disease virus (IBDV)(Azad et al., 1987). The smaller genome RNA, Segment B, encodes the α (VP1) protein (MacDonald and Dobos, 1981) which is believed to be a RNA polymerase (Cohen, 1975; Mertens, Jamieson and Dobos, 1982). The β and γ1 proteins are found in the capsid with slight variations in size. The γ2 protein also occurs in two forms, and while it is present at substantial levels in infected cells, there are no reports of its detection in purified virions.
Figure 1. Features of the IPNV RNA genome and proteins.

The viral genome is composed of two segments of double-stranded RNA, A and B. The B segment encodes the \( \alpha \) protein, a putative RNA polymerase. The A segment encodes the \( \beta \) (63-51.5 kD) and \( \gamma_1 \) (32 and 28.5 kD) proteins which are the major structural proteins of the virion and the \( \gamma_2 \) proteins (28.5 and 27 kD) which are found only in infected cells.
A Segment A cDNA of the Jasper isolate of IPNV has been sequenced (Duncan and Dobos, 1986) and found to contain a single large open reading frame (ORF). This ORF is long enough to account for the combined molecular weights of the $\beta$, $\gamma_2$ and $\gamma_1$ proteins. This finding strongly supports the theory that these proteins are produced through cleavages of a polyprotein precursor but no such polyprotein molecule has been detected in infected cells (Dobos, 1977; Dobos and Rowe, 1977) or by in vitro translation of Segment A genome RNA (Mertens and Dobos, 1981). Translation of full length A Segment RNA transcripts made in vitro using plasmid transcription vectors also fails to produce a polyprotein (Huang et al., 1986; Nagy et al., 1987). Furthermore, it has been shown that infected cells do not contain virus-specific RNAs of less than genome length and thus, it appears that a separate RNA is not produced for each of the proteins (Somogyi and Dobos, 1980). Two hypotheses provide the most likely explanations for these results: 1) a precursor peptide is produced but is processed so rapidly that it is very difficult to detect; or 2) there are independent internal initiations for the translation of the two downstream proteins.

In this thesis evidence is provided for the existence of a Segment A precursor polyprotein by production of truncated polyproteins in bacterial expression and in vitro translation systems. Evidence is provided that the $\gamma_2$ (NS) protein is the protease which processes the precursor polyprotein. The results also indicate that independent internal initiations of translation may be used as an alternative mechanism for the production of the A Segment proteins. Conclusions similar to these have been reached in
recently published reports (Azad et al., 1987; Nagy et al., 1987; Duncan and Dobos, 1987; and Jagadish et al., 1988).

The disease caused by IPNV poses a significant threat to the aquaculture industry. One of the goals of this research was to explore the possibility of using molecular cloning techniques to create a practical vaccine for IPN.

The first reported outbreak of IPN occurred in 1941 (McGonigle) and affected a hatchery population of juvenile brook trout (Salvelinus fontalis). In 1960, the viral nature of the disease was confirmed (Wolf et al.) and since that time there have been isolations of the virus in a variety of fish species, at locations throughout the world (Table 1). While the virus appears to have a broad host range and is virtually ubiquitous in the aquatic environment, acute disease has been reported primarily in a limited group of salmonid species. Most notable among these are brook trout (Salvelinus fontalis), rainbow trout (Salmo gairdneri), brown trout (Salmo trutta) and cutthroat trout (Salmo clarkii). These species are of growing economic importance and since mortalities range as high as 90%, an IPN outbreak in a hatchery can be an economic disaster for the aquaculturist.

Currently, there are no methods for controlling IPN except the destruction of infected stocks and the decontamination of facilities. There has been a great deal of interest in developing a vaccine for IPNV, but despite investigation of a variety of approaches, little progress has been made towards a truly practical vaccine. A number of difficulties must be overcome for successful implementation of an IPN vaccine. First, sufficient quantities of the immunogen must be produced at a reasonable
cost. Second, because of the high virulence of the virus the vaccine must be completely free of viable virus. Finally, since young fish are most susceptible (Wolf et al., 1960), the immunogen must be administered to fish as early as possible. This is particularly problematic because injection is not practical for fry and antigen uptake is poor by other methods.

Dorson (1982) has reviewed the work that has been done on live vaccines for IPN. Unfortunately, the attenuated strains tested thus far either failed to infect the fish or failed to induce protection. Strains with low virulence have been tested as vaccines for more virulent strains, but mortality from the vaccinating strain was either too high or protection was only moderate (Hill, Dorson, and Dixon, 1980).

A number of studies have been conducted to determine the efficacy of various killed virus vaccines. Dorson (1977) demonstrated that formalin inactivated virus was immunogenic when injected intraperitoneally into four week post-hatch fry, but immersion or oral immunization of the same vaccine was not effective. Similar results have been obtained in other studies (Hill, Dorson, and Dixon, 1980; Hill et al., 1980). Some investigators (Hill et al., 1980; Hill and Dixon, 1983) have suggested that the uptake of the antigen by immersion might be improved if the virus was disrupted into smaller, sub-viral components but disruption methods have resulted in loss of antigenicity.

Described in this thesis are studies in which a bacterial expression system has been used to produce viral antigens for use as a subunit vaccine. In a preliminary trial, these antigens were used as an immersion vaccine and provided substantial protection against subsequent viral
challenge. This approach has several advantages. First, large quantities of the proteins can be produced inexpensively. Second, since the use of viable pathogen is avoided completely, there is no possibility of infection resulting from immunization. Finally, the viral proteins can be manipulated to optimize antigenicity. In our laboratory, we have previously demonstrated the efficacy of a bacterially expressed subunit vaccine for another viral fish disease, IHN (infectious hematopoietic necrosis) (Gilmore et al., 1988).
LITERATURE REVIEW

History, pathology and epizootiology.

Infectious pancreatic necrosis virus is the etiological agent of a disease which effects a variety of fish species at locations around the world. A brief review of the history, pathology and epizootiology of this disease is presented here.

The first description of the disease was in 1941 (McGonigle). It was reported that young brook trout (*Salvelinus fontinalis*) exhibited symptoms that are now considered characteristic of the disease: violent whirling, horizontal "corkscrewing" and high mortality. When a second outbreak occurred in 1953, a viral etiology was suspected (Snieszko et al.). In 1955, Wood et al. reported histopathological studies on infected fish which resulted in the descriptive name, infectious pancreatic necrosis. It was not until 1960, however, that Wolf et al. reported the viral nature of the disease.

In addition to whirling, corkscrewing and high mortality, there are a number of other symptoms associated with the disease (Wolf, 1966; Pilcher and Fryer, 1980). These include darkening in color, abdominal distention, exophthalmia and hemorrhages at the base of the fins. Internally, the liver, spleen, and stomach are pale. The stomach is void of food and contains a clear or milky mucus. The pyloric caeca may exhibit petechial hemorrhages. The pancreatic acinar cells are necrotic with intracytoplasmic inclusions, pyknosis and karyorrhexis.
Pathological changes may also occur in the hematopoietic tissues of the kidney.

Most outbreaks of infectious pancreatic necrosis have been reported among young hatchery reared salmonid species, but the virus has been isolated in a variety of fish and other aquatic species at locations throughout the world. Table 1 provides a partial list of reported IPNV isolations illustrating the wide host range and geographic distribution of the virus. Isolations of IPNV from non-salmonid species have usually occurred in asymptomatic fish. There are, however, a number of economically significant non-salmonid species which are susceptible to the disease (Hill, 1976). Occurrence of the virus in asymptomatic non-commercial fish is also of indirect economic importance since these species may act as reservoirs for horizontal transmission to commercial fish populations.

The existence of a carrier state is thought to play an important role in the epizootiology of infectious pancreatic necrosis. Mortality in juvenile brook and rainbow trout (Salvelinus fontalis and Salmo gairdneri) due to IPNV infection decreases with age (Wolf et al., 1960; Fantsi and Savan, 1971), but older juveniles which survive infection usually become carriers (Billi and Wolf, 1969). Virus can be detected in the fecal and sex products of carrier fish throughout their lives (Billi and Wolf, 1969; Yamamoto, 1975; Reno et al., 1978). A number of reports have provided evidence for contaminated eggs transmitting the virus (Wolf, Quimby and Bradford, 1963; Bullock et al., 1965). Yearling brook and rainbow trout that are challenged with the virus are only transiently infected, with no carrier state being established (Wolf and
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Table 1. A partial listing of reported isolations of infectious pancreatic necrosis virus.
Quimby, 1969). Disappearance of the virus coincides with increasing levels of neutralizing antibody.

The persistent infection of fish cell lines with IPNV has been studied as a possible model for the carrier state. Hedrick et al. (1978) and Ahne (1977) have both demonstrated persistent infection of fish cell lines with IPNV. Electron micrographs of persistently infected cells revealed the presence of numerous, apparently incomplete virions which were considered to be defective interfering (DI) particles. It was suggested that the ability of IPNV to persist in carrier trout may depend on the balance between production of DI particles and complete infectious virions.

Another factor that plays an important role in the transmission of IPNV is its stability in the environment. Boudouy and Castric (1977) demonstrated that it retains substantial infectivity after three months storage in river water at 10°C. Desautel and MacKelvie (1975) found that storage of the virus in sea water at 4 or 10°C for 10 weeks caused negligible loss in infectivity and that the virus could survive drying at laboratory temperatures for as long as eight weeks. They also demonstrated that IPNV is stable across a surprisingly broad pH range, from pH 2.0 to 9.0. They suggested that the virus's resistance to heat and pH might lend substance to the proposal of an oral-fecal route in the epidemiology of IPN. Virus stability may also account for its survival in a number of possible "mechanical vectors" in which the virus probably does not replicate but can persist and potentially be transported. When owls, gulls and mink were inoculated with IPNV, the virus could be reisolated from their feces as long as seven days later (Sonstegard and
McDermott, 1972; Eskildsen and Jorgensen, 1973). It has also been shown that IPNV can survive ingestion by the marine ciliate *Miamensis avidus* (Meowus-Kobb, 1963).

**Serotyping.**

Classification of the growing number of IPNV isolates into strain groupings has become an increasingly complicated task. Although a number of studies have been conducted in which cross-neutralization by rabbit antisera has been compared for panels of isolates (Wolf and Quimby, 1971; Hill, 1976; MacDonald and Gower, 1981 and Okamoto et al., 1983), a substantial degree of uncertainty exist regarding the antigenic relatedness of the various isolates. Despite this uncertainty, several general conclusions are agreed upon. First, most of the isolates have some degree of cross-reactivity with the each other, but very significant differences do exist. Second, the European isolates fall into two distinct groups. These are the Sp stain which is highly pathogenic and the Ab strain which is much less pathogenic. Third, the North American isolate, VR299, is significantly different from either of the European strains. Finally, several of the North American strains, including Buhl, Powder Mill and West Buxton, are significantly cross-reactive with both VR299 and the Sp strains.

It has been suggested (by Dorson, 1982) that the standardization of methods used by the groups working in this area or the use of monoclonal antibodies (MAbs) might help further clarify the antigenic relationships between isolates. Caswell-Reno, Reno and Nicholson (1986) have produced monoclonal antibodies (MAbs) against the West
Buxton isolate of IPNV, and they have used these MAbs to compare the antigens of a number of isolates. Their MAbs identified at least four and possibly five structurally and/or functionally different epitopes on the virion. One of the MAbs detected an epitope present on the large capsid protein, \( \beta \) (VP2), and another recognized epitopes on the smaller capsid proteins, \( \gamma_1 \) and \( \gamma_{1a} \) (VP3 and VP4). The protein or proteins containing the epitopes recognized by the remaining MAbs could not be determined. Five MAbs were used in ELISA and neutralization tests to compare the reactivity of 14 isolates including representatives of the Ab, Sp and VR299 groups. At least nine distinct patterns of reactivity were demonstrated. Two of the MAbs recognized only West Buxton isolates, while the others recognized epitopes that were more common among the isolates.

**Immunity and host resistance factors.**

The presence of serum antibodies capable of neutralizing IPNV has been demonstrated in rainbow and brook trout. When virus was injected into adult fish, neutralizing activity in the serum rose markedly for three months (Wolf and Quimby, 1969). Injection of a killed virus vaccine into four week post-hatch fry has also been shown to result in the production of detectable levels of neutralizing activity in the serum (Dorson, 1977). Agniel (1975) demonstrated passive transfer of immunity by injecting fry with serum from adult donors immunized with the virus.

IPNV neutralizing activity is also often found in the serum of fish
with no known exposure to the virus (Jorgensen, 1973; Dorson and de Kinkelin, 1974a; Dorson and de Kinkelin, 1974b).

Dorson and de Kinkelin (1974b) have shown that there are two distinct components in the fish serum which are capable of neutralizing IPNV. The first is an IgM-like antibody which is the component known to account for a rise in neutralizing activity when induced by immunization or infection (Dorson, 1977a). The second is a poorly characterized protein which has a sedimentation coefficient of 6S and is simply referred to as the 6S component. It is found as a normal constituent of trout sera and it may be this component that is responsible for the detection of neutralizing activity in fish which have not been exposed to the virus. It has not been demonstrated that the serum concentration of the 6S component is responsive to immunogenic stimuli.

The neutralizing activity of normal trout sera (presumably due to the 6S component) is dependent on the strain of virus and other factors. Dorson et al. (1975) found that, for the Sp strain, neutralization does not occur with fresh isolates of the virus, but that isolates that have been repeatedly passaged in tissue culture are readily neutralized by normal trout sera. Virus that has been repeatedly passaged decreases in virulence as well. If the virus is passaged in tissue culture in the presence of normal trout sera, however, virulence and insensitivity to neutralization are maintained. Kelley and Nielson (1985) have demonstrated that adsorption of the virus to fish cells is inhibited by the presence of the 6S component of normal serum.

The action of interferon in the pathology of IPNV has been investigated but its role is not yet clearly understood. De Kinkelin,
Dorson, and Hattenberger-Baudouy (1982) have provided evidence that IPNV is sensitive to interferon *in vivo*. They found that fry could be protected from viral challenge by injection of serum fractions with interferon activity. Hedrick and Fryer (1981 and 1982) have investigated the activity of interferon in persistently infected cells. They were able to detect interferon in culture fluids of persistently infected RTG-2 (rainbow trout gonad) cells, but not in persistently infected STE-137 (steelhead trout) or CHSE-214 (chinook salmon embryo) cells. MacDonald and Kennedy, 1979, have reported that CHSE-214 cells are defective in interferon production.

**Vaccination studies.**

There has been a great deal of interest in developing a vaccine for IPNV. Although a variety of approaches have been investigated, there has been little progress towards a truly practical vaccine. Development of such a vaccine poses some unique problems. Since most of the mortality due to the disease occurs in young fish, the vaccine must be administered at a very early age. Injection of such small fish is impractical and, therefore, any successful vaccine must be administered either orally or by immersion. Uptake of killed-virus vaccine preparations is poor under these conditions. Attenuated vaccines can be administered by these methods, but results with attenuated vaccines have been disappointing and the possibility of reversion to virulence may be an unacceptable risk.

Dorson (1982) has reviewed and cited work on live vaccines for IPNV. The most obvious candidates for a live vaccine were
non-pathogenic variants that had been obtained by passage in tissue culture (Dorson, Castric and Torchy, 1978). These variants infected fry, but failed to induce protection (Hill and Dorson, unpublished). The Ab strain has been tested as a vaccine for induction of immunity against the more pathogenic Sp strain (Dorson, unpublished). The Ab strain itself caused 20% mortality and failed to induce protection against the Sp strain. The authors speculate that this was because of the low antigenic similarity between the strains. Attenuation of the Sp strain has been attempted by serial passages at supraoptimal temperatures (20 and 21°C) in the presence of normal trout serum (Dorson and Hill, unpublished). Only a single non-pathogenic variant was obtained and it was completely unable to infect and therefore protect fry. Hill, Dorson and Dixon (1980) have used a strain isolated from perch (74/53) as a vaccine, but protection was only moderate.

A number of studies have been conducted to determine the efficacy of various killed-virus IPNV vaccines (Sano et al., 1981; deKinlelin and Beatzotti, 1981; Hill, Dorson, and Dixon, 1980), but vaccination has not yet been found to be successful by any method other than injection. Most of the killed-virus vaccine preparations that have been tested have been formalin or 2-propolactone inactivated whole virus (reviewly Dixon and Hill, 1983). Early studies demonstrated that treatment of IPNV with a 1:4000 dilution of formalin at 4°C for up to 14 days (MacKelvie and Desautels, 1975) or with a 1:500 dilution of formalin for 1 hour at 37°C (Elliot and Amend, 1978) also failed to completely inactivate IPNV. Jorgensen (1973) reported that a 1:50 dilution of formalin caused a total loss of IPNV infectivity in 5-10
minutes. However, Jorgensen tested for residual infectivity by plaque titration which may not be an entirely satisfactory method, since formalin treatment of IPNV has been found to delay appearance of the cytopathic effect caused by surviving virus (MacKelvie and Desautels, 1975).

Dorson (1977) reported that IPNV inactivated with 1:200 dilution of formalin was immunogenic for trout fry when injected intraperitoneally. In this study, hyperosmotic shock followed by bath in concentrated vaccine suspension did not induce any protection. Oral immunization was also ineffective. The same vaccine preparation was injected into four week post-hatch fry and at two weeks post-immunization, these fry were resistant to virus challenge. This protection occurred despite the fact that the production of neutralizing antisera could not be detected. When fry were injected five weeks after hatching they produced detectable neutralizing antibodies and were also protected from challenge. Dorson explains these results by suggesting that young fry can be immunologically "primed" without synthesizing detectable antibody levels and that a complete immune response with antibody synthesis occurs later in the course of development. In a subsequent study Hill, Dorson and Dixon (1980) obtained similar results using virus that had been inactivated by a three hour treatment with 2-propiolactone at a 1:250 dilution.

Sano et al. (1981) carried out a study in which adult fish were vaccinated. Their strategy was to eliminate vertical transmission of IPNV by immunizing broodstock fish which would then produce virus-free eggs and sperm. The study demonstrated that a single
interperitoneal injection of 0.5 ml of concentrated, formalin neutralized virus into 500 to 800 gram rainbow trout resulted in a ND$_{50}$ (50% neutralizing dose) of 1000 in the fish sera within 60 days. When fish with ND$_{50}$ titers equal to or greater than 1000 were challenged by intravenous injection of homologous virus via the cuverian duct, no reisolation of the virus could be made from the spleen or the kidney three weeks after the challenge. In unpublished results, Sano found that antisera titer peaked about four months after vaccination. From these results it was suggested that if vaccination was performed three to four months before spawning, the brood stock would produce enough neutralizing antibody to assure virus-free sex products. The efficacy of this strategy has not been tested in a hatchery situation.

Dixon and Hill (1983) have performed the most thorough study on the inactivation of IPNV for use as a vaccine. In previous studies no attempt was made to use in vitro methods to quantitate the effects of viral inactivation on antigenicity. Dixon and Hill addressed this issue for both formalin and 2-propiolactone inactivation. Antigenicity was measured by double diffusion of dilutions of the inactivated virus and anti-IPNV serum in 1% agarose gels. Loss of infectivity was determined by plaque assay. Immunogenicity was tested by collection of sera from immunized fish and titration of this sera by neutralization of virus in tissue culture. Their results demonstrated that IPNV can be completely inactivated by treatment with 2-propiolactone, but that loss of antigenicity ranged from moderate to high depending on specific
conditions. On the other hand, treatment with formalin (1:200) for four days resulted in complete inactivation with little loss of antigenicity.

Hill et al. (1980) compared various methods of vaccinating fry with formalin-killed IPNV. In this study fry were protected by injection of the antigen, but vaccination by immersion was not effective. Dorson (1977) obtained similar results. Hill et al. (1980) suggested that the uptake of antigen by the immersion method might be improved if the virions were disrupted into smaller, subviral components; however, they found that chemical methods of disruption destroyed antigenicity.

Dixon and Hill (1983) began work on another approach for obtaining subviral components for vaccination. They investigated the possibility of obtaining viral antigen prior to assembly into complete virions, thus avoiding the use of disrupting agents. This was achieved by harvesting cells during an early stage of infection. Although preparations still required inactivation, antigenicity was retained after inactivation. This study failed, however, to clearly demonstrate whether vaccine prepared by this method was more effective or that antigen uptake during immersion was more efficient with smaller particles.

In our laboratory, we have demonstrated the efficacy of a subunit vaccine for another viral fish pathogen, infectious hematopoietic necrosis virus (IHNV) (Gilmore et al., 1988). A fragment of cDNA coding for 104 amino acids of the viral glycoprotein was inserted into a TrpE gene expression plasmid and the resulting fusion protein accounted for 10% of the bacterial (Escherichia coli) protein.
When fish were immersed in crude lysates of cells expressing the fusion protein, protective immunity was induced.

**Molecular biology.**

In the early studies describing the genome structure and replication events of IPNV, a controversy arose regarding the basic characteristics of the virus genome. To a large degree this controversy resulted from attempts to include IPNV in one of the established animal RNA-virus families. IPNV is now considered to be a member of a newly defined family, the Birnaviridae. This group has two other members, infectious bursal disease (IBD) virus of chickens and Drosophila X virus (DXV). Developments in the current understanding of the molecular biology of these viruses are reviewed here.

**General characteristics and nucleic acid composition.**

Malsberger and Cerini (1963a) conducted one of the first studies on IPNV. The latent period was established in one step growth analyses to be approximately five hours followed by the exponential release of virus for seven hours. No hemagglutination or hemadsorption could be demonstrated. The virus was stable at +4°C and -70°C.

In a subsequent study, Malsberger and Cerini (1963b) demonstrated "burst-like", rather than continuous, kinetics of virus release. They concluded that the virus matures intracellularly rather than at the cell surface. Virus yield per infected cell was 300-1000 TCID$_{50}$ (50% tissue culture infective dose). They also tested the effects of nucleic acid metabolism inhibitors on IPNV replication.
Thymine antagonists, that inhibit multiplication of DNA viruses, had no effect on IPNV. Inhibitors of RNA synthesis, however, caused as much as 90% inhibition of IPNV replication.

Cerini and Malsberger (1965) also conducted the first electron microscopic studies of IPNV. This report indicated that the virus had a diameter of 25 to 29 nm and a picornavirus-like morphology. This description was later refuted by Moss and Gravel! (1969), who examined both purified virions and thin sections of infected cells. When the thin sections were negatively stained it was found that virus particles could first be observed six hours after infection. Virus crystals were occasionally observed. More typically, individual particles or small clusters were found randomly throughout the cytoplasm. Virions were not enveloped or membrane associated. Early in infection (five to seven hours), doughnut shaped particles and tubules, both 45nm in diameter, were observed. Negatively stained purified virions had cubical symmetry with average diameters of 65 nm. Four capsomeres were observed on each capsid edge, suggesting that the capsid is composed of 92 capsomeres. Moss and Gravel concluded that, on the basis of the size and morphology of the virions, IPNV was probably a reovirus.

Nicholson (1971) conducted studies in which macromolecular synthesis in IPNV infected cells was followed by autoradiographic techniques. DNA synthesis within infected cells (as measured by $^3$H-thymidine incorporation) was sharply inhibited four to five hours after infection. An increase in RNA synthesis (measured by $^3$H-uridine incorporation) was observed at five to six hours post-infection. This
increase reached a maximum 11 hours after infection and soon declined. Treatments with RNAse, which degrades single but not double-stranded RNA, failed to demonstrate an enzyme resistant double-stranded RNA fraction similar to that observed in reovirus infected cells. Acridine orange staining also failed to reveal dsRNA in infected cells.

In 1972, Kelly and Loh published a study of the electron microscopic and biochemical characteristics of IPNV. In this study isopycnic sedimentation of $^{32}$P-labeled IPNV in CsCl resulted in a single band of radioactivity with a density of 1.33 $g/cm^3$. Infectivity was restricted to this band. Negatively stained preparations of the CsCl banded virus revealed non-enveloped, cubically symmetrical structures with an average particle diameter of 74 nm. Reovirus type 2 was stained for comparison and yielded an average particle size of approximately 77 nm. The IPNV particle, however, lacked the readily discernible inner capsid structure characteristically present in reoviruses. Confirming Moss and Gravell's observation (1969), four structural components per facet edge were observed, again suggesting that the capsid is composed of 92 capsomeres.

Kelly and Loh (1972) also characterized the viral nucleic acid by a number of methods. Viral RNA labeled with either $^{32}$P- or $^3$H-uridine was prepared from purified virus. The base composition was examined and was found to be noncomplementary with a purine to pyrimidine ratio of 0.88 to 0.90. These results suggested that IPNV RNA was not double-stranded like reovirus RNA. Ribonuclease degradation of IPNV RNA and reovirus RNA was compared in 1X and 0.01X SSC (standard
saline citrate, 0.15M NaCl, 0.015 M sodium citrate). In 1X SSC reovirus RNA was found to be completely resistant to the enzyme. IPNV RNA was 75% hydrolyzed under the same conditions. Both RNAs were hydrolyzed in 0.01X SSC. These observations were taken as further indication that IPNV did not have a double-stranded RNA genome. It was also observed that the ribonuclease susceptibility of IPNV RNA was dependent on the presence of Mg++ ions. In buffers containing 0.5mM Mg++ the RNA became resistant to nucleases. This was shown to be a substrate rather than enzyme effect.

Sedimentation analysis of IPNV RNA on sucrose gradients revealed a single component sedimenting at approximately 16S. This sedimentation was slower than expected for a single stranded RNA of this size. The authors concluded that the IPNV RNA was behaving as a rigid molecule with properties similar to those expected for double-stranded RNA. This 16S RNA was completely susceptible to ribonuclease digestion. The RNA was also found to be soluble in LiCl which was suggestive of a double-stranded structure. Since these results failed to point clearly to either a single- or double-stranded structure, the authors suggested that IPNV might contain single-stranded RNA with a great deal of double-stranded secondary structure. Based on their findings Kelly and Loh (1972) concluded that IPNV was distinctly different from the Reoviridae and should be placed in a new family or group.

Cohen, Poinsard and Scherrer (1973) carried out physiochemical analysis on the properties of the IPNV nucleic acids in an attempt to resolve some of the conflicting results of these earlier studies. A
central issue in this study was the purity of the virus from which RNA was extracted. It was their contention that many of the conflicting results in previous studies (particularly Kelly and Loh, 1972) may have been due to contaminating cellular RNA. They demonstrated that different RNA extraction methods might affect the outcome of subsequent analyses. It was their observation that if IPNV RNA preparation did not include protease digestion prior to phenol extraction, a significant portion of the RNA was trapped at the interphase. Since Loh and Kelly (1972) did not use protease treatment, the RNA they recovered from their phenol extractions might not be "representative of the composition that existed in the viral particle". In studies that will be discussed later (Persson and MacDonald, 1982 and Muller; Nitschke, 1987) a genome-linked protein was demonstrated that might account for the necessity of protease digestion prior to phenol extraction.

Cohen, Poinsard, and Scherrer (1973) demonstrated that IPNV RNA has a bouyant density of 1.615 in CsSO\textsubscript{4} equilibrium density gradient centrifugation. This is equal to values reported for the dsRNA of reovirus (Shatkin, 1965). It was also found that if the RNA was heated at 100°C for 10 minutes and then rapidly cooled, it banded at the same density (1.65) as rRNA. They demonstrated that IPNV RNA has a sharp melting profile with a T\textsubscript{m} value of 89°C. This is slightly higher than the value reported for reovirus RNA (Shatkin, 1965). Disputing the results of Kelly and Loh (1972), it was found that IPNV RNA has a base composition of complete complementarity (A/U and G/C equal to 1) and
that the RNA is RNAse resistant at molarities greater than 0.1X SSC. The sedimentation coefficient was calculated to be about 14S and was uneffected by ionic strength over a wide range. All of these results were taken as strong indication that IPNV RNA was double-stranded. Additionally it was found that electrophoresis of IPNV RNA on acrylamide gels resulted in resolution of two bands with molecular weights of 2.85 x 10^6 and 2.55 x10^6. If the RNA was heat denatured and quickly cooled before electrophoresis, two bands were observed migrating about twice as fast as the native forms. This was also considered to be consistant with a primarily double-stranded structure.

In 1976, Dobos used slightly different methodologies in an attempt to resolve some of the dispute over the nature of the IPNV genome RNA. In this case, the extravirion nucleic acids were eliminated from 32P-labelled semi-purified virus concentrates by digestion with nuclease prior to final purification. Purified virions were treated with proteinase K and SDS to release the genome RNA. The RNA was then recovered as a single 14S component by sucrose gradient centrifugation. This 14S RNA was analyzed by gel electrophoresis and autoradiography. Two distinct bands of equal intensity were observed. Their molecular weights were estimated to be 2.3 x 10^6 and 2.5 x 10^6, slightly lower than the values obtained by Cohen et al. (1973). Dobos also investigated the RNAse sensitivity of the IPNV RNA. He found that RNAse digestion resulted in an initial loss of 15% of the RNA, but the remainder of the RNA was resistant to even high concentrations of RNAse. It was suggested that this result might indicate that the genome RNA contains short single standed tail
regions. Denaturation studies were also carried out on the sucrose gradient purified RNA. The relative rates of electrophoretic mobility and sedimentation were compared for native and formamide/heat denatured RNA. The relative electrophoretic mobility of the denatured RNA indicated an approximate molecular weight of $1.5 \times 10^6$, slightly more than half that estimated for the double stranded molecules. The relative sedimentation of the denatured RNA increased from the 14S to 24S. RNA that had been pretreated with RNAse prior to denaturation was found to have the same sedimentation and electrophoresis behaviours as untreated RNA. This was taken as an indication that there were no internal single-stranded loops in the genome RNA. Dobos concluded that his data confirmed the primarily double-stranded nature of the IPNV genome RNA, but left open the possibility of short single-stranded regions at the ends of the molecules.

MacDonald and Yamamoto (1977) confirmed the evidence that Cohen, Poinsard and Scherrer (1973) and Dobos (1976) provided for the IPNV RNA being double-stranded. In addition to obtaining similar results for RNAse resistance, heat denaturation, CsSO$_4$ buoyant density, and pyrimidine to purine ratios, MacDonald and Yamamoto were able to provide further evidence by direct visualization of the IPNV RNA under the electron microscope. IPNV RNA appeared as a uniform linear strand with a width equal to that of DNA. Since single-stranded RNA appears as a collapsed "bush" by this method, the observations indicated that the RNA must be double-stranded.

Somogyi and Dobos (1980) conducted studies in which IPNV infected cells were pulse labelled with $^3$H -uridine. It was found that
virus specific RNA could be preferentially labelled if actinomycin D was added to the cultures one hour post-infection. Maximal virus-specific RNA synthesis occurred 8 to 10 hours after infection and was completely diminished by 12 to 14 hours. Three forms of RNA were detected. The first form to appear in abundance was a putative transcription intermediate (TRI). The TRI was LiCl precipitable, sedimented faster and broader (14 to 16S) than the 14S virion double stranded RNA and yielded genome sized pieces of double stranded RNA after digestion with RNAse. This data suggested a structure composed of a full length double-stranded RNA molecule and one or more partially completed single-stranded RNA transcripts. The second form of RNA to become abundant in the infected cells was a 24S genome length mRNA. No subgenomic length mRNAs could be demonstrated. It was shown that some of the radiolabeled 24S RNA could be chased into the third form of RNA, a 14S double-stranded RNA that had properties identical to the virion dsRNA. Dobos proposed that the TRI for each segment transcribed plus sense RNA strands in a conservative manner and that these plus strands served as mRNA or as templates for synthesis of the complementary strands of the double-stranded RNA. Dobos also pointed out the significance of detecting only two genome length mRNAs. This would suggest that there were no separate mRNA species for each of the four viral proteins. If this data were accurate, some of the proteins had to arise as the result of post-translational cleavage of a large precursor protein or as the result of multiple initiation and termination of translation within the genome length mRNAs.

By comparing the properties of IPNV genome RNA extracted from
virions either with or without protease treatment, Perrson and MacDonald (1982) were able to demonstrate the presence of a genome-linked protein. RNA that was obtained by dissociation of virions with 2% SDS and 5% 2-mercaptoethanol at 100°C was found to behave differently from RNA that was obtained by protease digestion and phenol extraction. The RNA which had not been protease treated exhibited a slower electrophoretic mobility, had a slightly lower buoyant density and demonstrated a marked tendency to circularize and aggregate. When the RNA which had not been protease treated was subjected to sequential RNase III and RNase A digestion, a 110,000 dalton protein was liberated. The strength with which the protein was linked to the RNA was considered suggestive of covalent bonding. Electron micrographs indicated that the proteins occurred only at the ends of the segments, promoting end to end attachments. The authors speculated that the genome-linked protein may serve as a primer for replication in a fashion similar to poliovirus and adenovirus genome-linked proteins.

Similar evidence for a genome-linked protein has been obtained for IBDV virus (Muller and Nitschke, 1987) and DXV (Revet and DeLain, 1982). In both cases the authors suggested that these proteins may be RNA-dependent RNA polymerases.

The presence of a polymerase activity, within the virion, capable of catalyzing the synthesis of single-stranded RNA has been demonstrated by Cohen (1975). If all four nucleosides were present, purified virions were capable of incorporating radiolabelled nucleotides into a form which was RNAse susceptible, presumably
single-stranded RNA. Polymerase activity increased as the temperature was increased from 10°C to 40°C. This was surprising since no detectable viral RNA synthesis occurred in infected cells at temperatures above 28°C. The polymerase activity was insensitive to actinomycin D and rifampicin and was completely dependent upon the presence of Mg++ (5-20mM).

Mertens, Jamieson and Dobos (1982) further characterized the activity and product of the IPNV virion-associated RNA polymerase. While they generally confirmed the characteristics of the enzyme activity, they arrived at different conclusions regarding the nature of the polymerase product. In this study the major reaction product was found to be a RNAse resistant 14S RNA. A genome-length 24S single stranded RNA could be detected in small quantities if bentonite was added to the reaction as an RNAse inhibitor. The discrepancy between these results and Cohen's (1975) was attributed to the limiting amounts of 3H-UTP used in the earlier study. An attempt was also made to determine whether synthesis of the single-stranded RNA occurs by a conservative mechanism, whereby the nascent RNA strand is released as single stranded RNA, or by a semi-conservative displacement mechanism, whereby the nascent strand displaces one of the two parental strands. IPNV virions were 3H-labelled and the polymerase reaction was conducted in the presence of unlabelled nucleotides. The data indicated that while most of the 3H-labelled virion RNA remained as 14S virus genome, some of the labelled RNA was found in the 24S single-stranded form. These results favored a semi-conservative mechanism. Attempts to confirm these results by
density labelling were not successful because of the inhibitory effects of the nucleotide analog (5-bromo UTP) on the polymerase activity.

Proteins.

Cohen, Poinsard and Sherrer (1973) were the first to report an analysis of the proteins of the IPNV virion. $^{14}$C-labeled virus was analyzed on SDS-urea gels. Proteins were observed at 80, 50 and 30 kilodaltons which accounted for 3%, 68% and 29% of the virion protein, respectively.

In 1977, Chang, MacDonald and Yamamoto compared the proteins of 10 isolates of IPNV. Coomassie blue stained SDS-PAGE analysis revealed nearly identical patterns for all of the isolates, with three proteins of molecular weights 50, 30 and 27 kD. The proportions of the 30 and 27 kD peptides varied slightly but the sum of the two consistently constituted 40% of the total protein. A large peptide in the 80-100 kilodalton range was not observed. This may have been a consequence of the inadequate sensitivity of the staining method.

Dobos (1977) conducted a study on the virus-specific protein synthesis in cells infected by IPNV. The time-course of protein synthesis was followed by pulse labeling infected cells with $^{35}$S-methionine and analyzing the labeled proteins by SDS-PAGE and autoradiography. Three size classes of virus-specific proteins were synthesized. They were designated the $\alpha$ proteins, molecular weights 90 and 100 kD; the $\beta$ proteins, molecular weights 59, 56, 54 and 50 kD; and the $\gamma$ proteins, molecular weights 32, 30 and 28 kD. In general, the relative proportions of synthesis for each class of proteins was the
same throughout the infectious cycle. A minor exception to this was the slightly greater level of α protein synthesis early in the infection.

This study demonstrated that the α protein did not have a precursor relationship to the β and γ proteins. The label could not be chased from the α protein to the smaller proteins. Additionally, experiments using amino acid analogues, protease inhibitors, ZnCl₂ and supraoptimal temperatures as inhibitors of protein processing were not successful in revealing the presence of a common precursor for the β and γ proteins. Processing was, however, observed within the β protein family. The 59 and 56 kD proteins were the first to appear in the infective cycle and the label in these could be chased to the 54 and 50 kD forms. The 50 kD form was the major form found in the purified virion. It was also demonstrated that the 30 and 28 kD γ₂ proteins were lost in chase experiments and did not occur in purified virus. Another observation of the study was that the frequencies of translation were inversely proportional to the molecular weights, i.e., the smaller proteins were produced more frequently than the larger proteins. This observation was remarkable since only two genome-length mRNAs had been observed and this data suggested that these two mRNAs are able to translate four proteins at differing rates. The authors pointed out that polycistronic mRNAs are rare in eucaryotes.

In 1977, Dobos and Rowe conducted peptide mapping experiments on the proteins of purified virions and the virus-specific proteins of IPNV infected cells. In each case the proteins were labeled with ³⁵S-methionine and separated by SDS-polyacrylamide gel
electrophoresis. Autoradiograms of the gels were used as templates for the excision of the viral proteins. Two-dimensional tryptic peptide maps were obtained by standard methods. Comparisons of the peptide maps revealed that the α (90kD), β₁ (59kD), γ₁ (29kD) and γ₂ (27kD) proteins were primary gene products and consequently had maps which were distinctly different from each other. All of the β size-class proteins had similar peptide maps, with β₁ apparently being the precursor for the smaller β peptides. The intracellular γ₁ (29kd) appeared to be the precursor for the intracellular γ₁a (27kd) and these peptides each had maps matching those of the γ₁ (29kd) and γ₁a (27kd) of the virion. The intracellular protein γ₂ (26kd) appeared to be the precursor of the intracellular protein γ₃ (25kd). The peptide maps of intercellular proteins γ₂ and γ₃ bore no resemblance to the maps of any of the virion proteins. The authors concluded that the coding capacity of the two segments of genome RNA was roughly equivalent to that required for the four primary gene products observed in infected cells. The smallest of these proteins, γ₂ and its derivative, γ₃, were not incorporated into the virion and were termed nonstructural.

MacDonald and Dobos (1981) identified which of the IPNV proteins were coded by each of the genome RNA segments. This was accomplished by reassortment studies with two temperature sensitive serotypes that differed significantly in the apparent molecular weights of both proteins and RNAs. Experiments with mixed infections and
subsequent selection for wild type temperature insensitivity led to the identification of only two assortment groups. It was found that the larger segment from each serogroup (designated A) encoded the three smaller primary gene products- $\beta$, $\gamma_1$, and $\gamma_2$. The smaller RNA (designated B) encoded the large $\alpha$ protein.

Darragh and MacDonald (1982) were able to demonstrate that a host range restriction in IPNV maps to the A segment. This was done by genetic reassortment studies using two strains of IPNV with differing abilities to replicate in host cell lines. The JV strain can replicate in both chinook salmon embryo (CHSE-214) cells and in fathead minnow (FHM) cells. The OV strain can replicate only in the CHSE-214 cells. Recombinants containing the OV A segment and the JV B segment were unable to infect FHM cells, while recombinants containing the OV B segment and the JV A segment were able to infect these cells. By using OV RNA transincapsidated with the JV capsid, the OV RNA was able to enter the FHM cells and produce OV antigens. This was taken as an indication that the host restriction block involved attachment to the cell.

To address the question of whether the three proteins encoded by the A segment of the IPNV genome resulted from multiple initiation and termination sites for translation or through processing of a previously undetected protein precursor, Mertens and Dobos (1982) conducted studies in which denatured virion RNA was used as mRNA for translation in a rabbit reticulocyte lysate system. The two genome segments were separated by preparative polyacrylamide gel electrophoresis. One problem that was encountered was the
contamination of the A segment by trailing of the B segment in the gels. As a result of this contamination, translation products from the A segment were always contaminated by small amounts of products from the B segment. Translation of the homogeneous B segment resulted in a 105 kd polypeptide and several smaller peptides which were apparently the result of premature termination of translation. Translation of the A segment resulted in three proteins having electrophoretic mobilities identical to the virus-specific infected cell proteins- $\gamma_2$, $\gamma_1$ and $\beta_1$. Tryptic peptide maps confirmed that the in vitro translation products were identical to their equivalent virus-specific proteins in infected cells. In an attempt to detect a common precursor for these proteins, a time course of the in vitro translation was conducted, with the reaction terminated at intervals between 0 and 60 minutes. A large molecular weight precursor was not detected. It was, however, found that the order of appearance of the proteins was $\gamma_2$, $\gamma_1$ and $\beta_1$. The time for completion of the polypeptide chains was proportional to their length, the smaller proteins being completed sooner. Pulse-chase experiments were also conducted and it was found that even during the shortest pulse (2 minutes) all three proteins became labeled. These results were consistent with simultaneous initiation of translation at three independent sites on a polycistronic mRNA.

Duncan and Dobos (1986) have reported the nucleotide sequence for a complete cDNA copy of the A segment of IPNV (Jasper strain). They found a single open reading frame large enough (2916bp) to encode
the three proteins of the A segment. The presence of this large open reading frame was highly suggestive of a common precursor for these proteins, despite the failure of investigators to detect this precursor. Sequence analysis also revealed that the open reading frame was flanked on the 5' and 3' ends by 119- and 62-bp of noncoding sequences. These regions may be binding sites for the RNA polymerase.

In our laboratory, cDNA clones of both the A and B segments have been constructed (Huang, et al., 1986). For each segment, clones containing the full coding region were assembled by splicing together two overlapping partial clones at a central restriction endonuclease site. The full length cDNAs were inserted into the plasmid transcription vectors, pT7-1 and pT7-2. These plasmids contain the promoter for the T7 bacteriophage RNA polymerase which can be used to synthesize RNA transcripts in vitro. Insertion of the B segment cDNA into pT7-2 resulted in the plasmid pT72/B. Insertion of the A segment cDNA into pT7-1 and pT7-2 resulted in the plasmids pT71/A and pT72/A (Figure 21). These two plasmids, pT71/A and pT72/A, contained the A segment cDNA insert in opposite orientations with regard to the T7 promoter. The cDNA containing transcription vectors were linearized by restriction enzyme digestion at a site at the end of the insert opposite from the promoter. Each linearized plasmid was then used as a template for run-off transcription of RNA beginning at the T7 promoter and ending at the site of the restriction endonuclease cleavage. When the RNA transcribed from pT72/B was translated in vitro, a protein comigrating with the virion α protein (approximately 95 kD) was produced. Translation of the RNA form pT71/A did not result
in the production of any virus-specific proteins, but translation of the RNA from pT72/A resulted in synthesis of proteins corresponding in size to β₁, γ₁ and γ₂. The translated proteins were confirmed as virus-specific by immunoprecipitation with antisera raised against purified virions. Antisera specific for either the β proteins or the γ proteins were also used to specifically identify the proteins of these classes. Interestingly, the γ₂ protein, which has not been found in the virion, was precipitated by both the serum raised against purified virions and the serum raised against the virion γ proteins.

To determine the coding order of the A segment proteins, three deletions were made from the A segment insert in pT72/A (Figure 2). The first deletion plasmid, p5′ Eco, was missing approximately 1.55 kb of the 3′ end of the A segment. Translation of the p5′ Eco RNA transcript resulted in a single protein with an apparent molecular weight of slightly less than that of the β₁ protein. Immunoprecipitation of this protein with anti-β indicated that this protein was a truncated form of β₁. Thus, the coding region for β₁ was within the first half of the A segment and extended slightly beyond the EcoRI restriction site.

The second deletion plasmid, p3′ Eco, was the complement of p5′ Eco. It contained 1.55 kb of the 3′ end and lacked 1.35 kb of the 5′ end. Translation of the RNA transcribed from this plasmid resulted in proteins comigrating with γ₁ and γ₂. Both of these proteins were precipitable with the anti-γ serum. It was concluded that γ₁ and γ₂ proteins must be encoded within the 3′ end of the A segment.
Figure 2. Map of Segment A restriction sites and coding regions used to determine gene order. The top bar shows the location of restriction enzyme sites which occur only once in the Segment A cDNA. The locations of the sites are given in terms of the number of base pairs from the the first methionine codon of the large open reading frame and were deduced from the nucleotide sequence of the Jasper strain (Duncan and Dobos, 1986) and partial sequencing of the Sp strain conducted in our laboratory (unpublished results). The lower bars indicate the coding region of the A Segment contained by transcription vectors used to determine the A Segment gene order (Huang et al., 1986). Translation of RNA produced from the plasmid p5'Eco resulted in production of only the β protein. Translation of the RNA transcripts from p3' Eco produced the γ₁ and γ₂ proteins. Translation of proteins from pΔSma RNAs resulted in the β and γ₂ proteins but not γ₁, thus establishing the gene order as 5'-β-γ₂-γ₁.
The third deletion plasmid, pΔSma, contained a small deletion of approximately 430 bp from the 3' end of the A segment. Translation of RNAs made from this plasmid resulted in production of the β₁ and γ₂ proteins, but not the γ₁ protein. This data indicated that the γ₁ protein must be encoded at the extreme 3' end of the A segment and the gene order must then be $^5\beta-\gamma_2-\gamma_1$.

Nagy et al. (1987) confirmed the $^5\beta-\gamma_2-\gamma_1$ gene order by hybrid arrested translation studies. Segment A cDNA fragments of defined regions were hybridized to purified plus strands of the genomic A segment RNA and the effect of these hybridizations on the translation products in vitro were determined. Hybridization conditions which blocked the two thirds of the A+ RNA at the 5' end allowed synthesis of only γ₁ (or VP3). Hybridization with a cDNA representing the 5' half of the segment A RNA allowed the synthesis of both γ₂ (NS) and γ₁ (VP3), but not β₁ (preVP2). Thus, the gene order for the A Segment was $^5\beta-\gamma_2-\gamma_1$. Furthermore, these results indicated internal initiation of translation could take place.
MATERIALS AND METHODS

Materials and reagents.

All chemical reagents were obtained from Sigma Chemical Company unless otherwise specified. Minimal essential media (MEM), Earle salts, penicillin and streptomycin were obtained from Grand Island Biological Company. Gentamicin sulfate was obtained from Whittaker M. A. Bioproducts. Fetal calf serum was purchased from HyClone. [\(^{35}S\)]-methionine was obtained from New England Nuclear and ICN. Pharmacia Fine Chemicals was the source of Sephadex-G200. Prestained and unstained protein molecular weight markers were obtained from Bethesda Research Laboratories (BRL) and Diversified Biotech. FMC provided SeaKem GTG and Sea Plaque (low melting temperature) agarose. Schleicher &Schuell was the source for nitrocellulose membranes. Freund's adjuvant and bacterial growth media were obtained from Difco. BioRad Laboratories was the source of gelatin and Tween-20. Boehringer Mannheim Biochemicals was the source for goat anti-rabbit-horse radish peroxidase conjugated (GAR-HP), ABTS, IPTG (isopropylthio-β-D-galactoside), and CIP (calf intestine phosphatase). Ampicillin, DNasel, lysozyme and Protein A-sepharose CL-4B were purchased from Sigma Chemical Company. Restriction enzymes were obtained from Bethesda Research Laboratories (BRL), New England Biolabs and International Biotechnologies Inc (IBI). T4 Kinase, SP6 RNA polymerase and T7 RNA polymerase were obtained from BRL. Promega Biotech was the source of the RNAsin. The universal terminator and pATH vector
sequencing primer oligodeoxynucleotides were prepared by the Center for Gene Research-Central Facility, O.S.U.

**Electrophoresis conditions.**

All electrophoretic protein separations were conducted on 9% separating and 3% stacking SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels by the method of Laemmli (1970). β-mercaptoethanol (BME) was included in the loading buffer as a reducing agent.

Electrophoretic separation of DNA fragments was by agarose gel electrophoresis in either Tris-acetate or Tris-borate buffer as described by Maniatis, Fritsch and Sambrook (1982). Typically, 1% gels were used with HindIII digested λ bacteriophage DNA as a molecular weight marker.

**Virus growth and purification.**

The Sp strain of IPNV was obtained from R.P. Hedrick, University of California, Davis. The Buhl strain of IPNV was obtained from N.E. Wood, International Aquaculture Research Center, Hagarman, Idaho. The Buhl strain was taken directly from infected fish tissue isolated and plaque purified three times before use as a viral stock. The virus was propagated in monolayer cultures of CHSE-214 cells (Fryer, Yusha and Pilcher, 1965) at a multiplicity of infection (MOI) of 0.05 to 0.1. Cells were grown at 16°C in Eagle minimal essential medium (MEM) and Earle salts supplemented with 0.15% bicarbonate, 5% fetal bovine serum, 100 IU of penicillin per ml, 100 µg
streptomycin per ml, and 10 μg of gentamicin sulfate per ml. Tissue
culture media from infected cells was harvested approximately five
days after infection. Cellular debris was removed by centrifugation
at 5000 rpm, 4°C, for 5 min in a Sorvall GSA rotor. Virus was
concentrated by centrifugation in a Beckman Type 35 rotor at 30,000
rpm for 90 min and resuspended in TBS (20 mM Tris, 500 mM NaCl, pH
7.5). Initial purification was by centrifugation through a step
gradient consisting of 5 ml of CsCl (ρ =1.4 g/cm³), 3 ml of CsCl (ρ=1.4
g/cm³), and 1.7 ml of 20% sucrose in TBS in a Beckman SW41 rotor at
35,000 rpm for 90 min. The virus banded at a density of 1.33 g/cm³
and the top component which are empty capsids (Stephens and
Hetrick, 1983) banded at a density of 1.29 g/cm³. Both bands were
collected by side puncture. The virus was further purified by
isopycnic density gradient centrifugation in CsCl (ρ=1.33 g/cm³) at
35,000 rpm for 16 h, 4°C. The virus band was again collected by side
puncture and dialyzed three times against a thousand-fold greater
volume of TBS. The purity of the virus was confirmed by SDS-PAGE
and silver stain analysis (Allen, 1980). The molecular weights of the
IPNV<sub>sp</sub> virion proteins were also determined by comparison to protein
molecular weight standards on silver stained SDS-PAGE gels (Figures
7a and 7b). The concentration of virion proteins in purified virus
preparations was determined by BioRad's protein quantitation
system.
[\textsuperscript{35}S]-methionine labeling of viral proteins.

Radiolabeled virion proteins were produced by infecting monolayer cultures of CHSE-214 cells at a multiplicity of infection (MOI) of 0.1. Infected cells were incubated at 18\degree\ C until 10\% cytopathic effect (CPE) was observed and then the media was removed and replaced by methionine deficient MEM containing 1\mu Ci per ml [\textsuperscript{35}S]-methionine. Incubation was continued until CPE was complete and then the virus was harvested and purified as described above.

Labeled virus specific infected cell proteins (ICP) were produced in CHSE-214 cells infected at 90\% confluency with IPNV\textsubscript{Sp} (MOI=100) for 1h. At that time the media was replaced with methionine free MEM containing 1\mu Ci per ml [\textsuperscript{35}S]-methionine. Cells were incubated at 18\degree\ C for 8 h, then harvested with a rubber policeman. The cells were concentrated by centrifugation at 5000 rpm for 5 min at 4\degree\ C in a Sorvall GSA rotor. The cell pellet was resuspended in TEP (100 mM Tris, pH 7.4, 10 mM EDTA, 10 mM Phenyl-methyl-sulfonyl fluoride) and sonicated three times for 10 sec at 40 watts (Heat Systems Ultrasonic, Inc., Sonifier Cell Disruptor).

Production of group-specific rabbit sera.

Purified IPNV\textsubscript{Sp} (1.5 mg) was suspended in 2 ml of 6 M guanidine hydrochloride-0.1 BME. The solution was incubated at 25\degree\ C for 20 h and then 37\degree\ C for 1 h. Dissociated proteins were separated on a Sephadex G-200 column with 6 M guanidine
hydrochloride-0.1 BME as the elution buffer. Samples from the column fractions were analyzed on silver stained (Allen, 1980) SDS-PAGE gels (Figure 8). Fractions containing viral proteins from the β, γ, and α size classes were pooled separately and fractions containing proteins from more than one size class were discarded. The pooled fractions were concentrated to a final volume of 0.5 ml, and emulsified with an equal volume of Freund's complete adjuvant. Each emulsion was injected subcutaneously at three sites along the back of a New Zealand White rabbit. The fractionation procedure was repeated with 0.6 mg of viral protein. The resulting fraction pools were emulsified in Freund incomplete adjuvant and injected four weeks after the initial immunization. Sera samples were collected two weeks after the second immunization. The resulting sera were designated anti-α, anti-β and anti-γ and were tested for reactivity with the viral antigens by Western blot analysis.

**Western blot analysis of group specific sera.**

Western blot analysis was used to demonstrate the specificity of the group specific antisera (Figure 9). IPNV<sub>sp</sub> virion proteins were separated on a SDS-PAGE gel and then transferred to nitrocellulose by the method of Towbin (1979). The membrane was treated with 3% gelatin in TBS (20 mM Tris-pH 7.5, 500mM NaCl) for 20 min and then rinsed three times in TBS. Separate membrane strips containing the transferred viral proteins were incubated for 1.5 h in the antisera (anti-α, anti-β, and anti-γ) which had been
diluted 200-fold. A membrane strip was also incubated in antiserum raised against whole virions, anti-IPNV<sub>Sp</sub> (Huang et al., 1986), which had been diluted a thousand-fold. The strips were rinsed three times in TBS. The strips were then incubated for 30 min in a 1:1000 dilution of goat anti-rabbit IgG conjugated to horse-radish peroxidase (GAR-HP), washed three times and developed in substrate solution 4CN (10 ml of 3 mg / ml 4-chloro-1-napthol in methanol, 50 ml TBS, 60 µl H<sub>2</sub>O<sub>2</sub>). The developed membranes were rinsed in water and dried.

**ELISA and Neutralization titration of group specific sera.**

The titer of the antisera (anti-α, anti-β, anti-γ and anti-IPNV<sub>Sp</sub>) was determined by two methods, serum neutralization and ELISA (enzyme-linked immunosorbent assay)(Table 2).

The serum neutralization titers were determined by the plaque reduction method. Briefly, 50 PFU (plaque forming units) of virus were mixed with a dilution of serum and incubated at room temperature for 1 h. The neutralization reaction was then mixed with an equal volume of 2X MEM containing 1% liquified low melting temperature agarose and overlayed on confluent CHSE-214 cells. The plates were incubated for six days, after which the agarose was removed and the cell monolayer was stained and the number of plaques counted. The end-point was the highest serum dilution to reduce the number of plaques by 50%.

ELISA titers (Figure 10) were determined by standard methods
in which 96 well microtiter plates (Falcon, Probind) were coated with purified virus (1 μg/ml in TBS) for 1 h at room temperature. The plates were washed three times with TBS-tween (TBS plus 0.05% Tween 20) and then antisera which had been serially diluted in TBS-tween were added. The plates were allowed to incubate for 1 h at room temperature. The wells were then washed and a 1:1000 dilution of GAR-HP in TBS was added and reacted for 20 min. After three final washes the color substrate solution, ABTS (30 ml 0.2% citric acid, pH 4, 18 μl H₂O₂, 225 μl 40 mM ABTS) was added and the OD₄₀₅nm was measured after 10 min.

Plasmid preparation and restriction enzyme analysis.

Plasmids were prepared by a modification of the alkaline lysis method of Birnboim and Doly, 1981 (Maniatis, Fritsch and Sambrook; 1982). A scaled up version of this method was used for 1 L of bacterial cells. Restriction enzyme digests were conducted under the conditions specified by the manufacturer.

Plasmid constructions.

The construction of pT72/A (Figure 17) has been described (Huang et al., 1986). This plasmid contains a cDNA for the entire coding region of the A Segment of IPNV. The cDNA is located immediately downstream from the T7 bacteriophage promoter. A deletion was generated in this plasmid, pT72/A, by digestion with Accl which removed approximately 1000 bp of the 3' end of the cDNA (Figure 17). The plasmid was purified from the deleted fragment by
agarose gel electrophoresis. The band containing the digested plasmid was excised, melted, and the plasmid religated in-gel as described by Struhl (1985). The ligation mixture was then transformed into *Escherichia coli* strain JM107 (Yanisch-Perron, Vieira and Messing, 1985). Plasmid DNA was purified from several of the resulting transformants and the construction was confirmed by restriction analysis with Accl and PstI. The deleted plasmid was designated pT72/AAA (Figure 21).

The entire A Segment cDNA insert was recloned into the plasmid vector pUC19 (Yanisch-Perron, Vieira and Messing, 1985) (Figure 3). This was accomplished by excision of the cDNA from pT72/A by digestion with PstI, isolation by electrophoresis on a 1% agarose gel and ligation in-gel with PstI digested pUC19 which had been treated with calf intestinal phosphatase (CIP). The ligation reaction was transformed into competent JM107 cells. Transformants were plated on LB-amp plates (LB agar plates with 120 μg/ml ampicillin) and after overnight incubation at 37°C, the resulting colonies were replica plated onto nitrocellulose membranes. These membranes were overlayed onto LB-amp plates with 0.5mM IPTG as an inducer of the *LacZ* promoter. Induced transformants were screened for expression of viral proteins by the colony blot immunoassay. Only four transformants gave strong positive signals by the immunoassay and these were analyzed further.

Plasmid preparations were made for each clone and initial restriction analysis was conducted by separate digestions with PstI and Accl. These digests indicated that all of the clones analyzed
contained the cDNA insert oriented so that the nonsense strand was under LacZ promoter control. One of these clones was designated pUC19/A(-) (Figure 3) and additional restriction enzyme analyses with Kpnl, Ndel, Xhol and EcoRl confirmed the orientation of this construction. Since none of the clones analyzed contained the A Segment cDNA in the positive sense orientation to the LacZ promoter, it was suspected that induction of viral gene expression might be toxic to the cells. Thus, the construction of pUC19 recombinants containing the A Segment cDNA insert was repeated under conditions in which induction of the LacZ promoter was restricted. Transformants inoculated onto nitrocellulose membranes for immunoassay were grown on noninducing media until colonies were approximately 1 mm in diameter. The membranes were then transferred to inducing media containing IPTG for 4 hours before being assayed. Six darkly staining colonies by immunoassay were chosen for further analysis and four were found to be in the positive sense orientation to the LacZ promoter. One of these clones was designated pUC19/A(+) (Figure 3) and additional restriction enzyme analyses confirmed this construction.

The cDNA was also cloned into the transcription vector pGEM-3Z (Promega)(Figure 4). This vector is a derivative of pUC19 which has the transcriptional promoters from bacteriophages T7 and SP6 inserted on either side of the multiple cloning site. The A Segment cDNA was excised from pUC19/A(-) by digestion with BamHI and HindIII, isolated on a 1% agarose gel and then ligated in-gel with pGEM-3Z which had been digested with BamHI, HindIII and
subsequently, alkaline phosphatase. The ligated DNA was then transformed into competent JM107 cells. Five transformants were selected for restriction analysis. All were found to contain the A Segment in the opposite orientation to the LacZ promoter. One of these clones was designated pGEM/A(-) (Figure 18). The cDNA insert in this plasmid was oriented so that the SP6 promoter could be used to transcribe a positive sense viral messenger RNA.

In each of the described plasmids, restriction sites which occurred once in the Segment A cDNA and once in the flanking multiple cloning site permitted the construction of ordered deletions in the cDNA. A series of 5' deletions of the A Segment were made with pUC19/A(-). These deletions were to the EcoRI, Kpnl, Xbal and AccI sites. The resulting plasmids were designated pUC19/A(-)ΔE, pUC19/A(-)ΔK, pUC19/A(-)ΔXb, and pUC19/A(-)ΔA, and retained approximately 1560, 1435, 1195 and 897 base pairs (bp) of the 3' end of segment A, respectively (Figure 11).

A similar set of 5' deletions were constructed with pGEM/A(-) (Figure 22). These were also made to the EcoRI, Xbal and AccI restriction sites. A Kpnl fragment deletion was not used in this series. The plasmids were designated pGEM/A(-)ΔE, pGEM/A(-)ΔXb and pGEM/A(-)ΔA and retained the same A Segment region as the corresponding pUC19/A(-) deletions.

Prior to construction of a 3' deletion series, an oligodeoxynucleotide containing translational stop codons in all three reading frames was inserted into the SstI site of pUC19/A(+) near the 3' end of the segment A insert (Figure 5). This was done to
prevent the fusion of the cDNA open reading frame with downstream vector sequences. The oligomer contained the sequence 5'TAATTAATTAAGCT and self annealing resulted in double-stranded fragments with 5'AGCT as a 3' overhang at each end. The oligomer was kinased and ligated onto SstI digested pUC19/A(+) DNA that had been treated with alkaline phosphatase. Eight transformants were screened for the presence of the oligomer by the loss of the SstI site and the creation of a new HindIII site. The creation of the HindIII site indicated the insertion of more than one oligomer since ligation of two or more of the oligomers together will result in the formation of this site. All eight of the transformants had lost the SstI site, but only one had the addition of a HindIII site. The clone containing the additional HindIII site was designated pUC19/A+S ("S" for stop codon).

To construct the 3' deletions, pUC19/A+S was digested separately with Smal, Accl, Xbal, Xhol/Sall, KpnI and EcoRI. The digested plasmids were separated from the fragments on agarose gels, ligated in-gel and transformed into competent JM107 cells. Transformants were analyzed by restriction enzyme digestion to confirm the construction of the desired deletion. The segment A cDNA coding regions remaining in each of the plasmids are shown in Figure 12. The deletants were designated pUC19/A+SΔS, pUC19/A+SΔA, pUC19/A+SΔXb, pUC19/A+SΔXo, pUC19/A+SΔKpnI, and pUC19A(+)ΔE. The plasmid, pUC19/A(+)ΔE, did not contain the translational terminator sequence and, as a result, the A Segment ORF was fused with 54 codons of out of phase LacZ sequence.

The Segment A cDNA was cloned into the TrpE-fusion
Figure 3. Construction of the plasmids pUC19/A(-) and pUC19/A(+). The Segment A cDNA insert (heavy line) was released from the plasmid pT72/A by digestion with PstI and was then isolated by agarose gel electrophoresis. The isolated cDNA was ligated, in-gel, to PstI digested pUC19 that had been treated with calf intestine phosphatase (CIP). The resulting plasmids, pUC19/A(-) and pUC19/A(+), contained the Segment A cDNA in opposite orientations to the LacZ promoter (small arrow). In pUC19/A(+) the coding region (+ sense) was in the same orientation as the LacZ promoter. In pUC19/A(-) this region is in the opposite orientation to the LacZ promoter.
Figure 3

pT72/A
5.7 kb

pUC19
2.69 kb

pUC19/A(+)
5.59 kb

pUC19/A(-)
5.59 kb

PstI, gel isolation of A Segment cDNA

T4 Ligase
Figure 4. Construction of the plasmid pGEM/A(-). The entire A Segment cDNA was released from the plasmid pUC19/A- by digestion with BamHI and HindIII and was then isolated by agarose gel electrophoresis. The isolated cDNA fragment was ligated into pGEM-3Z which had been BamHI/HindIII digested and then treated with calf intestine phosphatase (CIP). The resulting plasmid contained the cDNA large ORF in an orientation that permitted transcription of a sense strand RNA from the SP6 bacteriophage promoter.
Figure 4

BamHI, HindIII, gel isolation of the A Segment cDNA

T4 Ligase

BamHI, HindIII, CIP

pUC19/A(-) 5.59 kb

pGEM-3Z 2.7kb

pGEM/A(-) 5.65 kb

BamHI, HindIII, gel isolation of the A Segment cDNA
Figure 5. Insertion of a translational terminator oligonucleotide into pUC19/A(+) to create pUC19/A+S. The translational terminator oligonucleotide, which contained stop codons in all reading frames, was kinased and then ligated into pUC19/A(+) that had been digested with SstI and treated with calf intestine phosphatase (CIP). One of the resulting plasmids was designated pUC19/A+S. The presence of the translational terminator oligonucleotide in pUC19/A+S was confirmed by the loss of the SstI site and creation of a HindIII site. The presence of the HindIII site indicates that at least two of the oligonucleotides were inserted. Although the sequence at the bottom of the figure shows the insertion of three repeats of the oligonucleotide, the exact number of repeats is not known.
Figure 6. Construction of the plasmid pTA1. The plasmid pT72/A was linearized with HindIII, digested with BAL31, and then treated with $T_4$ DNA polymerase to create blunt ends. The plasmid was then digested with PstI and the pT72/A DNA fragments were ligated with Smal and PstI digested pATH2. The resulting plasmids were transformed into competent HB101 cells and screened immunologically for expression of viral proteins.
Figure 6

**Diagram Description:**

- **pT72/A** (5.7 kb)
  - + sense
  - PstI
  - HindIII

- **pATH2** (3.78 kb)
  - HindIII
  - Slow BAL31
  - T<sub>4</sub> pol blunt
  - PstI
  - Smal/PstI

- **pTA1** (6.58 kb)
  - Trp<sub>E</sub><sub>p</sub>
  - EcoRI
  - KpnI
  - XhoI
  - XbaI
  - NdeI

**Ligation Process:**

- PstI
- HindIII
- Sall
- XbaI
- Smal
- KpnI
- SstI
- Trp<sub>E</sub><sub>p</sub>
- Trp<sub>E</sub><sub>p</sub> blunt
- PstI
- 4 HindIII
- 1 ligation
expression vector pATH2 as shown in Figure 6. The 5' end of the A Segment cDNA was first digested with BAL31 to remove noncoding regions and to randomly generate fusions in each reading frame. To accomplish this, pT72/A was cut with HindIII and then digested with slow BAL 31 under condition specified by the manufacturer (IBI) to obtain deletions of between zero and 200 bp. Then, following phenol extraction and ethanol precipitation, the BAL 31 digested plasmid DNA was treated with T4 DNA polymerase to assure blunt ends. After phenol extraction and ethanol precipitation, the DNA was digested with PstI and cloned into the PstI/Smal digested pATH2. The resulting plasmids were then transformed into E. coli HB101 cells and screened immunologically for expression by the colony blot assay described below. A single colony giving a very strong positive signal was obtained and designated pTA1 ("TA" for TrpE-A Segment fusion) (Figure 30). The construction was confirmed by restriction analysis and the fusion junction was sequenced as described below.

The plasmid pATH1/A was constructed as a negative control for expression of the A Segment cDNA in the pATH1 vector. The pATH1 and pATH2 vectors are identical except for a slightly different organization of restriction enzyme sites in the MCS. The A Segment cDNA was cloned into pATH1 as a BamHI and HindIII fragment from pUC19/A(-) and resulted in an out of reading frame insertion.

**Phosphorylation of the oligonucleotide.**

The translational terminator oligonucleotide (400 pmol) was treated in a final volume of 100 μl containing 10 μM ATP, 10 mM
dithiothreitol, 10 mM MgCl$_2$, 50 mM Tris-HCl, pH 8.0 and 10 U T4 Kinase. The reaction was incubated at 37°C for 30 min and then extracted with phenol. Approximately 40 pmol of phosphorylated oligonucleotide was used for ligation into approximately 5 pmol of phosphatase treated pUC19/A+S.

**Plasmid sequencing of pTA1 gene junction.**

The construction of the plasmid pTA1 resulted in loss of convenient restriction sites for subcloning into the bacteriophage M13 for sequence analysis. In order to circumvent this difficulty, a 19 base pair primer was synthesized to TrpE sequences 5' of the junction between the TrpE and A Segment and plasmid sequencing was conducted by the method of Chen and Seeburg (1985). The primer (5'GCGTCGCGAAAGCTCGC) contained TrpE DNA sequence located (Yanofsky et al., 1981) located 35 base pairs from the Smal site of the pATH2 multiple cloning site.

**Production of antisera to the truncated polyprotein, t-pp(Accl).**

JM107 cells containing the plasmid pUC19A+SΔA were grown to mid-log phase in LB broth (Miller, 1972) and then induced by the addition of IPTG to a final concentration of 0.5 mM. The cells were passed three times through a French press (Carver, Model C) at 1.5 metric tons/cm$^2$. A 1 ml sample of the bacterial lysate was centrifuged in a Beckman Microfuge E for 2 minutes. The pellet was washed 2 times with TBS. The supernatant fluid was retained and
subjected to sequential precipitations with increasing concentrations of saturated ammonium sulfate (SAS) from 1 to 50% saturation. Protein samples from each of the SAS precipitations and from the initial insoluble material were analysed by SDS-PAGE and Western blotting. A substantial portion of the 83 kD truncated polyprotein [t-pp(Accl)] was retained in the initial insoluble fraction.

The remaining bacterial lysate was centrifuged at 5,000 rpm for 10 min in a Sorvall GSA rotor. The pellet was washed 3 times in TBS and then resuspended in SDS-PAGE loading buffer. The sample was boiled for 3 min and then separated by preparative SDS-PAGE. A broad band in the region of t-pp(Accl) was excised from the gel and electro-eluted with a Schleicher and Schuell Elutrap device. A sample of the eluted proteins was analyzed by SDS-PAGE, Coomassie blue staining, and Western blotting. Comparisons of these analyses revealed that t-pp(Accl) was well separated by SDS-PAGE and easily identifiable by Coomassie staining. Thus, the remaining eluted protein preparation was then separated by SDS-PAGE and stained. The t-pp(Accl) band was excised and electro-eluted. The eluant was dialysed three times against 1000 volumes of PBS and then concentrated to a final volume of 1.5 ml by dehydration across a dialysis membrane with dry polyethylene glycol, (PEG)-6000. A rabbit was immunized with the protein as previously described. After the second booster immunization a sample of serum was harvested and was tested by Western blot analysis for activity against virion, top component and infected cell proteins (Figure 20).
Colony blot assay.

Immunological screening of bacterial colonies was by a modification of the method of Helfman et al. (1983). Colonies were replicated onto 82 mm nitrocellulose sheets and the replicas were grown overnight at 37°C on LB agar supplemented with 120 μg ampicillin/ml and 1 mM IPTG. Bacterial colonies were lysed by exposure to CHCl₃ vapors for 30 min, followed by incubation for 1 hour in blocking/lysis solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 3% gelatin, and 1 μg DNase and lysozyme 40 μg per ml). The membranes were then rinsed two times in TBS followed by incubation for 1 h in a 1:1000 dilution of anti-IPNV sera in TBS. Membranes were rinsed again in TBS and then incubated for 30 min in 1:1000 dilution of goat anti-rabbit IgG-horse radish peroxidase conjugate. After two final rinses in TBS, the membranes were developed by incubation in 4CN. Developed membranes were rinsed in water and air dried.

Deletion analysis using bacterial expression and Western blot analysis.

Bacterial cultures were inoculated into LB with ampicillin (120 μg/ml) and grown overnight at 37°C. A 100 μl sample of the each overnight was inoculated into 5 ml of fresh LB with ampicillin and grown to mid-log phase (approximate OD₆₀₀nm of 0.7). Cultures of pUC19A(+) and its derivatives were induced by addition of IPTG to a final concentration of 1 mM. Cultures of pUC19A(-) and its
derivatives were not induced. Incubations were continued into early stationary phase (approximate OD$_{600\text{nm}}$ of 1.6). Cultures were diluted to an OD$_{600\text{nm}}$ of approximately 1.0 and 1 ml of culture was centrifuged for 3 min in a Beckman Microfuge E. The supernatant fluids were aspirated off and the cells were resuspended in 150µl of SDS-PAGE loading buffer. After boiling for 3 min, 50µl samples were separated by SDS-PAGE and analyzed by Western blotting with anti-IPNV serum (Huang et al., 1986) or the monospecific sera to the β and γ proteins (Figures 14, 15, 16, 17 and 18).

**In vitro transcription.**

RNA transcripts of various lengths were made from the Segment A cDNA by *in vitro* run-off transcription of the plasmids pT72/A, pT72/AΔA, pGEM/A(-)ΔE, pGEM/A(-)ΔX and pGEM/A(-)ΔA which were linearized at various restrictions sites (Figures 21 and 22). After digestion to completion with the restriction enzymes, the linearized plasmids (approximately 1µg) were extracted with phenol and precipitated with ethanol. The plasmid DNA pellets were resuspended in 95 µl of transcription buffer (40 mM Tris-HCl, pH 8.0, 8 mM MgCl$_2$, 25 mM spermidine, 5mM dithiothreitol and 500 µM each ATP, GTP, CTP and UTP in diethylpyrocarbonate [DEP] treated water). RNasin was added to a final concentration of 1 U/µl and then 25 U of T7 bacteriophage RNA polymerase were added and the reaction was incubated at 37°C for 1 h. The reactions were extracted with phenol and precipitated with ethanol. The RNA pellets were resuspended in
100 µl DEP-treated water with RNasin at 1 U/µl. Samples of the RNAs were analyzed on nondenaturing agarose gels.

**In vitro translation.**

The RNA transcripts were used to prime synthesis of $[^{35}\text{S}]$-methionine labeled proteins in a rabbit reticulocyte lysate translation system. A NEN Translation Kit was used according to the manufacturers specifications. For each translation reaction, 2 µl of the RNA solution was added to 10 µl of lysate and 13 µl of a reaction mixture (containing 50 µCi $[^{35}\text{S}]$-methionine). The final potassium and magnesium ion concentrations were 80 mM and 0.65 mM, respectively. Translation reactions were incubated at 37°C for 1h. Samples of the translations (5 µl) were analyzed by SDS-PAGE and autoradiography. Brome Mosaic Virus (BMV) RNA was translated as a positive control and BMV proteins were used as molecular weight markers.

To determine whether the viral encoded protease could act in trans, the 3' Accl deleted RNA which yeilds translation of an unprocessed truncated polyprotein, t-pp(Accl) was co-translated with RNA transcripts encoding all or part of the coding region for the putative protease-$\gamma_2$. Equal amounts of each RNA (1 µl) were added to the translation reactions and the translation products were analyzed by SDS-PAGE and autoradiography.

In an attempt to arrest processing of the viral polyprotein, purified Immunoglobbulins from each of the serum (anti-IPNV, anti-$\beta$, anti-$\gamma$, anti-t-pp(Accl) and normal) were added to translation
reactions primed with complete A Segment RNA transcripts. The translation reactions were conducted as described above but with the addition of 1\(\mu\)l of purified immunoglobulin (approximately 1\(\mu\)g/\(\mu\)l) per 25 \(\mu\)l translation reaction.

**Immunoglobulin purification and immunoprecipitations.**

A sample (3.7 ml) of each the sera described above was mixed with Buffer A (7.3 ml of 20mM Tris-HCl, pH 7.8 and 40 mM NaCl) and centrifuged for 30 min at 20,000 rpm in a Beckman SW40.1 rotor. The supernatant fluids were mixed with an equal volume of saturated ammonium sulfate (SAS), incubated on ice for 60 min and then centrifuged for 10 min at 6000 rpm in a Sorvall SS-34 rotor. The pellets were resuspended in 2 ml buffer A, then dialyzed 3X against 1 L of Buffer B (20 mM Tris-HCl, pH 7.8 and 20 mM NaCl). The suspensions were then passed three times through a 200 \(\mu\)l Protein A-Sepharose CL-4B column. The columns were washed with 40 ml of Wash Buffer (100 mM NaCl, 10 mM KPO4, pH 7.0). A 100 \(\mu\)l sample of the Protein A-Sepharose column with immunoglobulin still bound was removed for use in immunoprecipitation of translation products. Immunoglobulins were eluted from the remainder of the columns with 2 ml of 0.1 M Acetic acid. Tris-base (pH 8.5) was added to a final concentration 100 mM and the pH was adjusted to approximately 7.5 with NaOH.

**Radio-immune precipitation analysis (RIPA).**

A 20 \(\mu\)l sample of each of the *in vitro* translation reactions
described above was diluted to a final volume of 500 µl in RIPA Buffer (0.1% SDS, 0.5% NP40, 0.2% deoxycholate, 0.15 M NaCl and 10 mM Tris, pH 8.0) and centrifuged for 15 min at 4°C in a Beckman E microfuge to remove precipitable material. The supernatant fluids were removed and 100 µl aliquots were then mixed with 10 µl of each of the Protein A-Sepharose-immunoglobulin complexes described above. The mixtures were incubated overnight at 4°C with gentle agitation and then the immunoprecipitate was collected by centrifugation for 2 min in a Beckman microfuge E. The pellets were washed three times in RIPA buffer and then analysed by SDS-PAGE and autoradiography.

**Western Blot analysis of pTA1 expressed proteins.**

Cultures of JM107/pTA1 and JM107/pATH2 (5ml each) were grown at 37°C in supplemented M9 to an OD$_{600nm}$ of 0.6 and then induced by addition of Indoleacrylic acid (IAA) to a final concentration of 5 µg/ml. The incubation continued overnight and then the cells were harvested by centrifugation for 5 min at 5000 rpm in a Sorvall SM24 rotor. The cell pellets were each resuspended in 2 ml SDS-PAGE loading buffer and boiled for 3 min. Each sample was separated on a preparative SDS-PAGE gel and then transferred electrophoretically to nitrocellulose. The membranes were blocked with 3% gelatin in TBS and then cut into strips.

To determine the identity of the proteins expressed by pTA1, Western blot strips were reacted with each of the mono-specific antisera: anti-β, anti-γ and anti-t-pp(AcCl) (Figure 31).
Production of antigens for vaccine trial.

Overnight cultures of JM107/pUC19, JM107/pUC19A+S, JM107/pUC19A+SΔA and HB101/pTA1 were grown in LB-amp at 37°C. A 1 ml sample of each of the overnight cultures was inoculated into 1 L of growth media. For the pUC19, pUC19/A+S and pUC19A+SΔA cultures the growth media was LB-amp with twice the normal concentration of tryptone. The pTA1 culture was grown in supplemented M9 media as described previously. All cultures were grown to an OD$_{600\text{nm}}$ of 0.6 before induction. The pUC19 plasmids were induced by the addition of IPTG to a final concentration of 2.5 mM. The pTA1 culture was induced by the addition of IAA to a final concentration of 2 µg/ml. Incubation of the cultures was continued overnight at 37°C. The cultures were centrifuged for 5 min at 5000 rpm in a Sorvall GSA rotor. The supernatant fluid was decanted and the cells were resuspended in 1/100 volume lysis buffer (10 mM MgCl$_2$, 5 µg/ml DNase, 20 µg/ml lysozyme and 100 mM NaPO$_4$ buffer, pH 7.5) and incubated on ice for 1 hour. The cell suspensions were then sonicated three times for 20 seconds each at 40 watts (Heat Systems Ultrasonic, microtip). The bacterial lysates were assayed for the presence of viral antigens by Western blot analysis (Figure 32). Samples of 1, 5 and 20 µl from each of the lysates were separated by SDS-PAGE, Western blotted and analyzed with anti-IPNV serum.
Vaccine trial.

Rainbow trout (*Salmo gairdneri*) fry were obtained from Oak Springs Hatchery, Maupin, Oregon (Fall, 1987). The average weight of the fry was 0.13 g at the beginning of the experiment. The fry were divided into groups of 25 fish and each group was held in 18 L aquaria throughout the course of the experiment. The fish were fed Oregon Biodiet #2 at 2-4% body weight per day. Water temperature was 10-11°C.

Vaccination was by immersion. The water level in the tanks was lowered to approximately 800 ml and 25 ml of the bacterial lysate (described above) was added. Exposure to the vaccine was for 20 min at this concentration; the water level was increased to approximately 6 L for 15 min and then to 18 L for 20 min before water flow was resumed.

Fish were challenged with the Buhl strain of IPNV at 20 days after immunization. Virus was added to tanks at a concentration $10^{-6}$ plaque forming units per ml in 3L of water and maintained at this level for 18 h. The water level was then raised to 18L and the challenge was continued in static water (except that flow would be resumed for several hours once a week to clean tanks). Moribund fish were collected on a daily basis. The presence of virus in these fish was confirmed by the demonstration of CPE in tissue culture. The experiment was terminated 54 days after challenge.
RESULTS

Molecular weight determination of virion proteins.

The molecular weights of the IPNV virion proteins were established by comparison to molecular weight protein standards on silver stained SDS-PAGE gels (Figures 7a and 7b.). The α protein has an apparent molecular weight of 95 kD. A protein band migrating slightly above the α band is reportedly a dimer of β₃ (Dobos and Rowe, 1977). The β₁, β₂, β₃ and β₄ proteins have apparent molecular weights of 63, 61, 56.5 and 51.5 kD, respectively. The γ₁ and γ₁a proteins have molecular weights of 32 and 28.5 kD. In Figure 7a, Lane A, an additional virion protein with an apparent molecular weight of 26 kD is present. This protein cannot be detected on heavily loaded Coomassie stained gels, but may be detected on Western blots of virion proteins assayed with anti-IPNV serum (see Figures 17 and 32). The 26 kD protein may be γ₂ which had not been observed by other investigators (Dobos and Rowe, 1977; Dobos, 1977) in purified virion preparations. Anti-IPNV serum raised to purified virions, reacts with γ₂ proteins produced by in vitro translation (Huang et al., 1986). This indicates that γ₂ may be present in virions in small quantities. The electrophoretic migration of γ₂ is variable (Dobos, 1977) and its detection may be obscured by other virion proteins.
Figure 7a. Silver stained IPNV virion proteins separated on SDS-PAGE gels. The electrophoretic migration of the virion protein of IPNV was analyzed after silverstaining: (A), approximately 1μg of purified IPNV; (B), approximately 10 μg of purified IPNV. The molecular weight protein markers (C) were phosphorylase b (97 kD), bovine serum albumin (66.2 kD), ovalbumin (42.6 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD). The question mark indicates the putative γ2 which is present in lane (A).
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Figure 7b. Graph of the relative migrations of IPNV virion and molecular weight standard proteins. The molecular weights of IPNV virion proteins (Figure 7a., Lane B) were estimated by comparing their migrations to those of molecular weight standards (Figure 7a., Lane C). The molecular weight of the α protein was determined to be approximately 95 kD. The β₁, β₂, β₃ and β₄ proteins have apparent molecular weights of 63, 61, 56.5 and 51.5 kD, respectively. The γ₁ and γ₁α proteins have molecular weights of approximately 32 and 28.5 kD.
Production of group-specific antisera.

The virion proteins from each group, α, β, and γ were separated chromatographically and used as antigens to produce group-specific antisera. To accomplish this, purified virions were disrupted with 6 M guanidine-hydrochloride and 0.1 M BME. The proteins were then separated by Sephadex-G200 column chromatography. Fractions were collected and analysed by SDS-PAGE and silver staining (Figure 8). Some lower molecular weight polypeptides (35-45 kD) were observed in the fractions containing the β proteins. These were β protein degradation products. Fractions from each size class, α, β and γ₁, were pooled. The pooled fractions were dialyzed against PBS and then used to immunize rabbits.

The resulting sera were tested for reactivity with virion proteins by Western blot analysis (Figure 9). The anti-α serum failed to react with any of the virion proteins; the anti-β serum reacted only with the β proteins; and the anti-γ serum produced a strong signal with γ₁ and γ₁ₐ proteins. The anti-γ serum also exhibited some reactivity with the virion β proteins. In studies published by Huang et al. (1986), the anti-γ serum was shown to react with γ₂ peptides produced by in vitro translation.

The ability of each of the antisera to neutralize virus was tested by plaque reduction assays (Table 2). None of the three antisera neutralized virus infectivity, even at high concentrations. The anti-β
Figure 8. SDS-PAGE analysis of virion proteins separated by column chromatography. After denaturation with 6 M guanidine hydrochloride and 0.1 M BME, IPNV virion proteins were separated on a Sephadex G200 column. The eluted fractions were analysed on SDS-PAGE gels and silver stained. The column fractions are denoted on the bottom and the virion proteins are indicated on the side. Fractions 1 and 2 were pooled for the $\alpha$ protein. Fractions 4 through 9 were pooled for the $\beta$ proteins and fractions 12 through 16 were pooled for the $\gamma$ proteins. The pooled fractions were used for the production of group-specific antisera.
**Figure 9.** Western blots of anti-IPNV, anti-γ, anti-β and anti-α sera reacted against IPNV virion proteins. The reactivity of antisera prepared to purified α, β and γ proteins were compared by immunoblotting to SDS-PAGE separated IPNV proteins. The antisera used for each blot is given at the bottom and the respective virion proteins are noted on the side.
Table 2. Determination of antisera titers. The titer of each of the sera, anti-α, anti-β, anti-γ and anti-IPNV, was determined by serum neutralization and ELISA. Serum neutralization titers were determined by the plaque reduction method. The determination of ELISA titers is detailed in Figure 10.
Figure 10. ELISA determination of antisera titers. ELISA titers were determined by incubating dilutions of each antisera, anti-\(\alpha\), anti-\(\beta\), anti-\(\gamma\), anti-IPNV and normal serum, with purified virus bound to microtiter plate wells. Spectrophotometric determination of the color produced by an enzyme-labelled second antibody (goat anti-rabbit-horseradish peroxidase) was used to quantitate antibody binding. The titer was considered to be the greatest dilution of serum which resulted in an absorbance value greater than that of a 1/10 dilution of normal, non-immune, serum. The titer cut-off point is indicated by a bar across the lower region of the graph.
and anti-γ in combination also failed to neutralize virus infectivity.

An estimate of the antisera reactivity with virion proteins was made by enzyme-linked immunosorbent assay (ELISA) (Table 2 and Figure 10). The titer of the serum was the lowest dilution that resulted in an absorbance value greater than that of a 1/10 dilution of normal serum. The anti-α serum titer was very low, 1/50. The titer of the anti-β serum was 1/500 and the anti-γ serum titer was 1/5000. The serum raised against whole virus, anti-IPNVsp, had a titer of 1/50,000 by this assay.

Deletion analysis of the Segment A coding regions by bacterial expression.

Construction of pUC19/A(-) and pUC19/A(+).

The Segment A cDNA from pT72/A (Huang et al., 1986) was recloned into pUC19 as a PstI fragment (Figure 3). The transformants were plated on inducing media and screened immunologically for the expression of viral polypeptides. Several positive colonies were selected for restriction analysis and all were found to contain the A Segment cDNA ORF in the opposite orientation to the LacZ promoter. One of the resulting plasmids was designated pUC19/A(-) (Figure 3). Since there were no immuno-positive colonies with plasmids containing the A Segment cDNA inserted in the positive orientation to the LacZ promoter, it was suspected that this construction may have resulted in expression of toxic levels of viral gene products. Thus, the cloning was repeated and this time transformants were allowed to
grow to visible colonies before transfer to inducing media. Approximately one half of the immunologically positive transformants from this cloning were shown by restriction analysis to be in the positive orientation in regard to the \textit{LacZ} promoter. One of the plasmids containing the cDNA in the positive orientation was designated pUC19/A(+) (Figure 3).

**Western blot analysis of virus specific polypeptides produced by pUC19/A(+), pUC19/A(-), pT72/A and pT71/A cultures.**

The virus-specific polypeptides produced by JM107 cells containing the plasmids pUC19/A(+), pUC19/A(-), pT72/A and pT71/A (Huang et al., 1986) were compared by Western blot analysis (Figure 11). The plasmids pT72/A (Figure 21) and pT71/A contained the Segment A cDNA in opposite orientations. These plasmids were used as negative controls since the A Segment cDNA was not controlled by an inducible bacterial promoter. A bacterial protein (indicated by a B) gave a weak background signal in all the lanes containing the bacterial cultures. The cells containing the plasmid pUC19/A(+) (Lane 3) produced a very weak signal for a polypeptide corresponding to $\beta_1$ in molecular weight (indicated by arrow) and a stronger signal for a polypeptide corresponding to $\gamma_1$. The pUC19/A(-), pT72/A and pT71/A cultures each produced an immunoreactive peptide of 38 kD in addition to a polypeptide corresponding in molecular weight to $\gamma_1$. This was surprising since, in these plasmids the viral cDNA was not under the influence of any known bacterial promoter, nor had a 38 kD protein been
Figure 11. Western blot of IPNV proteins expressed by pUC19/A(-), pUC19/A(+), pT72/A and pT71/A. Lysates of E. coli JM107 carrying pUC19/A(-), pUC19/A(+), pT72/A, or pT71/A were prepared and analyzed by SDS-PAGE and Western blotting with anti-IPNV serum as described in Materials and Methods. The virus specific polypeptides are noted on the right. The lanes contained the following: (1) pT72/A; (2) pT71/A; (3) pUC19/A(-); (4) purified IPNV; (5) molecular weight markers in descending order of size 85, 75, 45, 35, 30 and 20 kD; and (5) pUC19/A(+). The putative β₁ polypeptide in Lane 3 is indicated by an arrow. A bacterial protein (indicated by a B) gave a weak background signal and can be seen in control lanes (see Figures 16 and 18) of cultures containing pUC19 without the viral cDNA.
described in either infected cells or purified virions.

Construction of plasmids with 3' deletions to the A Segment cDNA.

The plasmid pUC19/A+S (Figure 5) was used for construction of plasmids with sequential deletions to the 3' end of the cDNA. Deletions were to the Smal, Accl, Xbal, Xhol, KpnI and EcoRI sites and the resulting plasmids were designated pUC19/A+SΔS, pUC19/A+SΔA, pUC19/A+SΔXb, pUC19/A+SΔXo, pUC19/A+SΔK and pUC19/A+SΔE. The cDNA region remaining in each of the plasmids was followed by a termination codon provided by the oligonucleotide. The plasmid pUC19/A+SΔE was an exception in that the oligonucleotide had been deleted and the A Segment ORF was fused to LacZ sequences that provided another 54 codons before a termination codon was reached. Restriction enzyme maps for the plasmids pUC19/A(+) and pUC19/A+S are shown in Figure 12 along with bar maps showing the cDNA regions remaining in each of the deleted plasmids.

Western blot analysis of virus-specific polypeptides expressed by cultures containing pUC19/A(+) and its derivatives.

The polypeptides from induced cultures of JM107 cells containing pUC19/A(+), pUC19/A+S and pUC19/A+S deletion plasmids were compared by Western blot analysis with anti-IPNV, anti-β and anti-γ sera (Figures 13, 14 and 15, respectively). The pUC19/A(+) and pUC19/A+S cultures (Lanes 3 and 4 in each figure) contained immunoreactive polypeptides corresponding to the γ₁ and γ₁ₐ proteins.
Figure 12. Restriction maps of pUC19/A(+) and plasmids derived from it. The upper circular plasmid map is of pUC19/A(+) which contains the Segment A cDNA (heavy line) cloned into pUC19 (thin line). The positive sense transcriptional direction of the cDNA large open reading frame (large arrow) is in the same orientation as the LacZ gene promoter (small arrow). The initiation codon of the cDNA large open reading frame (bold faced ATG) is preceded, 20 codons upstream, by the in-phase initiation codon of the LacZ gene (underlined ATG). PstI sites are at the boundaries of the cDNA and vector. Other restriction sites which are unique or occur once within the cDNA and once within the vector are indicated. The lower circular plasmid map is of pUC19/A+S. This plasmid is similar to pUC19/A(+) except that an oligonucleotide with translational stop codons (TAA) in all reading frames has been inserted at the SstI site (Figure 5). The plasmid pUC19/A+S was used for construction of plasmids with sequential deletions to the 3' end of the cDNA as described in Materials and Methods. The resulting plasmids were designated pUC19/A+SΔS, pUC19/A+SΔA, pUC19/A+SΔXb, pUC19/A+SΔXo, pUC19/A+SΔK and pUC19/A+SΔE and retained approximately 2440, 2020, 1720, 1510, 1480 or 1360 base pairs of the 5' end of the cDNA, respectively. The bars at the bottom indicate the regions of the A Segment cDNA remaining in each plasmid. The deleted cDNA in each of the plasmids is followed closely by the termination signals of the oligonucleotide described above. The only exception to this is the plasmid
pUC19/A+SΔE in which the cDNA is followed by 54 codons of vector sequences before a translation termination codon is reached. The additional codons are out of phase LacZ gene sequences.
Figure 12

Lac_p ··· ATG/ACC/ATG/ATT/ACG/CCA/AGC/TGG/GAT/GCC/TGC/AGG/-
PstI
GGG/GGG/GGT/CTA/TAT/CAA/TGC/AAG/ ATG.....A Segment ORF...

pUC19/A(+)
5.59 kb

pUC19/A+S
5.60 kb

pUC19/A(+)

pUC19/A+SΔS

pUC19/A+ΔΔ

pUC19/A+SΔXb

pUC19/A+SΔXo

pUC19/A+ΔK

pUC19/A+ΔE

54 codons TGA
Figure 13. Western blot using anti-IPNV serum to detect polypeptides expressed by pUC19/A(+), pUC19/A+S and pUC19/A+S deletion plasmids. Equal quantities (based on absorbance) of induced E. coli JM 107 cells containing the plasmids were harvested and the total cell proteins were separated by SDS-PAGE and analyzed by Western blot with the anti-IPNV serum as described in Materials and Methods. Lane 1 contains purified virion proteins. The migration of molecular weight marker proteins are shown at the right. Below each lane is the name of the respective plasmid. The bars at the bottom are representations of the A Segment cDNA region contained by each plasmid.
Figure 13

ATG-1  NdeI  EcoRI  XhoI  XbaI  AccI  SmaI  TAA

pUC19/A(+)  pUC19/A+S  pUC19/A+SΔS  pUC19/A+SΔA  pUC19/A+SΔXb  pUC19/A+SΔXo  pUC19/A+SΔk  pUC19/A+SΔE

54 codons  TGA
Figure 14. Western blot using anti-β serum to detect IPNV polypeptides expressed by pUC19/A(+) , pUC19/A+S and pUC19/A+S deletion plasmids. A duplicate of the Western blot shown in Figure 13 was assayed using anti-β serum. Lane 1 shows the detection of the virion β protein with this serum. The migration of molecular weight marker proteins are shown at the right. Below each lane is the name of the respective plasmid. The bars at the bottom are representations of the A Segment cDNA region contained by each plasmid.
Figure 14

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ATG-1 NdeI EcoRI XbaI AccI SmaI KpnI

TAA TAA TAA TAA TAA TAA TAA TAA

54 codons TGA
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Figure 15. Western blot using anti-$\gamma$ serum to detect IPNV polypeptides expressed by pUC19/A(+), pUC19/A+S, and pUC19A+S deletion plasmids. A duplicate of the Western blots shown in Figure 13 was assayed with anti-$\gamma$ serum. Lane 1 shows the detection of the virion $\gamma_1$ and $\gamma_{1a}$ proteins with this serum. The migration of molecular weight marker proteins are shown at the right. Below each lane is the name of the respective plasmid. The bars at the bottom are representations of the A Segment cDNA region contained by each plasmid.
These polypeptides reacted with both the anti-IPNV sera and the anti-γ sera. The polypeptide comigrating with γ₁ appears to be a bacterially expressed γ₁, but the polypeptide comigrating with γ₁a may be either γ₁a or γ₂ since these proteins migrate near each other and the antisera reacts with both (Huang et al., 1986).

The culture containing the plasmid deleted to the Smal site, pUC19/A+SΔS, produced a polypeptide that migrated slightly faster than γ₁ (approximately 30 kD) and was recognized by the anti-γ serum (Figure 15, Lane 5). Since approximately 470 bp of the γ₁ protein coding region were deleted in this plasmid, this polypeptide must contain primarily γ₂ peptide sequences.

The pUC19/A+S culture also contains two polypeptides of approximately 50 and 55 kD that were detected weakly with both the anti-IPNV and the anti-β sera (Lane 3, Figures 13 and 14, respectively). The presence of these polypeptides was unaffected by the 3′ deletions (Lanes 5-10, figures 13 and 14) and thus, they appear to be β polypeptides that terminate prior to the EcoRI site.

The pUC19/A+SΔA culture (Lane 6) contained a polypeptide of approximately 83 kD that reacted with all three antisera. This polypeptide was significantly larger than any of the virion proteins encoded by the A Segment and the reaction of both the anti-β and anti-γ sera with this polypeptide strongly suggested that it was a bacterially expressed truncated precursor polyprotein (t-pp). This peptide will subsequently be referred to as t-pp(Accl). The production
of a truncated polyprotein by pUC19/A+SΔA but not by pUC19/A+SΔS suggested that deletion of the region between the Smal and Accl sites had resulted in the loss of the viral protease activity responsible for processing the polyprotein.

The plasmids pUC19/A+SΔXb, pUC19/A+SΔXo and pUC19/A+SΔK produced polypeptides that, along with t-pp(Accl), formed a series with sequential decreases in molecular weight corresponding to the decreasing region of the cDNA that remained in each plasmid. The plasmid pUC19/A+SΔXb produced a polypeptide of approximately 72 kD which was recognized by all three of the antisera. The plasmid pUC19/A+SΔXo produced a polypeptide of approximately 66.5 kD which was detected weakly with the anti-IPNV and anti-β sera but not with the anti-γ serum. The plasmid pUC19/A+SΔK produced a polypeptide of approximately 62 kD which also reacted with only the anti-IPNV and anti-β sera. Since pUC19/A+SΔXo produced a polypeptide slightly larger (66.5 kD) than β₁ (63 kD) and pUC19/A+SΔK produced a polypeptide slightly smaller (62 kD) than β₁, it would appear that the end of the β coding must lie between the KpnI and Xhol restriction sites.

The pUC19/A+SΔE culture contained a polypeptide of approximately 65 kD that produced a strong signal with the anti-IPNV and anti-β sera but was not recognized by the anti-γ serum. This polypeptide was larger than the one produced by pUC19/A+SΔK although pUC19/A+SΔE contained less of the cDNA coding region. The larger size of this polypeptide can be accounted for by the 54 additional codons
provided by out of phase LacZ gene sequences (Figure 12).

**Construction of plasmids with 5' deletions to the A Segment cDNA.**

A series of plasmids with 5' deletions to the A segment cDNA were constructed from pUC19/A(-) to determine what viral sequences were required for expression of the 38 kD and \( \gamma_1 \) polypeptides (Figure 11). The deletion plasmids were constructed by a strategy similar to that described above for deletion of pUC19/A+S. In this case, 5' deletions were made to the EcoRI, KpnI, XbaI and Accl sites. The resulting plasmids were designated pUC19/A(-)ΔE pUC19/A(-)ΔK, pUC19/A(-)ΔXb and pUC19/A(-)ΔA, respectively. A circular restriction map of pUC19/A+ is shown in Figure 16 along with a bar representation of the cDNA coding region retained by each of the plasmids.

**Western blot analysis of virus-specific polypeptides expressed by cultures containing pUC19/A(-) and its derivatives.**

Cultures of JM107 cells containing each of the plasmids were grown to late log phase, the cell concentrations were equalized and the cells were harvested. The proteins from each culture were analysed by SDS-PAGE and Western blot analysis with anti-IPNV (Figure 17), anti-\( \gamma \) (Figure 18) and anti-\( \beta \) sera. None of the polypeptides expressed by any of these cultures were detected by the anti-\( \beta \) serum (data not shown).

The pUC19/A(-) culture (Lane 3 in Figures 17 and 18) produced a polypeptide corresponding to \( \gamma_1 \) in molecular weight and a polypeptide
Figure 16. Restriction maps of the plasmid pUC19/A(-) and deleted plasmids derived from it. A representation of the restriction sites of the plasmid pUC19/A(-) is shown on a circular map. The location of restriction sites that are unique within the Segment A cDNA (heavy line) or occur once in the cDNA and once in the multiple cloning site of the vector are indicated. The positive sense direction of the cDNA large open reading frame (large arrow) is oriented opposite to the LacZ gene and its promoter (small arrow). The plasmid pUC19/A(-) was used for construction of plasmids with sequential deletions to the 5' end of the cDNA as described in Materials and Methods. Deletions were made to the 5' EcoRI, Kpnl, Xbal and Accl sites. The resulting plasmids were designated pUC19/A(-)ΔE, pUC19/A(-)ΔK, pUC19/A(-)ΔXb and pUC19/A(-)ΔA and retained approximately 1560, 1435, 1195 and 900 base pairs of the 3' end of the cDNA, respectively. Below the circular plasmid map, bars are shown that indicate the regions of the cDNA which remain in each of the deleted plasmids.
Figure 16

pUC19/A(-) 5.59 kb

ATG-1 Ndel EcoRI XhoI XbaI Accl Smal TAA

pUC19/A(-)  pUC19/A(-)ΔE  pUC19/A(-)ΔK  pUC19/A(-)ΔXb  pUC19/A(-)ΔA

Lac _p_
Figure 17. Western blot using anti-IPNV serum to detect IPNV polypeptides expressed by pUC19/A(-) and deletion plasmids derived from it. Proteins from *E. coli* JM107 cultures containing each of the plasmids were separated by SDS-PAGE and analyzed by Western blot with anti-IPNV serum. Lane 1 contains purified virion proteins. The migration of molecular weight marker proteins are shown at the right. Below each lane is the name of the respective plasmid. The bars at the bottom are representations of the A Segment cDNA region contained by each plasmid.
Figure 17

- Proteins: α, β, γ₁, γ₁α

- Enzymes: KpnI, NdeI, EcoRI, XhoI, XbaI, AccI, SmaI

- Genes: pUC19/A(-), pUC19/A(-)ΔE, pUC19/A(-)ΔK, pUC19/A(-)ΔXb, pUC19/A(-)ΔA
Figure 18. Western blot using anti-\(\gamma\) serum to detect IPNV proteins expressed by pUC19/A(-) and deletion plasmids derived from it. A duplicate of the Western blot shown in Figure 17 was assayed with anti-\(\gamma\) serum. Lane 1 contains purified virion proteins. The migration of molecular weight marker proteins are shown at the right. Below each lane is the name of the respective plasmid. The bars at the bottom are representations of the A Segment cDNA region contained by each plasmid.
of 38 kD as described earlier (Figure 11). This preparation, however, also contained an additional virus-specific peptide of approximately 25 kD. All four of these polypeptides reacted with both the anti-IPNV and anti-γ sera.

The cultures containing the plasmids pUC19/A(-)ΔE, pUC19/A(-)ΔK and pUC19/A(-)ΔXb (Lanes 4, 5 and 6) each produced only the 38 kD polypeptide which reacted with both the anti-IPNV and anti-γ sera.

The plasmid pUC19/A(-)ΔA (Lane 7) which retained only about 900 bp of the 3' end of the cDNA failed to produce any immunoreactive polypeptides.

Production and analysis of antisera raised to the 83 kD virus-specific polypeptide, t-pp(Accl).

Purification of t-pp(Accl) and production of anti-t-pp(Accl) serum.

The production of a stable truncated precursor polyprotein, t-pp(Accl) by pUC19/A+SΔA provided the opportunity to produce an antiserum that would react with γ2 (and β) but not γ1 polypeptides.

Analysis of soluble and insoluble fractions from induced cultures of JM107 cells containing pUC19/A+SΔA revealed that a substantial portion of t-pp(Accl) remained in the insoluble fraction of the lysed cells. The polypeptide was purified from the insoluble fraction by two cycles of separation on SDS-PAGE, excision from the gel and electroelution. Figure 19 shows a silver stained SDS-PAGE gel containing a sample of the final eluant that was used to immunize a
Figure 19. Silverstained SDS-PAGE of the purified t-pp(Accl) polypeptide. The 83 kD virus-specific polypeptide, t-pp(Accl) expressed by pUC19/A+SΔA (Figure 10, Lane 6) was partially purified as described in Materials and Methods. Lane 1 shows a sample of the purified t-pp(Accl) that was used to immunize a rabbit. Lanes 2 and 3 contain IPNV virion proteins and molecular weight markers, respectively.
rabbit. The resulting antiserum was designated anti-t-pp(Accl).

**Western blot analysis comparing the specificities of anti-t-pp(Accl) anti-β, anti-α and anti-IPNV sera.**

Four duplicate Western blots were prepared, each containing electrophoretically separated lanes of virion, infected cell and top component proteins. Top component proteins are a by-product of IPNV purification and reportedly contain only β virion proteins (Stephens and Hetrick, 1983). Each of the antisera was reacted with one of the Western blots. The resulting patterns of reactivity are shown in Figure 20.

The anti-t-pp(Accl) serum recognized the γ2 and γ2a of the infected cell proteins (ICP) (Lane 3) and failed to recognize the γ1 and γ1a proteins in the virion (Lane 2). This result indicated that the anti-t-pp(Accl) serum could distinguish between the γ proteins immunologically. The β3 protein (56.5 kD) was the only virion protein recognized by this antiserum (Lane 2), whereas β1 (63 kD), β2 (61 kD), γ2 (28 kD), and γ2a (25 kD) were recognized in the ICP lane (Lane 3). Two additional bands were detected as well at 38 and 45 kD. In Lane 4, which contained the proteins from the top component preparation, two high molecular weight dimers of the β proteins (approximately 120-135 kD) were observed.

The anti-γ serum recognized the γ1 and γ1a in Lane 6 of the
Figure 20. Western blot analysis of virion, infected cell and top component proteins with anti-t-pp(Accl), anti-γ, anti-β and anti-IPNV sera. Four duplicate Western blots containing separated IPNV virion (IPNV), infected cell proteins (ICP) and top component proteins (TC) were reacted with either anti-t-pp(Accl), anti-γ, anti-β or anti-IPNV sera. Lanes 2-4 show the pattern of reactivity produced by anti-t-pp(Accl); Lanes 5-8, anti-γ serum; Lanes 9-12, anti-β serum; and Lanes 13-15, anti-IPNV serum.
Figure 20
virion proteins. This serum also detected a faint band corresponding to \( \gamma_{2a} \). Three proteins in the \( \gamma \) region of the infected cell proteins (Lane 7) were detected by the anti-\( \gamma \) serum. A broad band of \( \gamma_1 \) was detected; the two bands migrating below \( \gamma_1 \) were \( \gamma_2 \) and \( \gamma_{2a} \). Proteins migrating at 38 kD and 45 kD were also detected very weakly by the antiserum. There was no reaction with the \( \beta \) proteins in the top component lane (Lane 8).

The anti-\( \beta \) serum detected \( \beta_3 \) in each protein preparation (Lanes 10,11 and 12). In Lanes 11 (ICP) and 12 (top component) strongly reactive bands were detected at 70 kD. These bands were presumably protein contaminants present in the purified virus preparation used in generating the antiserum (Lane A, Figure 7). The 45 kD protein in the infected cell proteins was also detected by the anti-\( \beta \) serum.

The reaction of the anti-IPNV serum with virion proteins (Lane 14) was similar to that described previously. In the ICP lane (Lane 15), \( \alpha, \beta_1, \beta_2, \beta_3, 45 \text{ kD}, \gamma_1, \gamma_{1a}/\gamma_2 \) and \( \gamma_{2a} \) proteins were detected. The reaction of the anti-IPNV serum with top component was not determined.

The identification of the 45 and 38 kD proteins was tentatively addressed in these studies based upon the reactivity of the proteins with each respective antisera. Both proteins were recognized by the anti-t-pp(Accl) serum. The 45 kD protein was recognized by the anti-\( \beta \) and anti-IPNV sera but only weakly by the anti-\( \gamma \) serum. The 38
kD protein was recognized by the anti-γ but not the anti-β serum. Thus it appears that the anti-t-pp(Accl) serum recognized the 45 kD protein because it contained β peptide sequences and recognized the 38 kD protein because it contained γ₂ peptide sequences. These results paralleled those obtained in Figure 17 where a 38 kD protein was detected in lysates of bacteria containing the plasmids pUC19/A(-)ΔE, pUC19/A(-)ΔK and pUC19/A(-)ΔXb. These plasmids contained only γ-specific sequences. While a virus-specific polypeptide of approximately 38 kD has been demonstrated in bacterial expression (Figure 11) and in vitro translation systems (Figure 23; Huang et al., 1986), the immunological detection of the 38 kD protein in the infected cell proteins provided the first evidence that it was a viral protein produced during virus replication in the cell.

Deletion analysis of the Segment A coding regions by in vitro transcription and translation.

Transcription vectors: construction and use.

Plasmid transcription vectors were used to create positive sense RNA transcripts of various regions of the A Segment. The transcription vectors contained bacteriophage promoters (T7 or SP6) upstream from the large open reading frame. Bacteriophage RNA polymerases were used for in vitro transcription of RNAs from the plasmid DNAs which had been linearized with restriction enzymes. The transcripts began at the bacteriophage promoter and ended at the site at which the DNA had been linearized with the restriction enzyme. The
Figure 21. Production of 3' deleted A Segment RNA transcripts from transcription vectors pT72/A and pT72/AΔA.

The plasmid pT72/A contains the large open reading frame of the A Segment cDNA (heavy line) down stream from the bacteriophage T7 promoter. This plasmid was used as template for in vitro transcription of positive sense A Segment RNA transcripts with the T7 RNA polymerase (Huang et al., 1986). The plasmid pT72/A was digested with either BamHI or Smal and then used as template for run off transcription. The BamHI digested plasmid yielded full length transcripts. The Smal digested plasmid yielded transcripts truncated by approximately 500 bases at the 3' end. The first two bars at the bottom of the figure illustrate the coding regions retained by the transcripts. The transcription vector pT72/AΔA was derived from pT72/A by digestion with Accl, isolation of the plasmid by agarose gel electrophoresis, and religation, in-gel. The plasmid pT72/AΔA was digested with Accl, Xbal, Xhol or EcoRI and then used as template for run-off transcription. The resulting transcripts were deleted at the 3' end by approximately 1000, 1200, 1400, or 1560 bases, respectively. The coding regions retained by each of the transcripts are illustrated by the lower four bars at the bottom of the figure.
Figure 21

**pT72/A**

5.7kb

- **pT72/AΔA**

4.7kb

**ATG-1**

NdeI EcoRI Xhol XbaI

- **3' Smal**
- **3' Accl**
- **3' XbaI**
- **3' Xhol**
- **3' EcoRI**

**Accl, gel isolate, T4 ligase**

**Figure 21**

ATG-1

NdeI EcoRI Xhol XbaI

- **3' Smal**
- **3' Accl**
- **3' XbaI**
- **3' Xhol**
- **3' EcoRI**

**Full length**

- **TAA**
Figure 22. Production of 3' and 3'/5' deleted A Segment RNA transcripts from transcription vectors pGEM/A(-)ΔE, pGEM/A(-)ΔXb, and pGEM/A(-)ΔA. The plasmids pGEM/A(-)ΔE, pGEM/A(-)ΔXb, and pGEM/A(-)ΔA were constructed by digestion of pGEM/A(-) with EcoRI, Xbal, or Accl, isolation of the plasmids by agarose gel electrophoresis, and then religation. The plasmid pGEM/A(-)ΔE was linearized with HindIII, SmaI or Accl and used as templates for run-off transcription of deleted A Segment RNA transcripts. The HindIII digested plasmid yielded RNA transcripts beginning at the EcoRI site and extending to the end of the coding region. The SmaI and Accl digested plasmids yield transcripts which began at the EcoRI site and ended at each of the respective restriction sites and thus, were deleted at both 5' and 3' ends. The plasmids pGEM/A(-)ΔXb and pGEM/A(-)ΔA were linearized with HindIII and used as template for transcription of RNAs beginning at the Xbal and Accl sites, respectively, and extending to the end of the coding region. Bars at the bottom of the figure illustrate the coding regions contained by each of the transcripts.
Figure 22

Diagram of restriction enzyme digestion sites and cloning strategies for pGem plasmids.

- pGem/A(-)
  - 5.65kb
  - Cloning sites: + sense
  - Restriction sites: XbaI, KpnI, EcoRI
  - Gel isolation; T<sub>4</sub> ligase

- pGem/A(-)ΔA
  - 3.75kb
  - Cloning sites: + sense
  - Restriction sites: XbaI, KpnI, EcoRI
  - Gel isolation; T<sub>4</sub> ligase

- pGem/A(-)ΔE
  - 4.3kb
  - Cloning sites: + sense
  - Restriction sites: XbaI, KpnI, EcoRI
  - Gel isolation; T<sub>4</sub> ligase

- pGem/A(-)ΔXb
  - 3.9kb
  - Cloning sites: + sense
  - Restriction sites: XbaI, KpnI, EcoRI
  - Gel isolation; T<sub>4</sub> ligase
resulting RNA transcripts functioned as mRNA and although, they were not capped or polyadenylated, they could be translated in vitro.

Five plasmids were used as templates for the transcription of RNAs from the A Segment cDNA. These plasmids and the RNA transcripts produced from them are described in Figures 21 and 22.

The plasmid pT72/A was linearized at the BamHI site to produce full length transcripts and linearized at the Smal site to produce transcripts deleted by approximately 500 bases at the 3' end. The deleted RNA transcripts are referred to by the end of the A Segment coding region that had been deleted and the restriction site that was used to make that deletion. For example, the transcripts from the Smal linearized pT72/A are referred to as 3' Smal deleted transcripts.

The plasmid pT72/AΔA was used to produce 3' Accl, 3' XbaI, 3' XhoI and 3' EcoRI deleted RNA transcripts. The plasmid pGEM/A(-)ΔE was used to produce 5' EcoRI, 5' EcoRI/3' Smal and 5'EcoRI/3' Accl deleted RNA transcripts. The plasmids pGEM/A(-)ΔA and pGEM/A(-)ΔXb were used to produce the 5' Accl and 5' XbaI transcripts.

**In vitro translation of A Segment RNA transcripts.**

The RNA transcripts containing various portions of the Segment A coding region were used to prime translation of [35S]-methionine labeled polypeptides in a rabbit reticulocyte lysate system. The products of these translations were analyzed by SDS-PAGE and autoradiography (Figure 23). To confirm the identity of the
translation products, samples were immunoprecipitated with anti-IPNV, anti-β, anti-γ and anti-t-pp(Accl) sera and the precipitates analyzed by SDS-PAGE and autoradiography (Figures 24, 25, and 26, respectively).

The translation products of the full length RNA transcripts included polypeptides comigrating with β₁, γ₁ and γ₁a/γ₂. Polypeptides of approximately 15, 20 and 38 kD were also produced. All of these polypeptides were precipitated by anti-IPNV serum and were, therefore, virus-specific. The polypeptide migrating at 63 kD was identified as β₁ by immunoprecipitation with the anti-IPNV, anti-β (Figure 25, Lane 2) and anti-t-pp(Accl) sera but not by the anti-γ serum. The 38 kD polypeptide and the polypeptide comigrating with γ₁ appeared in all of the immunoprecipitations of translation products of the full length transcripts. This was curious because the 38 kD protein was shown previously to be γ-specific and should not have been detected by the anti-β serum. It may be that these polypeptides were precipitated nonspecifically or were difficult to wash from the precipitates. The polypeptide band comigrating with γ₁a/γ₂ at approximately 28.5 kD was efficiently precipitated by the anti-t-pp(Accl) serum and thus, the presence of γ₂ in this band was confirmed. The 15 and 20 kD polypeptides were only discernible in the anti-IPNV immunoprecipitation. Polypeptides of the same molecular weight were produced by the 5' Accl deleted RNA transcript which retained only the final 900 bp of of the large open reading frame.
Since this region had been shown to code for the $\gamma_1$ protein (Huang et al. 1986), the 15 and 20 kD proteins were probably truncated $\gamma_1$ peptides.

Translation of the 3' Smal deleted RNA transcripts resulted in polypeptides corresponding to $\beta_1$ and $\gamma_{1a}/\gamma_2$ in molecular weight. Both of these polypeptides were immunoprecipitated with anti-IPNV serum. The polypeptide migrating with at 63 kD was identified as $\beta_1$ by its reaction with anti-$\beta$ and anti-t-pp(Accl) sera but not the anti-$\gamma$ serum. The polypeptide band comigrating with $\gamma_{1a}/\gamma_2$ was identified as $\gamma_2$ by its reaction with anti-t-pp(Accl).

A polypeptide of approximately 83 kD was the only translation product of 3' Accl deleted RNA transcripts. This polypeptide was precipitated by anti-IPNV, anti-$\beta$, anti-$\gamma$ and anti-t-pp(Accl) and was analogous to the bacterially expressed t-pp(Accl). The 3' Xbal, 3' Xhol and 3' EcoRI deleted RNA transcripts each produced a single polypeptide of 72, 66.5 and 57 KD, respectively. Each of these polypeptides was precipitated by the anti-IPNV, anti-$\beta$ and anti-t-pp(Accl) sera and represented a truncated polyprotein.

The translation products of the 5' EcoRI deleted RNA transcripts were identical to those produced by the full length RNA except that $\beta_1$ was not produced. Translation of the 5' EcoRI/3' Smal RNA transcript resulted in production of a single peptide comigrating with $\gamma_{1a}/\gamma_2$. This polypeptide was identified as $\gamma_2$ by immunoprecipitation with anti-IPNV and anti-t-pp(Accl) sera. The 5'
EcoRI/ 3' Accl deleted RNA produced polypeptides detected weakly at approximately 20 and 24 kD. These polypeptides were precipitated by anti-IPNV, anti-γ and anti-t-pp(Accl).

The 5' XbaI deleted RNA transcripts produced a polypeptide comigrating with γ1 and polypeptides of 41, 30, 29, 25 and 20 kD. All of these polypeptides were precipitated by anti-IPNV sera. The 38 kD and γ1 polypeptides had precipitation patterns similar to those of the corresponding translation products of the 5' EcoRI deleted RNA transcripts. The 29 kD protein was recognized by the anti-γ serum, but not the anti-β and anti-t-pp(Accl) sera. It was, therefore, a γ1a or a truncated γ1 polypeptide. The 41 kD polypeptide did not appear in any of the precipitations with anti-γ, anti-β or anti-t-pp(Accl), and thus, it was not possible to establish its identity by immunoreactivity. However, virtually all of the coding region of the 5' XbaI deleted RNA (approximately 1200 bases) would be required for a 41 kD protein (assuming 115 Daltons/amino acid). The 41 kD protein must then contain both γ1 and γ2 peptide sequences.

Translation of 5' Accl deleted RNA resulted in a very strong signal corresponding to the γ1 protein. The 20 and 25 kD polypeptides were also present along with a series of polypeptides which were slightly larger than γ1, the largest being approximately 34 kD. All of the polypeptides were recognized by the anti-IPNV serum. The polypeptide comigrating with γ1 was precipitated efficiently by the
Figure 23. *In vitro* translation products of deleted A Segment RNA transcripts. RNA transcripts containing various portions of the A Segment large ORF were translated in a rabbit reticulocyte lysate system. The $^{35}$S-methionine labeled translation products were analyzed by SDS-PAGE and autoradiography. Lanes 1 and 2 contain infected cell proteins (ICP) and virion proteins (IPNV), respectively. Lane 3 contains endogenous translation products that are produced when no RNA is added to the translation system. Lanes 4-14 contain the *in vitro* translation products. Below each lane is the name of RNA transcript that was used in the translation. The names are based upon the region that has been deleted. The coding region retained by each of the transcripts is shown as a bar at the bottom of the figure.
**Figure 23**

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</tr>
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<td>4</td>
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</tr>
<tr>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>10</td>
<td>5' EcoRI/3'Accl</td>
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<td>11</td>
<td>5' Xbal</td>
</tr>
<tr>
<td>12</td>
<td>5' Accl</td>
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**ATG-1**

<table>
<thead>
<tr>
<th>Restriction Enzymes</th>
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<tbody>
<tr>
<td>NdeI</td>
</tr>
</tbody>
</table>

**Gene Variants**

- **Full length**
- **3' Smal**
- **3' Accl**
- **3' Xbal**
- **3' XhoI**
- **3' EcoRI**
- **5' EcoRI**
- **5' EcoRI/3'Smal**
- **5' EcoRI/3'Accl**
- **5' Xbal**
- **5' Accl**
Figure 24. Radio-immune precipitation analysis (RIPA) of \textit{in vitro} translation products with anti-IPNV serum. The translation products of the deleted A Segment RNA transcripts (Figure 23) were immunoprecipitated with the anti-IPNV serum. Lanes 1 and 2 contain infected cell proteins (ICP) and virion proteins (IPNV) which were not immunoprecipitated. Lanes 3-13 contain the immunoprecipitated \textit{in vitro} translation products. Below each lane is the name of RNA transcript that was used in the translation. The names are based upon the region that has been deleted. The coding region retained by each of the transcripts is shown as a bar at the bottom of the figure. Lane 14 contains immunoprecipitated infected cell proteins (ICP).
Figure 24: anti-IPNV

ATG-1

Full length
3' Smal
3' AccI
3' XbaI
3' XhoI
3' EcoRI
5' EcoRI
5' EcoRI/3'Smal
5' EcoRI/3'AccI
5' XbaI
5' AccI

ICP IPNV
Full length 3' Smal
3' AccI 3' XbaI
3' XhoI 5' EcoRI
5' EcoRI/3'Smal
5' EcoRI/3'AccI
5' XbaI 5' AccI
Immunop. ICP

119
Figure 25. RIPA of *in vitro* translation products with anti-β serum. The translation products of the deleted A Segment RNA transcripts (Figure 23) were immunoprecipitated with the anti-β serum. Lanes 1 and 2 contain infected cell proteins (ICP) and virion proteins (IPNV) which were not immunoprecipitated. Lanes 3-13 contain the immunoprecipitated *in vitro* translation products. Below each lane is the name of RNA transcript that was used in the translation. The names are based upon the region that has been deleted. The coding region retained by each of the transcripts is shown as a bar at the bottom of the figure. Lane 14 contains immunoprecipitated infected cell proteins (ICP).
Figure 25: anti-β

Full length

3' Smal

3' Accl

3' Xbal

3' Xhol

3' EcoRI

5' EcoRI

5' EcoRI/3'Smal

5' EcoRI/3'Accl

5' Xbal

5' Accl

ATG-1

KpnI

Ndel

EcoRI

Xhol

Xbal

Accl

Smal

TAA
Figure 26. RIPA of *in vitro* translation products with anti-γ serum. The translation products of the deleted A Segment RNA transcripts (Figure 23) were immunoprecipitated with the anti-γ serum. Lanes 1 and 2 contain infected cell proteins (ICP) and virion proteins (IPNV) which were not immunoprecipitated. Lanes 3-13 contain the immunoprecipitated *in vitro* translation products. Below each lane is the name of RNA transcript that was used in the translation. The names are based upon the region that has been deleted. The coding region retained by each of the transcripts is shown as a bar at the bottom of the figure. Lane 14 contains immunoprecipitated infected cell proteins (ICP).
### Figure 26: anti-γ

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<td>5' EcoRI/3' AccI</td>
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<td>11</td>
<td>5' AccI</td>
</tr>
<tr>
<td>12</td>
<td>Immunop. ICP</td>
</tr>
</tbody>
</table>

**Enzymes Used:**
- ATG-1
- Ndel
- EcoRI
- KpnI
- XhoI
- Xbal
- AccI
- Smal
- TAA

**Restriction Sites:**
- 110 kDa
- 97 kDa
- 35 kDa
- 20 kDa

**Markers:**
- ICP
- IPNV

**Legend:**
- γ
- β
- α
Figure 27. RIPA of *in vitro* translation products with anti-t-pp(Accl) serum. The translation products of the deleted A Segment RNA transcripts (Figure 23) were immunoprecipitated with the anti-t-pp(Accl) serum. Lanes 1 and 2 contain infected cell proteins (ICP) and virion proteins (IPNV) which were not immunoprecipitated. Lanes 3-13 contain the immunoprecipitated *in vitro* translation products. Below each lane is the name of RNA transcript that was used in the translation. The names are based upon the region that has been deleted. The coding region retained by each of the transcripts is shown as a bar at the bottom of the figure. Lane 14 contains immunoprecipitated infected cell proteins (ICP).
Figure 27: anti-t-pp(Accl)

ATG-1

Full length
3' Smal
3' Accl
3' Xbal
3' Xhol
3' EcoRI
5' EcoRI
5' EcoRI/3'Smal
5' EcoRI/3'Accl
5' Xbal
5' Accl

ICP
IPNV

1 2 3 4 5 6 7 8 9 10 11 12 13 14

110 97 35 20

KpnI
Ndel
EcoR1
Xhol
Xbal
Accl
Smal
TAA
anti-γ serum but not by the anti-β or anti-t-pp(Accl) sera. None of the other polypeptides could be identified with either anti-γ, anti-β, or anti-t-pp(Accl) serum.

**Cotranslation of RNA transcripts encoding all or portions of γ₂ polypeptide with the RNA transcripts encoding t-pp(Accl).**

To test whether a trans-acting protease could be associated with all or parts of γ₂, the set of RNAs containing portions of the 3' half of the cDNA were cotranslated with RNA transcripts used to produce t-pp(Accl)(Figure 24). The polypeptide t-pp(Accl) should contain the junction between β and γ₂ and thus be a substrate for protease activity. In each cotranslation reaction t-pp(Accl) was produced along with the products from the 3' RNAs but there was no indication that t-pp(Accl) was cleaved at the β-γ₂ junction to produce smaller polypeptides (β and a truncated γ₂).

**Translation of A Segment RNA transcripts in the presence of specific immunoglobulins.**

The full length A Segment RNA transcripts, which produced all of the A Segment polypeptides (Figure 19), were translated in the presence of purified immunoglobulins made from each of the antisera, anti-IPNV<sub>SP</sub>, anti-β, anti-γ and anti-t-pp(Accl). This was done to determine whether specific antibody could arrest processing of the polyprotein. Figure 23 shows that in each case the protein products
Figure 28. Test for \textit{trans}-activity of the viral protease by cotranslation of the 3' Accl deleted RNA, encoding t-pp(Accl), with other deleted RNAs encoding $\gamma_2$ peptides. The t-pp(Accl) polypeptide has been shown to contain $\beta$ and $\gamma_2$ regions (Figures 20, 25 and 27) and thus, should contain the junction were the polyprotein is cleaved to form these proteins. RNA transcripts shown to yield translation products corresponding to complete or truncated $\gamma_2$ polypeptides were cotranslated with equal quantities of the 5' Accl deleted RNA transcript to see if any of the resulting $\gamma_2$ polypeptides were capable of cleaving the $\beta$-$\gamma_2$ junction of t-pp(Accl) in \textit{trans}. Bars showing the coding regions retained by each of RNA transcripts are at the bottom of the figure.
Figure 29. **Translation of full length RNA transcripts in the presence of purified immunoglobulins to test for the arrest of processing.** Purified immunoglobulin from anti-IPNV, anti-β, anti-γ, anti-t-pp(Accl) or normal sera was added to translation reactions to determine whether any of the antibodies were able to arrest the processing of the polyprotein. Lane 4 shows the translation products produced in the absence of immunoglobulin and Lane 9 shows the translation products produced in the presence of immunoglobulin purified from normal serum. These control lanes contained the same polypeptide pattern as the lanes containing the translation products obtained in the presence of the different antisera for viral proteins (Lanes 5, 6, 7 and 8).
were identical to those produced in the absence of immunoglobulin and thus, under these conditions, specific purified antibody did not arrest polyprotein processing.

Expression of viral antigens for use as a subunit vaccine.

Construction of the plasmid pTA1 and analysis of expression products.

The construction of the expression vector, pTA1, is described in Figure 6. The plasmid pTA1 contains the Segment A cDNA inserted into the \textit{TrpE} gene of the pATH2 vector. Five base pairs of the 5' end of the cDNA have been deleted, allowing the in frame fusion of the \textit{TrpE} gene with the the A Segment cDNA (Figure 30, B).

A colony containing the plasmid pTA1 produced a very strong signal when reacted with anti-IPNV serum in a colony blot immunoassay (Figure 30, C). To determine which viral polypeptides were responsible for this immunoreactivity, proteins from bacterial cultures containing pTA1 were separated by SDS-PAGE, transferred to nitrocellulose and then reacted with anti-\(\beta\), anti-\(\gamma\), or anti-t-pp(Accl) sera. The anti-\(\beta\) sera reacted with a series of bands ranging in molecular weight from approximately 40 to 90 kD. The anti-\(\gamma\) and anti-t-pp(Accl) sera each reacted with at least two low molecular weight bands of between 20 and 35 kD. Thus the \(\beta\) and \(\gamma\) proteins of IPNV were expressed in bacteria containing pTA1.
Figure 30. The pTA1 plasmid: structure and screening for expression. A circular restriction map of pTA1 is shown (A). Details of the construction of this plasmid are described in Figure 6. Briefly, the A Segment cDNA (heavy line) was digested with BAL 31 nuclease to remove sequences 5' to the initiation codon of the large open reading frame in order to generate open reading frame fusions with the trpE gene upon insertion into the pATH2 expression vector (light line). The transformants resulting from this construction were screened immunologically for the expression of viral proteins by colony blot assay with the anti-IPNV serum. A single strongly positive colony resulted (C). The plasmid from this colony was designated pTA1 and the construction was verified by restriction enzyme analysis and plasmid sequencing of the junction between the TrpE gene and the 5' end of the A Segment cDNA (B). Sequencing of the gene junction revealed that only 5 bp of the cDNA (bold letters) had been deleted but that a continuous open reading frame was formed between the TrpE gene (plain letters) and the large open reading frame of the A Segment cDNA. Seven codons (21 bp) of cDNA sequence upstream from the large open reading frame contribute to the continuous reading frame of the gene fusion. The sequence of the gene junction also revealed that the SmaI site of the vector had been restored by fusion to three guanidine residues from the cDNA.
Figure 30

A

B

C

colony blot immunoassay
Figure 31. Western blots of pTA1 expressed polypeptides reacted with anti-β, anti-γ, and anti-t-pp(Accl) sera. Bacterial cultures containing the pTA1 plasmid or the control plasmid, pATH2, were grown to mid-log phase, the TrpE promoter was induced with indoleacrylic acid and the incubation was continued overnight. The cells were harvested and the proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Purified IPNV virion proteins were also separated by SDS-PAGE and transferred to nitrocellulose. The electrophoresis conditions differed slightly for the virion proteins so the migrations of these proteins are not directly comparable to those of the bacterial proteins. (The migrations of the viral proteins expressed by pTA1 cultures is shown in relation to virion proteins and molecular weight standards in Figure 32.) Membrane strips containing each of the protein samples were incubated with each of the antisera. The anti-β serum reacted with a number of protein bands in the pTA1 culture. The anti-γ and anti-t-pp(Accl) each identify two low molecular weight protein bands.
Vaccine trial with bacterially expressed viral antigens.

Lysates of induced cultures of pTA1, pUC19/A+S and pUC19/A+SΔA were used in a preliminary study of the efficacy of bacterially expressed viral polypeptides as a vaccine for IPNV. Lysate samples of 1, 5 and 25 μl were separated by SDS-PAGE and analyzed by Western blot with anti-IPNV serum (Figure 32). The pUC19/A+S culture produced polypeptides corresponding with γ₁ and γ₁a. The pUC19/A+SΔA culture produced the t-pp(Accl) polypeptide. In the lane containing the 1 μl sample of the pTA1 lysate, virus-specific polypeptides comigrating with β₁, γ₁ and γ₁a were present. Additional peptides of approximately 75, 81, 87 and 93 KD were observed. The quantity of antigen present in the 5 and 25 μl samples was so great that individual bands were difficult to distinguish.

Each of the bacterial lysates was used for vaccination of 25 rainbow trout fry by immersion. Twenty days after the vaccination, the fry were challenged with $10^{-6}$ plaque forming units/ml of the Buhl Strain of IPNV. Moribund fish were collected on a daily basis for 54 days. The presence of virus in all of these fish was confirmed by the production of characteristic cytopathic effect in tissue culture. Figure 33 shows a plot of the cumulative mortality in each group versus the number of days after the challenge. In the mock (pUC19) treated group, the total cumulative mortality reached 44 % by the end of the challenge period. The final cumulative mortality of the pTA1 treated group was only 4%. The pUC19/A+S and pUC19/A+SΔA treated groups
had final cumulative mortalities of 36 and 20%, respectively. Although these results are preliminary, it appeared that vaccination with the bacterial expressed viral proteins and those of pTA1 in particular provided substantial protection from viral challenge.
Figure 32. Western blot analysis of the bacterially expressed viral antigens used for vaccination trial. The production of bacterial lysates used for vaccine trials is described in Materials and Methods. Briefly, cultures containing each of the plasmids pUC19, pUC19/A+S, pUC19/A+SΔA and pTA1 were grown to mid-log phase and then induced with either IAA for pTA1 or with IPTG for the other plasmids. Incubation was continued overnight and the cells were harvested and sonicated. Samples of 1, 5 and 25 µl of each of the sonicated cell lysates were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose and then analyzed with anti-IPNV serum. The pUC19 lanes show the background produced by bacterial proteins. In the pUC19/A+S lanes, the antisera identified two bands comigrating with γ1 and γ1a. There was also a weak signal from a band corresponding to β1. In the pUC19/A+SΔA lane, the antisera recognized the 83 kD t-pp(Accl) protein. The quantity of viral antigen present in pTA1 lanes was substantially greater than that of the pUC19/A+S and pUC19/A+SΔA lanes. The quantity of viral antigens present in the 5 and 25 µl lanes was so great that it was difficult to distinguish individual bands. In the lane containing the 1 µl sample of pTA1 lysate proteins, individual bands can be seen which correspond in molecular weights to β1 (63.5 kD), γ1 (32 kD) and γ1a (28.5 kD). Polypeptides of 75, 81, 87 and 93 kD were also detected by the antisera.
Figure 33. Preliminary vaccine trial: cumulative percent mortality over time. Each of the bacterial lysates, pUC19, pUC19/A+S, pUC19/A+SΔA and pTA1, were used to vaccinate a group of 25 rainbow trout by immersion. Twenty days after immunization the fish were challenged with $10^{-6}$ plaque forming units of the Buhl strain of IPNV per ml of water. The challenge was continued for 54 days with moribund fish being collected on a daily base. The graph shows the percentage of cumulative mortality at three day intervals.
DISCUSSION

Molecular biology.

One of the major questions in the replication cycle of the birnaviruses is how does the virus provide for the synthesis of the viral proteins $\beta$, $\gamma_1$ and $\gamma_2$, from a polycistronic messenger RNA with a single large open reading frame. Earlier reports by Dobos (1977) had indicated that a polyprotein precursor was not involved in birnavirus viral protein synthesis. Instead an alternative model of independent initiation and termination of translation on a single polycistronic messenger RNA was proposed (Nagy et al., 1987). More recently, the identification of a precursor polyprotein was made for IPNV (Duncan et al., 1987) and IBDV (Azad et al., 1987; Jagadish et al., 1988), and evidence for this precursor is also presented in this thesis. This evidence suggested that the IPNV viral proteins, $\beta$, $\gamma_1$ and $\gamma_2$, are synthesized initially as a polyprotein which is proteolytically cleaved by the $\gamma_2$ gene encoded protease. In addition, evidence is presented in this thesis for the alternative model of protein synthesis, internal initiation of translation.

Described in this thesis are the use of bacterial expression and cell free translation systems to conduct deletion analysis on the IPNV A Segment. The cDNA that was used in both of these systems was cloned from genomic RNA of the SP strain (Huang et al., 1986). The production of a truncated polyprotein, t-pp(Accl), was demonstrated in both systems.
Evidence also indicated that the γ2 protein has regions which are critical to the processing of the polyprotein. Polyvalent antisera were produced that allowed identification of the virus-specific polypeptides expressed in these systems.

Duncan et al. (1987) have used \textit{in vitro} translation to conduct similar deletion analysis studies on the A segment of the Jasper strain of IPNV. In their study the translation products were identified by SDS-PAGE analysis after partial proteolysis with N-chlorosuccinimide. They demonstrated a full length polyprotein as well as a truncated polyprotein analogous to t-pp(Accl). Their results implicated the γ2 protein as the virus-specific protease responsible for polyprotein processing.

Azad et al. (1987) used bacterial expression to conduct deletion analysis on the large (A) genome segment of IBDV. Monoclonal antibodies specific for γ1 (VP3) and β1 (VP2a) were used to confirm the identities of the bacterial expression products. They isolated a truncated polyprotein as a fusion with a bacterial protein and showed that deletions to γ2 (VP4) resulted in a failure to produce processed γ1 (VP3).

In this thesis, comparison of the results obtained for 3' truncations of the A Segment ORF at the Accl and the Smal sites illustrate the case for both the existence of a polyprotein and the assignment of the processing activity to the γ2 protein. Truncation at the Smal site left approximately 2445 bases of the coding region, while truncation at the Accl site left approximately 2019 bases. In both, the \textit{in vitro} translation (Figure 23) and bacterial expression systems (Figure13),
the Smal truncation resulted in the production of processed polypeptides. The Accl truncation, however, resulted in production of the unprocessed truncated polyprotein, t-pp-(Accl). Therefore, not only was the existence of a polyprotein demonstrated, but the sequence located between the Accl site and the Smal site was shown to be critical to the activity of the protease that processes the polyprotein. Although the exact boundary between the \( \gamma_1 \) and \( \gamma_2 \) proteins is not known, it must lie somewhere between 100 and 200 bases 3' of the Accl site (Figure 34). If this is the case, the Smal deletion eliminated more than half of \( \gamma_1 \) without interfering with processing, while the Accl deletion that includes about one fourth of \( \gamma_2 \) resulted in a complete cessation of processing. Therefore, \( \gamma_2 \) was implicated as the protease responsible for the cleavage of the polyprotein to \( \beta_1 \), \( \gamma_1 \) and \( \gamma_2 \).

Although the loss of processing which resulted when 3' deletions were made to the Accl site suggested that \( \gamma_2 \) was the virus specific protease, it was not conclusive proof that it, alone, was responsible for the protease activity. In an attempt to show more definitively which polypeptide or polypeptides were responsible for the processing, the 5' Accl deleted RNA transcripts were cotranslated with transcripts containing various portions the \( \gamma_1 \) and \( \gamma_2 \) coding regions. Among these were transcripts that resulted in complete \( \gamma_2 \) and \( \gamma_1 \) (5' Eco), complete \( \gamma_2 \) but not \( \gamma_1 \) (5' EcoRI/3' Smal) and complete \( \gamma_1 \) but not \( \gamma_2 \) (5' Accl)(Figure 22). In each case t-pp(Accl) and the \( \gamma \) proteins were produced but there
was no indication of protease cleavage of the b- γ2 junction of t-pp(Accl).

There are several possible explanations for these results. The t-pp(Accl) polypeptide may not be able to assume a confirmation that makes the cleavage site available to the protease or the protease may not be able to assume its active conformation independent of the polyprotein. The later possibility might be tested by cotranslating the full length transcript with the 5' Accl deleted transcript. If t-pp(Accl) were processed under these conditions it would demonstrate that the cleavage site was available and that the complete polyprotein produced a protease that could act in trans but if the t-pp(Accl) were not processed it would indicate that either the confirmation needed for recognition of the cleavage site was altered or that the protease from the complete polyprotein will only act in cis.

In a recent report, Jagadish et al. (1988) used site-directed mutagenesis to make modifications to the γ2 (VP4) coding region of an IBDV large segment (A) cDNA which expressed processed viral proteins in E. coli. They found that insertion of 4 codons into the central region of γ2 resulted in a complete loss of processing. Since the addition of four amino acids is unlikely to cause drastic changes in the overall secondary structure of the polyprotein, it would seem that the loss in processing is attributable to relatively minor changes localized to the γ2 region. The success of this study suggested that site-directed mutagenesis might be a valuable tool for detailed mapping the IPNV protease active sites and cleavage sites.

As mentioned earlier, Duncan et al. (1987) have been able to
produce a complete polyprotein by *in vitro* translation. In their study, translation of the full length transcript resulted in production of \( \gamma_1, \gamma_2 \) and \( \beta_1 \), along with seven to nine other polypeptide bands. The largest of these polypeptides was 101 kD. Comparisons of SDS-PAGE profiles of partial proteolysis products demonstrated that the 101 kD polypeptide was the complete polyprotein.

The *in vitro* translation of the full length transcript described in this thesis was conducted under conditions very similar to those used by Duncan et al. (1987), yet a complete polyprotein was not observed (Figure 23). Duncan et al. (1987) suggested that the production of the unprocessed polyprotein in their system may have been the consequence of mutations to the protease region which lowered the efficiency of processing. When they compared the sequence of the cDNA used in the deletion studies to that of another cDNA which was obtained independently, they discovered two differences in the sequence which would result in amino acid changes. These changes occurred 1697 and 2071 bases pairs downstream from the initiation codon of the large open reading frame and were, therefore, contained within the \( \gamma_2 \) coding region.

Specific antibody added to rabbit reticulocyte translation systems have been shown to inhibit the processing of proteins for a number of viruses (i.e. Carrington and Dougherty, 1987). Thus, an attempt was made to arrest processing of the polyprotein by translation of full length transcripts in the presence of purified antibodies specific for the IPNV proteins. If the antisera were able to interfere with processing of the polyprotein, further studies with monoclonal antibodies to specific
epitopes in the protease or at its cleavage sites might be possible. The products of these translations, however, showed no difference between the antibody treated and control reactions (Figure 29). The polyprotein processing may be so rapid that cleavage occurs before antibody can bind.

In this (Figure 23) and previous studies (Huang et al., 1986), we have demonstrated the ability of the γ2 and γ1 polypeptides to be produced by in vitro translation even from RNA transcripts having 5' deletions which removed the initiation codon of the large ORF. This demonstrated that γ2 and γ1 polypeptides could be produced by internal initiations of translation in vitro. The only reported evidence of internal initiations of translation in vivo was the observation of Mertens and Dobos (1982) in pulse chase experiments that the time required for synthesis of these proteins was proportional to their length, with the smaller proteins (γ1 and γ2) being completed sooner. These results were considered consistent with simultaneous initiation of translation at three independent sites on the polycistronic A Segment mRNA.

If internal initiation of translation does occur in vivo, what initiation sites are most likely used? Figure 34 shows the location of the in-phase methionine codons that might be used for internal initiation of the γ proteins. Since RNA transcripts which are deleted on the 5' end to the EcoRI site can produce complete γ2 by in vitro translation, the initiation codon for γ2 must be 3' of this site. There are two possible initiation codons which could be used for γ2 translation. One of these
occurs within the Ncol site. This places it within the predicted $\beta$-$\gamma_2$ boundary. This methionine codon is not conserved in the Jasper strain sequence, however, which sheds some doubt on its use in vivo. Since this codon lies within the Ncol site it would be relatively easy to eliminate it by site directed mutagenesis and test its role in initiation of translation in vitro. The other possible initiation codon for $\gamma_2$ translation is located approximately 40 bases downstream from the EcoRI site. This methionine codon does occur in the Jasper strain sequence, but it is located well within the predicted coding region of the $\beta$ protein. If initiation does occur at this site, than either $\beta$ and $\gamma_2$ share peptide sequences or the $\beta$ polypeptide region is cleaved off to produce the $\gamma_2$ protein.

Since in vitro translation of a 5' Accl deleted RNA transcripts resulted in the production of a complete $\gamma_1$ (Figure 23), the initiation sites for this protein must be located downstream of this site. There are four potential initiation codons in this region (Figure 34). The first is not conserved in the Jasper strain sequence and is located more than 100 bases upstream from the predicted $\gamma_2$-$\gamma_1$ boundary. It would, therefore, appear to be an unlikely candidate for the $\gamma_1$ initiation site. The three remaining sites are all clustered near the predicted $\gamma_2$-$\gamma_1$ boundary and are conserved in the Jasper strain sequence. Duncan et al. (1987) have predicted that, for the Jasper strain, it is the second initiation codon that is used. This prediction was based on homology of the sequences surrounding this codon and the initiation codon of the large ORF. Most
notably, a 5'-ATC-3' triplet was conserved at 5-10 bases upstream of each of the codons. This triplet is also conserved in the Sp strain sequence at both codons. Duncan et al. (1987) point out that the same triplet is found preceding the vesicular stomatitis virus ribosome binding site (Herman, 1986).

In contrast to the results which were obtained in vitro, bacterial expression of 5' deletions of the A Segment coding region did not result in internal initiations for the \( \gamma_1 \) polypeptide (Figure 17). An authentic \( \gamma_1 \) was produced only when a full length cDNA was present in the plasmid. A similar observation has been made by Azad et al. (1987) for the bacterial expression of IBDV large (A) segment proteins and it was their contention that this was evidence that internal initiation does not occur in vivo.

As a preface to further discussion of the results for the bacterial expression by plasmids containing 5' deleted A segment cDNAs, it should be pointed out that the transcriptional promoter for the virus specific RNA in this expression system is unknown. The 5' deletions were constructed in pUC19/A(-) which contains the A Segment cDNA inserted in the opposite orientation from the LacZ promoter (Figure 16). It is also in the opposite orientation to the other active plasmid promoter (amp'). Work is currently in progress to map by S1 nuclease digestion the 5' ends of the virus specific mRNAs produced from this plasmid and determine the site of their transcriptional initiation.

The virus-specific polypeptides detected in cultures containing pUC19/A(-), which has an undeleted A Segment cDNA insert, were the 38
kD and the $\gamma_1$ and $\gamma_{1a}$ polypeptides (Figure 17). The $\beta_1$ or $\gamma_2$ polypeptides were not detected but these polypeptides were difficult to detect even for induced cultures of pUC19/A(+) (Figure 13). Deletions of the 5' end of the cDNA to the EcoRI, XhoI and XbaI sites resulted in the expression of the 38 kD polypeptide but not the $\gamma_1$ polypeptide. Deletion to the Accl site resulted in the failure to produce any virus-specific polypeptides. These results are markedly different from those obtained by in vitro translation of the RNA transcripts containing the same coding regions. In that study a $\gamma_1$ polypeptide was produced. The presence of the 38 kD polypeptide, which was recognized by the anti-$\gamma$ serum, was proof that mRNA coding for the $\gamma_1$ polypeptide was being produced but internal initiation of translation at the site that was used in vitro did not occur in bacteria.

Although the 38 kD polypeptide was produced by both bacterial expression and in vitro translation of the IPNV Sp strain A Segment, it has not been reported for the in vitro translation studies of the IPNV Jasper strain (Duncan et al., 1987) or in the bacterial expression of IBDV polypeptides (Azad et al. 1987); nor had this polypeptide ever been detected in vivo for any birnavirus. Therefore, the production of the 38 kD polypeptide appeared to be an artifact of the bacterial expression and in vitro translation of IPNV Sp strain polypeptides. It was surprising, then, to find that it was identified in infected cell proteins and thus, it is produced in vivo (Figure 20). The 38 kD protein was produced even when the A Segment ORF was deleted from the 5' end to as far as the XbaI site which demonstrated that this polypeptide must have arose as a
consequence of internal initiation of translation. Thus, the 38 kD polypeptide detected \textit{in vivo} provided evidence for internal initiation of translation \textit{in vivo} as well. Whether this is a fortuitous event or this protein has a functional role in virus replication is unknown at this time. Figure 34 shows that there are three in-phase methionine codons between the Xbal and Accl sites that could be the initiation codons used for production of the 38 kD protein. Based on the molecular weight of the polypeptide and the average molecular weight per amino acid for all the A Segment polypeptides (125 Daltons/ amino acid) the last of these sites is the most likely initiation codon for the 38 kD protein. The 41 kD protein produced by \textit{in vitro} translation of the 5' Xbal deleted RNA (Figure 23) may be accounted for by the initiation at one of the other two upstream methionine codons.

The production of the 38 kD polypeptide also revealed some information about the activity of the viral protease. The 38 kD polypeptide presumably contained approximately 6 KD of the \( \gamma_2 \) polypeptide in addition to the 32 kD of the \( \gamma_1 \) polypeptide and thus contained the \( \gamma_2 - \gamma_1 \) junction. It therefore, provided a potential substrate for the \( \gamma_2 - \gamma_1 \) protease cleavage analogous to the \( \beta - \gamma_2 \) cleavage site provided by t-pp(Accl). When the 5' EcoRI deleted RNA transcript was translated \textit{in vitro}, the complete \( \gamma_2 \) polypeptide was produced as well as the 38 kD polypeptide. The \( \gamma_2 \) polypeptide, therefore, did not appear to act in \textit{trans} on the \( \gamma_2 - \gamma_1 \) cleavage site of the 38 kD polypeptide.
Figure 34. Summary of IPNV A Segment structural features. The restriction map shows sites that are unique within the IPNV, Sp strain, A Segment cDNA. The numbers below each restriction site indicate the approximate distance of that site, in terms of base pairs, from the initiation codon of the large open reading frame (Met 1). These were arrived at by comparisons of partial sequence data for this cDNA (sequencing in progress in our laboratory) with the published sequence of a Jasper strain cDNA (Duncan and Dobos, 1986). The grey bar above the restriction map indicates the regions of the Sp cDNA which have been sequenced. The regions sequenced, thus far, show greater than 85% amino acid homology within the large ORF. The arrows on the restriction map indicate the location of methionine codons that are in-phase with the large ORF. Only those in-phase methionine codons present in the sequenced regions of the Sp strain are shown. These regions, however, include those areas were initiations of translation are likely to occur. The locations of the methionine codons, in terms of base pairs from the
intiation codon of the large ORF, are approximately 1393, 1482, 1808, 1889, 1959, 2079, 2191, 2214 and 2250. The white arrows are methionine codons that occur within both the Sp and Jasper strains. The bars below the restriction map indicate the predicted boundaries between protein coding regions. The exact locations of the boundaries are not known. Deletion analysis, however, located the boundaries within the grey regions. Bacterial expression of Segment A cDNAs deleted at the 3' end to the XhoI and KpnI sites results in polypeptides which are slightly larger (66.5 kD for XhoI) or slightly smaller (62 kD for KpnI) than $\beta_1$ (63 kD) (Figure 13). Therefore, the 3' end of the $\beta_1$ coding region must lie somewhere within the 31 base pairs between the KpnI and XhoI sites. In a recent report, Jagadish et al. (1988) demonstrated that the processing of the IBDV $\beta_1$ protein to $\beta_3$ involved deletions to the carboxy terminus. The lower bar in the figure indicates the region included in the $\beta_3$ protein of IPNV, assuming that it is also processed from the carboxy terminus. In vitro translation of 3' EcoRI deleted RNA transcripts resulted in production of a polypeptide of 57 kD which is only slightly larger than the 56.5 kD $\beta_3$ (Figure 23). Therefore, the region encoding the $\beta_3$ protein ends somewhere just 5' of the EcoRI site. The figure shows arrows illustrating the protease activity of $\gamma_2$ cleaving the $\beta_1$-$\gamma_2$ and $\gamma_2$-$\gamma_1$ junctions. It is not known if the $\gamma_2$ protease is involved in the processing of $\beta_1$ to $\beta_3$ via $\beta_2$. The boundary between the $\gamma_2$ and $\gamma_1$ proteins was not as well defined by deletion analysis as the $\beta_1$-$\gamma_2$
boundary. In vitro translation of 3' Smal deleted RNA transcripts resulted in production of a complete $\gamma_2$ but failed to produce $\gamma_1$ (Figure 23). Conversely, translation of 5' Accl deleted RNA transcripts resulted in a complete $\gamma_1$ polypeptide but no $\gamma_2$. Therefore, the $\gamma_2$-$\gamma_1$ boundary must lie somewhere between the Accl and Smal sites. An estimate of the location of the $\gamma_2$-$\gamma_1$ boundary was made based upon the molecular weights of the A segment proteins. The total molecular weight of these proteins is approximately 122 KD ($\beta_1[63\text{KD}] + \gamma_1[32\text{KD}] + \gamma_2[27\text{KD}]$).

Since there are approximately 2916 bases in the large ORF, the average molecular weight per amino acid must be approximately 125 Daltons (122KD/2916 bases x 3 bases/amino acid). The coding region of the 32 KD $\gamma_1$ protein must, then, begin approximately 768 bases (32 kD x 3 bases/ 0.125 kD) before the termination codon (TAA) or in other words 2148 bases downstream from the first initiation codon. In the figure, a black line through the grey area marks this predicted $\gamma_2$-$\gamma_1$ boundary. Three conserved in-phase methionine codons lie within the Smal-Accl region just down stream of this location and presumably, one of these sites serves as the initiation codon for in vitro translation of the $\gamma_1$ polypeptide.
Bacterial expression of IPNV antigens for use as a subunit vaccine.

Presented in this thesis are preliminary results which demonstrate the potential efficacy of bacterially expressed antigens as a subunit vaccine for IPNV. Since there is presently no means of control for IPN except the complete destruction of the infected fish stocks, there has been a great deal of interest in developing a vaccine for this disease. Dorson (1977) demonstrated that four week post-hatch fry could be successfully immunized by intraperitoneal injection of a formalin inactivated virus. Immersion or oral immunization with the same vaccine was not effective, however. This study illustrated both the potential promise and the difficulty of vaccine development for IPN. It demonstrated that the fry were immunocompetent at an age that would make vaccination worthwhile but that as a practical matter the conventional inactivated vaccine was useless because it would require individual injections into thousands of tiny fry (less than 0.5g). Similarly, there have been difficulties with attenuated vaccines. The attenuated vaccines either failed to induce protective immunity or retained unacceptable levels of virulence (reviewed by Dorson, 1982).

The development of a subunit vaccine using bacterially expressed viral antigens provided several advantages. With the bacterially produced antigens, immersion vaccination would be more effective for two reasons. First, bacterial expression would allow the production of large quantities of viral antigen at a reasonable cost and therefore, a much higher concentration of the antigen could be used in the immersion vaccination.
Second, several authors (Hill et al., 1980 and Dixon and Hill, 1983) have proposed that uptake of antigen by immersion might be more effective if smaller, subviral particles were used. The proteins expressed in bacteria presumably do not assemble into complete capsids and therefore, may be presented as smaller immunogen particles.

Antigens produced by bacteria have several other advantages as vaccines. They contain no complete virions and, thus, there would be no possibility of inadvertent infections. Bacterial expression also allows for manipulation of the antigens in ways that are not possible with conventional vaccines. For example it is possible to alter the characteristics of the viral proteins by deletions or fusions with other proteins. These types of changes could possibly contribute to stability or immunogenicity of the antigens.

At the outset of this study it was necessary to determine which of the viral proteins were important to the induction of immunity. Stephens and Hetrick (1983) used top component, a by-product of IPNV purification that contains only the β proteins, to immunize rabbits and produce an antiserum that could neutralize virus infectivity in tissue culture. This indicated that β proteins bore neutralizing epitopes and therefore, the expression of the β proteins would be of particular importance in production of a bacterially expressed subunit vaccine. Following this study, however, Fahey et al. (1985), published a report in which the IBDV γ₁ (VP3) protein was effective as an immunogen but the β₃ (VP2) protein was not. Since IPNV and IBDV are so similar, it seemed unlikely that they would carry neutralizing epitopes on different proteins.
The production of the anti-γ and anti-β serum described in this thesis was, in part, done as an attempt to determine conclusively which of the IPNV proteins bore neutralizing epitopes. Unfortunately, neither of these antisera were neutralizing. This was probably because the purification procedure resulted in protein denaturation and may have destroyed the neutralizing epitopes. Recent studies using neutralizing monoclonal antibodies, have demonstrated, however, that for both IBDV (Azad et al., 1987) and IPNV (Caswell-Reno et al., 1986) the β proteins (VP2) contain the primary neutralizing epitopes. The failure to initially identify the IBDV β protein as an immunogen has been attributed to the denaturation of that protein during purification (Azad et al., 1987). Thus, it appears that the neutralizing epitopes on both the IPNV and IBDV β proteins are sensitive to denaturation.

In the first section of this thesis, bacterial expression was used as a tool to study the production and processing of IPNV A Segment proteins. The complete A Segment was expressed by the plasmids pUC19/A(+) and pUC19/A+S but the production of the β protein, as determined by Western blot analysis, was quite poor (Figures 13 and 14). On the other hand, several of the derivatives of these plasmids which contained 3′ deletions to the A segment cDNA produced polypeptides that reacted strongly when assayed with anti-β serum. Most notable among these were pUC19/A+SΔA which produced t-pp(Accl) and pUC19/A+SΔE which produced a polypeptide corresponding approximately to β3 but with amino acids coded for by vector sequences at the carboxy terminus. It is not known why these constructions resulted in production of substantial
quantities of $\beta_1$ antigen when pUC19/A(+) and pUC19/A+S failed to do so. It may be that the pUC19/A(+) and pUC19/A+S expression resulted in the production of $\beta_1$ via processing of the polyprotein and that the $\beta_1$ thus produced is not stable and is rapidly degraded. A number of studies (i.e. Gerard et al., 1986) have shown that the carboxy-terminal structure of bacterially expressed proteins affects their stability. Therefore, it may be that the carboxy termini of the $\beta$ polypeptides produced by pUC19/A+SΔA and pUC19/A+SΔE make these polypeptides more stable than the $\beta_1$ polypeptide.

The plasmid pTA1 was constructed specifically for bacterial expression of IPNV antigens for use as a vaccine. The plasmid contained the entire A Segment cDNA fused to the TrpE gene of the pATH2 expression vector. The pATH2 vector had been used previously in our laboratory to obtain high level expression of a fusion protein that was an effective vaccine for another viral fish pathogen, IHNV(Gilmore et al. 1988). Induced bacterial cultures containing pTA1 produced substantial quantities of virus specific polypeptides (Figure 32). The major virus-specific products had molecular weights of approximately 28.5, 32, 63.5, 75, 81, 87 and 93 kD. Western blot analysis with the anti-$\beta$, anti-$\gamma$ and anti-t-pp(Accl) sera showed that the lower bands (28.5 and 32 kD) contained $\gamma_1$, $\gamma_{1a}$ and $\gamma_2$ polypeptides. The 63.5 kD band reacted with the anti-$\beta$ serum and thus appeared to be $\beta_1$. The high molecular bands (75, 81, 87 and 93 kD) also reacted with the anti-$\beta$ serum and were presumably TrpE-$\beta_1$ fusion products. The variation of size observed in these polypeptides may be the
result of degradation or premature terminations of translation.

Lysates of induced cultures of pUC19/A(+), pUC19/A+SΔA and pTA1 were compared as immersion vaccines (Figure 33). Twenty days after immunization, the fry were challenged with the Buhl strain of IPNV. The Buhl strain was chosen because it produced more consistent levels of mortalities than the Sp strain.

The results of the vaccine trial must be considered preliminary because of the limited scope of the experiment. Despite this, they appeared quite promising. The mock treated group had a final cumulative mortality of 44%, while the pTA1 treated group had a final cumulative mortality of only 4%. The protection afforded by the pUC19/A(+) and pUC19/A+SΔA treatments was less dramatic, with final cumulative mortalities of 20 and 36%, respectively. As a general trend, it appears that the amount of protection was proportional to the amount of the β antigen present in each of the lysates.

Additional studies will be required to fully assess the effectiveness of these vaccines. Work is in progress to optimize the expression and preparation of the viral antigens in bacteria. The nature and duration of the immune response should be defined and the range of cross-protection with the various serogroups should also be determined.
BIBLIOGRAPHY


