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The two segments of double-stranded RNA (dsRNA) from infectious pancreatic necrosis virus (IPNV) were cloned into plasmid vector pUC8. Two distinct sets of overlapping clones were identified by restriction enzyme and Southern blot analysis. Each of these sets was shown, by Northern blot analysis, to be exclusively related to either segment A or B of genomic RNA.

The synthesis of complementary DNA (cDNA) from viral RNA segments A and B was less than full-length. Continuous sequences from each segment were represented by two overlapping inserts which were ligated together at a common restriction site. The entire lengths of the cloned segments A and B were estimated to be 2.9 and 2.6

kilobases, respectively.

The cDNA sequences from segements A and B were subcloned into the T7 RNA polymerase vectors, pT71 and pT72 and used to transcribe single-stranded RNA (ssRNA). This RNA was used to indirectly compare the lengths of the cloned sequences to those of the viral dsRNA by glyoxal denaturation and agarose gel electrophoresis. The electrophoretic mobilities of the single-stranded RNA's originating from cloned viral sequences were identical to those of the individual strands of ds genomic RNA.

The coding capacity of the viral cDNA was determined by cell-free translation analysis. The single-stranded RNA described above was active in a rabbit reticulocyte lysate translation system and did not require either 5'-capping or 3'-polyadenylation. The electrophoretic mobility of the proteins originating from the cloned viral segments was compared to those produced from viral dsRNA as well as the proteins found in purified virus. The four proteins reportedly encoded by the genome of IPNV were identified among the translation products of the individual cloned segments by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The coding assignments of the proteins produced from cloned segments A and B are shown to be identical to those previously reported for IPNV.

Molecular Cloning of Infectious Pancreatic Necrosis Virus and Characterization of the Coding Capacity of the Complementary DNA

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CONTRIBUTION OF AUTHORS

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MOLECULAR CLONING OF INFECTIOUS PANCREATIC NECROSIS VIRUS AND CHARACTERIZATION OF THE CODING CAPACITY OF THE COMPLEMENTARY DNA

I. INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is a primary pathogen of hatchery-reared rainbow and brook trout. The disease is manifested as an acute catarrhal enteritis which in extreme cases can lead to over 90% mortality of hatchery stocks (Pilcher and Fryer, 1980). IPNV can be found in aquatic environments worldwide and has become a significant concern within the aquaculture industry.

Early descriptions of IPNV identified three major serotypes which were separated geographically. The main North American serotype was designated VR299, and two European serotypes were characterized as Sp and Ab (Pilcher and Fryer, 1980). More recently, strains of IPNV have been isolated from fish in Japan and Taiwan (Hedrick et al., 1983a, 1983b; Okamoto et al., 1983). These new strains have been shown to be related to one of the three major serotypes by cross-neutralization and/or analysis of the purified viral nucleic acids and proteins. In addition, there are a number of isolates which do not

clearly lie within any of these serotype groups and that vary in terms of virulence and host range from strain to strain (Dorson, 1982).

IPNV is a member of the proposed family Birnaviridae (Fields, 1985) first suggested by Dobos et al., in 1979. The majority of biophysical data that is available for this family comes from studies with VR299 and is briefly described here. The viral capsid is a naked icosahedron, with an average diameter of approximately 60nm. Purified virions contain four proteins when analyzed on SDS polyacrylamide gels (SDS PAGE). The largest of these is the putative RNA polymerase. It has a molecular weight of approximately 105,000 Daltons (105kD) and represents 3% of the total viral protein (Cohen, Poinsard, and Scherrer, 1973). The remaining three proteins have molecular weights of 54kD, 31kD, and 29kD. Over 60% of the viral protein consists of the 54kD protein (Cohen, Poinsard, and Scherrer, 1973; Dobos, 1977). The 29kD protein has been shown by pulse-labeling of infected cells (Dobos, 1977) as well as tryptic peptide mapping (Mertens and Dobos, 1982) to be a breakdown product of the 31kD protein.

The viral nucleic acid consists of two segments of double-stranded RNA (dsRNA) that encode four proteins. Three of these are associated with purified virions. The fourth is present in infected cells only (Dobos and Rowe, 1977) and has a molecular weight of 29kD. Like the 31kD

protein, it also undergoes processing to a final size of 25kD. The dsRNA segments have molecular weights of approximately 2.3 and 2.5 X 10 D (Cohen, Poinsard, and Scherrer, 1973; Dobos, 1976; MacDonald and Yamamoto, 1977; Dobos, Hallett, Kells, Sorensen, and Rowe, 1977). The larger of these has been designated segment A and has been shown by genetic reassortment studies (MacDonald and Dobos, 1981) and by cell-free translation analysis of dsRNA (Mertens and Dobos, 1983) to encode the three smaller proteins described above. The smaller segment of RNA, B, encodes the putative RNA polymerase.

The work presented in this thesis describes the molecular cloning of the dsRNA for the Sp serotype of IPNV and the characterization of the coding capacity of the two cloned segments by cell-free translation analysis. The genomic dsRNA was converted into complementary DNA (cDNA) using standard cloning techniques and inserted into the plasmid vector pUC8. The origin of the cDNA clones with respect to the individual segments of dsRNA was verified by Northern blot analysis. Sizing of the cloned sequences was performed by subcloning the cDNA from each segment into the T7 RNA polymerase vectors, pT71 and pT72. These vectors are designed such that ssRNA can be transcribed by T7 RNA polymerase which initiates at a promoter site immediately upstream from the inserted cDNA. This allowed for the synthesis - in both reading

orientations - of ssRNA which was equivalent in size to the length of the viral cDNA. These RNA's were used in (1) RNA denaturing gels to determine their size relative to denatured genomic RNA, and (2) cell-free translation reactions to determine the coding capacity and sense orientation of the cDNA clones from each segment. The results confirm that the entire coding capacity has been obtained for the genome of Sp IPNV.

II. LITERATURE REVIEW

A. Infectious pancreatic necrosis disease

Infectious pancreatic necrosis virus (IPNV) is the etiological agent of a disease common to several species of juvenile trout. The first description of the disease is believed to have been made in Canada by McGonigle in 1941 who characterized the infection in young brook trout (Salvelinus fontinalis) as an "acute catarrhal enteritis." It was not until 1953 that a similar outbreak occurred in fingerling brook trout in West Virginia. Histopathological studies led Wood et al. (1955) to describe the condition as "infectious pancreatic necrosis" (IPN), although no causative agent was identified. Snieszko et al. (1959) later demonstrated the infectious nature of the disease by transmitting IPN to fish with tissue homogenates of infected fish. Characterization of IPN as a viral infection was later made by Wolf et al. (1960). Using bacterial- and cell-free homogenates of infected tissues, they were able to reproduce the disease in brook trout fry and observe cytopathic effect (CPE) in tissue cultures of trout origin.

The geographical distribution of IPNV is quite extensive. It has been isolated throughout Europe, North

America, and in several parts of Eastern Asia. It appears to be ubiquitous in the aquatic environment, found not only in susceptible and carrier fish, but also in molluscs and crustaceans. Despite the wide variety of carriers, the virus is primarily virulent for a restricted group of salmonid species: Salvelinus fontinalis, Salmo trutta, S. salar, S. gairderi, and S. clarkii (Pilcher and Fryer, 1980). Juveniles of three Onchorynchus species (O. tshawytscha, O. nerka, and O. kisutch) are resistant to the disease (Parisot et al., 1963) although IPNV has been isolated from adult coho salmon (McMichael et al., 1973). Two diseases of non-salmonids caused by IPNV-like viruses are branchionephritis of eels (Sano et al., 1981) and spinning disease of menhaden and shad (Newman, 1980; Stephens, 1980). Other fish from which IPNV has been isolated include carp, perch, roach, sea bream, pike (Hill, 1977) tilapia, and eels (Hedrick et al., 1983a, 1983b). More complete reviews of the distribution and occurrence of IPNV have been preapred by Scherrer (1973), Wolf (1976), Pilcher and Fryer (1980), and Dorson (1982).

The early clinical signs of IPN include an abnormal mortality among the fastest growing fish which display a characteristic corkscrew spiralling along the long axis of the body (Wolf and Quimby, 1967). Externally, fish develop darkened body pigments, exopthalmia, abdominal swelling, and hemorrhaging at the base of the ventral fins. An

internal analyisis reveals both a pale liver and spleen, hemorrhaging of the anterior viscera, ascites, and the presence of mucous-like material in the stomach and anterior intestine. Petechiae are common along the pyloric caeca - an area that contains pancreatic tissues - and the stomach is usually devoid of food.

Fish displaying clinical signs of IPNV infection can be assayed for the presence of virus in a number of ways. Most of these require the cultivation and identification of IPNV in tissue culture inoculated with homogenates of fish tissue. Cell lines of choice are usually rainbow trout gonad (RTG-2) or chinook salmon embryo (CHSE-214) cells. If CPE arises in these cells as a result of exposure to samples of infected tissues, the presence of IPNV is confirmed by neutralizing antibody (Wolf, 1976), complement fixation (Finlay and Hill, 1975), or fluorescent antibody (Tu et al., 1974).

A number of factors influence the severity of IPNV infections. Among the most significant are the strain of virus and the species and strain of the infected host. Two of the major serotypes display dramatic differences in pathogenicity and host range. Sp IPNV has been reported to cause over 90% mortality of young fry (Pilcher and Fryer, 1980), and has only been associated with epizootics in salmonids. Ab IPNV has a very low pathogenicity for trout (10%), yet appears to be very similar to the EVE strain

associated with branchionephritis in eels (Hedrick et al., 1983a; 1983b). In addition, Hill (pers. comm.) has found that the virulence of Sp IPNV is dependent upon the strain of trout that is infected. Other factors involved with virulence include the age or size of the fish, and environmental conditions such as water temperature and stress (Hill, 1982).

B. Characterization of IPNV

IPNV currently belongs to the proposed family Birnaviridae (Fields, 1985) which was first suggested by Dobos et al., in 1979. Members of this group have genomes consisting of two segments of double-stranded (ds) RNA. Previously, these viruses were included in the picornavirus group (Malsberger and Cerini, 1963) and the reovirus group (Moss and Gravell, 1969). In addition to IPNV, the birnaviridae contains drosophila X virus and infectious bursal disease virus of chickens. These three viruses are serologically distinct and have unique protein banding patterns determined from SDS PAGE (Dobos et al., 1979).

The majority of the biochemical analysis of IPNV has been performed using the VR299 serotype, or North American type strain. Structurally, this virus is a naked, icosahedral capsid comprised of four viral proteins (Cohen

et al., 1973; Dobos, 1977). The sizes of these proteins are 105,000 Daltons (105kD), 54kD, 31Kd, and 29kD. The 29kD protein has been shown by pulse-labeling analysis to be a breakdown product of the 31kD protein (Dobos, 1977). The 105kD protein is present as 3% of the total protein and functions as the viral RNA polymerase.

The two segments of dsRNA have estimated sizes of 2.3 and 2.5 x 10^6 D. Together these segments encode four viral proteins: the three virion associated proteins (which includes the 29kD breakdown product) plus a 29kD protein found only in infected cells (Dobos, 1977). The coding assignments of the two segments have been determined from both genetic reassortment studies (MacDonald and Dobos, 1981) as well as the analysis of the cell-free translation products of dsRNA isolated from purified virus (Mertens and Dobos, 1982). It has been determined that the larger RNA segment, A, codes for three proteins, while the smaller segment, B, codes for the putative viral RNA polymerase. The physical orientation of the three genes of segment A has not been determined.

Several attempts have been made to classify the major isolates of the IPNV which are pathogenic to fish (Wolf and Quimby, 1971; MacDonald and Gower, 1981; Okamoto et al., 1982; Hedrick et al., 1983a; 1983b). Three major serotypes are generally accepted. The type strain in North America is VR299, whereas the two major European serotypes

are Sp and Ab. Studies with different sets of reference antisera have led to the placement of the same strain of IPNV in different serotype groups (Dorson, 1982).

Difficulties have arisen in classifying the IPNV-like isolates from non-salmonid species and virulent strains isolated from within the same geographical area. Cross-neutralizing titers are not always conclusive for the non-salmonid isolates (Hill, pers. comm.), and there are several North American strains, such as Buhl and Powder Mill, which appear to be antigenically distinct from VR299 (Wolf and Quimby, 1971). A Japanese isolate, designateed EVE, is associated with branchionephritis in eels. EVE appeared after the importation of eels from Europe into a region which had no prior history of IPNV. Its close antigenic and structural similarities to Ab (Hedrick et al., 1983a; 1983b) imply that EVE is a strain of Ab. It is likely that a more precise classification of IPNV will include one or more additional serotypes.

C. Sensitivity of IPNV to normal rainbow trout serum

Isolates of Sp IPNV which are repeatedly passed in tissue culture acquire sensitivity to the serum of normal rainbow trout (Dorson and deKinkelin, 1974; Dorson, deKinkelin, and Torchy, 1975). Five strains of IPNV from North America and two isolates obtained from shellfish

have been tested and found to have varying degrees of serum sensitivity (Kelly and Nielsen, 1985). The antiviral activity of normal rainbow trout (RBT) serum is associated with a protein fraction of 6S which somehow prevents the virus from infecting normally susceptible cell cultures. Kelly and Nielsen (1985) demonstrated that adsorption to CHSE-214 cells by Sp IPNV is inhibited by the presence of 6S RBT serum. In addition, strains that could infect a large number of cell lines were also more sensitive to neutralization by the 6S component.

The 6S sensitive virus appears to be selected for in tissue culture, and in the process becomes avirulent (Dorson, deKinkelin, and Torchey, 1975). Both virulence and resistance to the 6S serum component can be maintained by passing the virus in the presence of normal trout serum. The 6S component may have a similar effect on virulence in vivo by selecting against a faster growing avirulent genotype. Further work is required to determine the biochemical nature of the the 6S component and the biological consequences of its interaction with IPNV.

D. Transmission of IPNV disease

The two primary sources of IPNV are infected juvenile fish and adult carriers that have acquired a persistent infection (PI). In both cases, the virus is shed into the

surrounding water via intestinal discharges, and it has also been demonstrated that adults release virus into both seminal and ovarian fluids. The combination of these two sources makes it very difficult to eliminate the virus from infected watersheds and facilitates transfer of the virus via eggs or brood stock to uninfected areas. Bullock et al. (1976) have demonstrated that vertical transmisssion via contaminated eggs occurs despite treatment of the eggs with concentrations of iodine in excess of that required to inactivate the virus.

Hedrick and Fryer (1981, 1982) examined three salmonid cell lines and carrier brook trout persistently infected with IPNV. They proposed a model for the persistent state whereby defective interfering (DI) particles and/or interferon are responsible for maintaining a low number of infected cells which continuously produce varying amounts of virus. Characteristics of the PI cell lines were consistent with DI mediated persistence described by Holland et al. (1980) in that (1) the culture media from PI-STE-137 and PI-RTG-2 cell lines (but not PI- CHSE-214) exhibited autointerference, (2) the cells were resistent to superinfection by homologous virus (including serologically distinct strains), yet (3) sensitive to heterologous infection, and (4) persistence was curable by viral specific antibody. However, these cell lines differ

from the general description of DI persistence in that PI-RTG-2 cells produced interferon and that the number of infected cells was extremely low (0.5-1.0%) rather than close to 100%. Their observations of PI brook trout were similar to those of the PI cell lines and consistent with their model of DI-mediated persistence. Interferon was not detected, and although antibody could be found, there was no corelation between the levels of antibody and the amount of virus shed by the fish.

Kennedy and MacDonald (1979) also examined PI in CHSE-214 cells. Their results were very similar to those reported by Hedrick and Fryer (1981) for PI-CHSE-214 cells except that all cells appeared to be infected. The nature of PI by IPNV in salmonids is thus dependent upon highly specific interactions between a particular strain of the virus and the cellular response of the host strain.

E. Prevention and control of IPN disease

The viral nature of IPN disease has made its prevention and therapy a difficult problem. The ability of IPNV to be transmitted vertically through infected eggs and to be carried by a diverse range of fish and invertebrates greatly enhances its distribution and survival. The most effective controls involve meticulous monitoring of transported eggs or broodstock, particularly

those from regions with a known history of the virus. IPNV is transmitted by infected fish via intestinal discharges into surrounding waters at concentrations of up to 1X10 infectious doses/ml (Wolf and Quimby, 1967). Since there are no therapeutic measures available to control the spread of IPNV in hatcheries, the consequences are severe and demand elimination of the hatchery stocks and complete disinfection of the facility and equipment.

Attempts to produce vaccines for IPNV by attenuation or inactivation have either been unsuccessful or impractical for use on a large scale. The major problems encountered thus far include (1) reliable and effective procedures for inactivating the virus; (2) methods for applying the vaccine to large numbers of fish; (3) suitable antigenic forms of the virus; (4) the need for a polyvalent vaccine in certain areas; (5) immunocompetence of young fry; and (6) cost effectiveness.

Several studies have been performed using formalin-treated IPNV as immunogen (Dorson, 1977; Sano et al., 1981; Dixon and Hill, 1983). In each case, the injection of formalin inactivated virus into fish resulted in the production of neutralizing antibody and a degree of protection against challenge by live virus. Significantly, the fish shown by Dorson (1977) to produce antibody were injected at 5 weeks post-hatching, thus demonstrating the immunocompetence of young fry. Two strategies for the

control of IPNV are thus being pursued. The first is to minimize the vertical transmission of IPNV by inducing immunity in brood stock. The potential for this approach is supported by the ability to cure persistent infections in tissue cultures by the addition of antibody (Hedrick and Fryer, 1981), and data demonstrating protection from IPNV in adult fish through immunization (Sano et al., 1981). The second strategy is to develop an effective vaccine for hatchery fish which can be applied without injection and will induce a long-lived immune response. Thus far, the only successful vaccination trials have been performed by injection. Although a recent report by McAllister (1984) claimed that an avirulent strain of IPNV could be used to vaccinate fry as early as 10 days post-hatching, there was no mention of the methods employed.

An alternative approach to vaccine preparation is the design and production of a subunit vaccine prepared by the molecular cloning of protein sequences responsible for eliciting a neutralizing antibody response. Examples of this technique include the production of cloned VP3 sequences of foot and mouth disease of swine and cattle (Kleid et al., 1981; Bittle et al., 1982) and the synthesis of hepatitis B surface antigen particles (HBsAg) (Valenzuela et al., 1985) or partial peptides of the major polypeptide derived from HBsAg (Dreesman et al., 1982).

The proteins or peptides produced in these systems were capable of reacting with antibodies prepared against whole virus, stimulating the production of anitbody in mammals which cross-reacts with native viral proteins, and in the case of foot and mouth disease, providing protection in cattle and swine against challenge by the virus.

The capsid structure of IPNV lends itself to this approach since it is comprised mainly of a single outer protein that has been shown to produce neutralizing anitbody in rabbits (Stephens, pers. commun.). In addition, a bacterial sysetem should be suitable for its production since this protein has not been shown to be glycoslyated and the virus lacks a membrane (thus, the processing which normally accompanies membrane-bound proteins in eucaryotes should not be required). It is also encouraging that in the normal course of infection, IPNV produces large quantities of defective particles. These particles are composed of the major capsid protein (Dobos, 1977) and apparently act in autointerference. A subunit vaccine consisting solely of the major capsid protein could mimic the effect of the defective particles and provide a more highly antigenic structure than the formalin or beta-propiolactone (Dixon and Hill, 1983) inactivated virions examined to date.

III. MOLECULAR CLONING OF IPNV GENOMIC RNA

INTRODUCTION

Infectious pancreactic necrosis virus (IPNV) of fish causes a highly acute, contagious disease in juvenile rainbow and brook trout (see Pilcher and Fryer, 1980 for review). Highly virulent strains can cause greater than 90% mortality of hatchery stocks, therefore making them a major concern within the aquaculture industry. At present, no preventative or therapeutic treatments exist other than the destruction of infected stocks and disinfection of the hatchery facilities.

IPNV is classified as a birnavirus (Dobos et al., 1979; Fileds, 1985). Members of this group are characterized by a genome consisting of two segments of double-stranded RNA (dsRNA) which code for four proteins. Genetic reassortment experiments have demonstrated that the larger RNA (segment A) codes for three proteins while the smaller RNA (segment B) codes for the putative RNA polymerase (MacDonald and Dobos, 1981).

IPNV was first cultivated by Wolf et al., (1960) and has since been characterized serologically by Wolf and Quimby (1971), Jorgenson and Kehlet (1971), MacDonald and Gower (1981), Okamoto et al. (1983), and Hedrick et al. (1983a, 1983b). The two predominant European serotypes are Sp and Ab , while the type strain in North America is VR299 . Precise placement of all IPNV isolates within one

of these groups is not possible since many of them show relatively low cross-neutralizing titres for more than one type of anitserum.

The three major serotypes, although closely related, show marked differences in terms of structure and virulence. Capsid structure varies in terms of the number of virion associated proteins, the molecular weights of these proteins, and the electrophoretic mobility of the RNA segments. The most predominant structural difference is seen in the protein profile of VR299 isolates. While other serotypes contain three capsid proteins, VR299 contains an additional protein derived from the processing of the 31kd protein (Dobos, 1977).

The geographical distribution of IPNV is worldwide and it has a surprisingly wide host range. Both VR299 and Ab-like isolates have been obtained from trout and tilapia in Taiwan (Hedrick et al., 1983a; 1983b) and the virus has been recovered from invertebrates such as molluscs and crustaceans (Hill, 1982).

We have cloned the viral genome of IPNV in order to establish a physical map of two RNA segments and to develop a subunit vaccine consisting of the major capsid protein. We report here the molecular cloning of the Sp serotype into the plasmid vector pUC8 and present a preliminary restriction map of the two segments of cDNA. We report in the accompanying paper the subcloning of the

cDNA from both segments into the T7 RNA polymerase plasmids pT71 and pT72 (Huang et al., 1985b). Using those subclones, it is shown here that single-stranded RNA synthesized by T7 RNA polymerase produces plus and minus strands of RNA which migrate alongside those of the genomic RNA when electrophoresed in glyoxal gels.

METHODS

Virus growth and purification. Six strains of IPNV were used in this study. VR299, Ab, and the Sp strain used to prepare cDNA (SpCS) were provided by Dr. R.P. Hedrick. Strains of Sp known to be sensitive (SpS) and resistent (SpR) to a 6S serum componant of normal rainbow trout serum were obtained from Dr. B. Hill. The Buhl, Idaho strain was obtained from Nancy Wood, International Aquaculture Research Center. The virus was propagated in CHSE-214 cells (Fryer et al., 1965) at a multiplicity of infection of 0.05-0.1. Cell monolayers were grown at 18C in glass bottles using Eagle's minimal essential media (MEM) with Earle's salts (Gibco) supplemented with 0.11% bicarbonate, 10% fetal bovine serum, 100IU/ml penicillin, 100 micrograms/ml (ug/ml) streptomycin (Gibco), and 10 ug/ml gentamicin sulfate (Sigma). SpR was grown in the presence of a 1:150 dilution of normal rainbow trout serum. Virus particles were harvested between 3-5 days post-infection. After an initial low speed centrifugation, the virus was pelleted in a Beckman T35 rotor at 30,000 (30K) RPM for 90 minutes and resuspended in S&M buffer (0.1 M NaCl; 8mM MgSO4; 20 mM Tris-HCl, pH7.5; 0.01% gelatin). The virus was initially purified by centrifugation through a step gradient consisting of 1.4 g/cc CsCl, 1.25 g/cc CsCl, and 20% sucrose in S&M in

either a Beckman SW41 or SW50.1 rotor at 35K for 90 minutes. The virus banding between the 1.4 and 1.25g/cc CsCl layers was collected by side puncture, layered onto a 1.33g/cc CsCl equilibrium gradient, and centrifuged for 14-16 hours at 35K RPM at 4C. Intact virus was collected by side puncture or from above using a Buchler Densi-flo IIC fraction collector. The volume of CsCl containing the virus was diluted with S&M and the virus pelleted at 35K for 90 minutes in a SW41 rotor at 4C.

Reagents. RNaseH, terminal deoxynucleotide transferase, and oligo-dT12 primers were obtained from Pharmacia. Poly-A polymerse, E. coli DNA polymerase I, all restriction endonucleases, and E. coli DNA ligase were purchased from Bethesda Research Laboratories, and avian myeloblastosis virus (AMV) reverse transcriptase from Life Sciences.

Preparation of viral RNA. The viral pellet was resupended in TE (10mM Tris-HCl, pH 8.0; 1 mM Na2EDTA) containing 0.5% sodium dodecyl sulfate (SDS) and 100 ug/ml proteinase K and left at room temperature overnight or at 65C for 2 hours. The RNA was extracted twice with an equal volume of phenol/0.1% 8-hydroxyquinoline and then twice with chloroform/isoamyl alcohol (IAA), followed by ethanol precipitation.

Poly-A tailing of viral RNA. Polyadenylate tails ranging between 40-80 bases in length were added to the 3'-ends of the dsRNA using E. coli poly-A polymerase (Sippel, 1973). The reaction was performed in 25 microliters (ul) at 37C (250 mM NaCl; 50 mM Tris-HCl, pH 8.0; 10 mM MgCl2; 2.5 mM MnCl2; 50 ug/ml bovine serum albumin; 0.1 mM 3H-ATP; 15 ug dsRNA; 3.5 units poly-A polymerase). After 40 minutes, the reaction mixture was extracted once with phenol and once with chloroform/IAA followed by ethanol precipitation in the presence of 2M ammonium acetate.

First strand cDNA synthesis. The poly-A tailed viral RNA was resuspended in 20 ul sterile distilled water (treated with diethylpyrocarbonate), boiled for three minutes, and then chilled on ice. The reaction volume was raised to 200ul containing 50 ug/ml oligo-dT12; 80 ug/ml actinomycin D; 2 mM each of dATP, dGTP, and TTP; 100 uM alpha-32P-dCTP (specific activity = 4 Ci/mMole); 50 mM Tris-HCl, pH 8.3; 10 mM MgCl2; 10 mM dithiothreitol (DTT); and 13 units/ug RNA of AMV reverse transcriptase. After incubation at 42C for 3-4 hours, the reaction was stopped by the addition of EDTA and SDS. The RNA/cDNA hybrid was extracted with phenol and then chloroform/IAA as described above. The nucleic acid was ethanol precipitated twice in the presence of 2M ammonium acetate prior to second strand cDNA synthesis.

Second strand cDNA synthesis. The RNA/cDNA hybrid nucleic acid was converted into dsDNA by a modification of the method of Okayama and Berg (1982). This method employes E. coli DNA polymerase I and RNase H at concentrations of 30u and 1 unit/ug cDNA, respectively, plus 50u/ml E. coli DNA ligase. The nucleic acid was resuspended in a reaction volume of 100 ul containing the enzyme concentrations indicated above in a solution of 100 mM Hepes, pH 6.9; 4 mM MgC12; 15 mM beta-mercaptoethanol (BME); 70 mM KC1; 2 mM each dCTP, dGTP, dATP; 50 uM alpha-32P-TTP (final specific activity = 1 Ci/mM); and 15 uM beta-nicotinamide adenine dinucleotide (BNAD). The reaction was incubated successively at 12C and room temperature for one hour each and terminated with EDTA and SDS. Ethanol precipitation in the presence of 2M ammonium acetate was performed twice to eliminate free dNTP from the ds cDNA prior to poly-dC tailing.

Transformation of cDNA. Complementary homopolymer tails of ca. 15-20 bases were added to the cDNA (poly-dC) and to Pst1 cut plasmid vector pUC8 (poly-dG) using terminal deoxynucleotide transferase (Tdt). Insert and vector DNA's were reannealed at 42C for several hours and transformed into calcium chloride treated E. coli host strain SC181 (Appleyard, 1954) by the method of Maniatis et al. (1982). Transformants were isolated by plating onto LB agar

supplemented with 120 ug/ml ampicillin.

Preparation of cDNA probes. 2 ug viral genomic RNA was 32P-labeled using AMV reverse transcriptase. The RNA plus calf-thymus primers (Taylor et al., 1976) was denatured by boiling in sterile distilled water for 2 minutes and quenching on ice. The reaction volume was raised to 30 ul containing 50 mM Tris-HCl, pH 8.3; 60 mM KCl; 10 mM MgC12; 5 mM DTT; and 50 uCi 32P-dCTP (sp. act = 3200 Ci/mM) and incubated at 42C for 2 hours. EDTA was then added and the labeled cDNA purified by Sephadex G-50 column chromotography.

Colony blots. Transformants were examined for viral sequences using the screening method of Maniatis et al. (1982). Bacterial colonies were picked onto nitrocellulose filter papers overlaying LB agar with 120 ug/ml ampicillin. The filters were incubated at 37C overnight and the colonies lysed by sequential treatment of the filters with 10%SDS; 0.5M NaOH and 1.5M Nacl; 0.5M Tris-HCl, pH 8.0 and 1.5 M NaCl; and 2X SSPE (0.36 M NaCl; 20 mM NaH2PO4, pH 7.4; 2 mM EDTA, pH 7.4). After baking for 2 hrs at 80C, the filters were treated with prehybridization buffer comprised of 6X SSPE, 50% formamide, 0.5% SDS, 1% glycine, 5X Denhardt's solution, and 200 ug/ml denatured salmon sperm DNA. Hybridization

was performed at 42C for 12 hours with the same buffer without glycine and containing approximately $1X10^6$ CPM/ml of the 32P-labeled probe.

Plasmid DNA preparation. Two techniques were employed for either the analysis of plasmid DNA by agarose gel electrophoresis or for the production of large quantities of DNA. Overnight bacterial colonies were treated with an alkaline/SDS craking buffer for direct gel electrophoresis (Kurath, 1985), whereas the boiling method of Holmes and Quigley (1981) was used for large scale DNA purification.

Restriction enzyme analysis. Restriction maps were determined for those transformants positively identified by colony blots using viral cDNA probes. An initial size screening was performed for the largest transformants. Pstl was used to release the entire tailed insert from pUC8. Sizing was performed by electrophoresis in 1% agarose gels in Tris-acetate buffer (Maniatis et al., 1982) using HindIII lambda DNA as markers. Afterwards, up to 20 different restriction enzymes were used to determine the location of overlapping sequences. Enzyme reactions were performed as described by the the manufacturer using 500 nanograms (ng) of plasmid DNA.

Cross hybridization analysis. Recombinant plasmids were cleaved with Pstl and electrophoresed in horizaontal 1% agarose gels as above to separate the cDNA sequences from pUC8 vector DNA. The DNA was then transferred to nitrocellulose filter paper by the method of Southern (1975) and the filter baked at 80C for two hours under vacuum. A nick-translated probe was prepared from a particular transformant and hybridized to the filter as described above. One to two ug of plasmid DNA was added to a 50 ul reaction containing 50 mM Tris-HC1, pH 7.5; 20 mM MgC12; 2 mM BME; 40 uM each dATP, dCTP, dGTP; 2 uM 32P-TTP (300 Ci/mM); 10u E. coli DNA Pol I; and DNase titrated for an optimal reaction time of 15 minutes at 15C. The labeled probe was passed over a Sephadex G-50 column prior to hybridization.

Non-denaturing RNA gel electrophoresis. IPNV genome RNA's were electrophoresed for 15 hrs at 100V in 5% polyacrylamide gels (Laemmli, 1970) in Tris-acetate buffer. The RNA was silver stained by the method of Allen (1980).

Northen blot analysis. RNA denaturing gels were run as described by Thomas (1982) with slight modifications for IPNV RNA. Both labeled and unlabeled genomic RNA's were denatured with glyoxal and electrophoresed in agarose gels

followed by transfer to nitrocellulose paper using 20X SSPE. Denaturation was performed for 1 hour at 65C in a solution containing 1.8M deionized glyoxal, 50% DMSO, and 10 mM phosphate buffer (PB) (pH 7.0). The denatured RNA was loaded into a 1.2% agarose gel in 10 mM PB (pH 7.0), and electrophoresed at 80V for 4-6 hours with circulating buffer.

Sp viral RNA markers were labeled prior to denaturation with cytidine 3',5'-bis(phosphate) (pCp). Approximately 1 ug of dsRNA was incubated with 0.4-1.0ug T4 RNA ligase in 50 mM HEPES, pH 7.5; 18 mM MgCl2; 3 mM DTT; 0.1 mM ATP; 10 ug/ml BSA; 10% DMSO; and 50 uCi pCp (sp. act. = 2900 Ci/mM) for several hours at 4C.

After transferring the RNA's to nitrocellulose filters, the lanes containing the unlabeled RNA were separated from the pCp labeled sample and the filter prepared for hybridization as described for colony blots, above. Nick-translated probes of individual cDNA plasmids were prepared and hybridized as described earlier.

In vitro RNA synthesis. Overlapping cDNA sequences for each segment of Sp IPNV were subcloned into the RNA polymerase plasmids pT71 or pT72 for the production of ssRNA (Huang et al., 1985b). Single stranded RNA's were synthesized using 32P-ATP. The single stranded RNA's were electrophoresed alongside 32P-labeled genomic RNA in a

glyoxal gel, as described above, after denaturation at 65C for either one or three hours. The gel was dried onto filter paper and exposed to Kodak XAR5 film at -70C using an intensifying screen.

RESULTS

Molecular cloning of Sp IPNV. The overall strategy for the cloning of the dsRNA genome of IPNV is outlined in Figure III.1. An autoradiograph of the first strand cDNA synthesis is shown in Figure III.2. This alkaline denaturing gel indicates that while the majority of the molecules produced by reverse transcriptase are less than full length, complete synthesis of both strands of RNA did occur (lane 2). The same analysis after second strand synthesis showed no significant change in the overall size distribution of the cDNA (not shown). The entire mixture of dscDNA was used to transform SC181 cells and resulted in 553 ampicillin resistant transformants. Colony blot analysis was performed using a cDNA probe prepared from Sp genomic RNA to identify transformants containing viral sequences. Each of these transformants was analyzed by the toothpick-alkaline lysis screening method and the largest plasmids were chosen for cross-hybridization and restriction enzyme analysis.

Cross-hybridization. Repeated cross-hybridization analysis was performed using different nick-translated plasmids as probes. The resulting hybridization patterns led to the identification of two families of related inserts. The individual inserts were then digested with up to 20

restriction endonucleases to form a preliminary restricion map (Figures III.3,4). Each set is represented by a minimum of two overlapping clones, although other smaller clones were obtained which lie within the boundries established by the two largest clones. The largest set of clones consists of pSp551 and pSp413 whose overall length is approximately 2.9 kilobases (kb). The second set of clones contains pSp424 and pSp234 and measures approximately 2.6 kb.

Northern blot analysis. Nick-translated probes were prepared from each of the overlapping plasmids and used to determine their relationship to genomic segments A and B. Each set of overlapping clones hybridized to only one of the genomic segments (Figure III.5): clones pSp551 and pSp413 hybridized to segment A RNA, while pSp 424 and pSp234 hybridized to segment B RNA.

Size analysis of overlapping cDNA clones. We report in the accompanying paper (Huang et al., 1985b) the subcloning of segment A and B cDNA's into the T7 RNA polymerase plasmids pT71 and pT72. It was determined by in vitro translation analysis that ssRNA's from pT71/A and pT72/A represent the (-) and (+) strands, respectively, and that the RNA from pT72/B represents the (+) strand. We compared here the electrophoretic mobility of these ssRNA's with

those of the genomic RNA under glyoxal denaturing conditions (Figure III.6). The ssRNA's (synthesized using 32P-ATP) and pCp labeled genomic RNA were denatured for either one or three hours at 65C with glyoxal and then electrophoresed for five hours at 80V. The electrophoretic mobility of the RNA's clearly differs with respect to the time of denaturation at 65C. Genomic RNA from both the one and three hour time samples separates into (+) and (-) strands, although both the degree of separation and the rate of migration are greater for the three hour sample. The electrophoretic mobility of the in vitro synthesized ssRNA's was also greater for the three hour time point.

The distance of migration for each band was measured and a comparison was made between the genomic RNA and ssRNA samples (Table 1). The three ssRNA's denatured for one hour and their corresponding bands from the genomic RNA denatured for three hours have identical migration patterns.

Comparison of RNA's from different IPNV strains. Purified viral RNA's from 5 strains of IPNV were compared by polyacrylamide gel electrophoresis (PAGE). The relative banding patterns for VR299, Ab, and Sp (Figure III.7) are similar to those reported by Hedrick et al. (1983a). Among the strains of Sp analyzed, two of these (SpS and SpCS) are sensitive to a component of the 6S serum fraction of

normal rainbow trout (RBT) serum (Dorsen, 1974, 1975). This sensitivity appears after several passages of the virus in fish cells in the absence of RBT serum. Subsequent addition of normal RBT to the culture media at dilutions ranging from 1:100 to 1:500 neutralizes viral infectivity *in vitro*. Resistance to normal RBT serum can be maintained in a given strain by cultivating the virus in the presence of normal RBT serum. Such strains (SpR) retain normal titre levels as well as virulence in fish. It is therefore of fundamental interest that the electrophoretic mobility of segment A RNA is significantly slower for the SpR strain (Figure III.7, lane 5) than for the 6S sensitive strains (Figure III.7, lanes 3&4), particularly since segment A encodes the major structural proteins of the virus.

Comparison of VR299, Ab, Buhl, and Sp by Northern blot analysis. RNA from IPNV strains VR299, Buhl, and the 6S sensitive and 6S resistant strains of Sp were denatured with glyoxal and used to prepare two identical nitrocellulose filters for Northern blot analysis (Figure III.8). One filter was hybridized to a nick-translated probe prepared from pT71/A (which contains the entire segment A cDNA sequence). The second was hybridized to a similar probe prepared from pT72/B (which contains the entire cDNA sequence of segment B). The results of each

probe were the same in that hybridization occurred with the RNA's from SpCS and SpR strains only. The RNA's from VR299 and Buhl, which are serologically distinct strains from North America, did not hybridize to either one of the Sp cDNA probes. These results provide support at the molecular level for the classification of these strains of IPNV by serological methods. They also indicate that minor changes in the nucleic acid sequences, if any, are responsible for the development of sensitivity to normal RBT serum since RNA from both SpR and SpCS strains strongly hybridized to these probes.

DISCUSSION

The cloning of the genomic RNA from Sp IPNV led to the construction of two groups of overlapping cDNA clones. All inserts within each group are related to each other by cross-hybridization analysis. In addition, each set of inserts is related to only one of the two genomic RNA segments by Northern blot analysis. The inserts from these groups have been designated segments A and B according to the terminology first established by Dobos (1981).

Restriction maps of the two cDNA segments were determined by comparing both the restriction and cross-hybridization patterns of individual cDNA inserts. Measurements of the resulting overlapping fragments (Figures III.3,4) give an estimated size of 2.9 kb for segment A and 2.6 kb for segment B. These values are significantly smaller than those which would be predicted from a previous report by Hedrick et al. (1983a). They compared the dsRNA's from VR299, Ab, and Sp in polyacrylamide gels and estimated the molecular weights of Sp segments A and B to be 2.2 and 2.0 megadaltons, respectively. Using 640D/base pair of RNA as a conversion factor, these values predict that segments A and B would be 3.75 and 3.44 kb, respectively, or approximately 850 bp larger than the cDNA clones in this study.

We have performed a similar PAGE gel analysis of

dsRNA's using VR299, Ab, and three strains of Sp IPNV (Figure III7). The relative migration pattern of the three serotypes is identical to that seen by Hedrick et al. (1983a) so we assume that our viral stocks of Sp are similar. Therefore, we cannot use restriction fragment size estimates to verify the full length cloning of dsRNA.

An alternative method was used to compare the cDNA clones to the genomic dsRNA by using in vitro synthesized ssRNA. T7 RNA polymerase was used to synthesize both the (+) and (-) strands from cDNA segment A and the (+) strand from cDNA segment B. Under appropriate denaturing conditions using glyoxal and DMSO, all three ssRNA's comigrated with their corresponding strands of genomic RNA (Figure III.6 and Table 1).

Since the electrophoretic mobility of all RNA samples increased with prolonged incubation, we assume that the covalent binding of glyoxal adds a net negative charge to the molecule proportional to the number of glyoxal adducts. This charge effect must be the primary determinant of electrophoretic mobility even though glyoxal also removes secondary structure. The fact that the genomic RNA required a longer period of time to attain the same degree of denaturation as the in vitro ssRNA's is thought to be due to its double-stranded configuration. With dsRNA, the guanosine residues are initially protected from glyoxal due to interstrand hydrogen bonding. Some

additional incubation time is therefore needed to overcome interstrand bonding such that the four genomic strands are as available to covalent binding by glyoxal as are the ssRNA samples.

The separation of IPNV dsRNA into (+) and (-) strands by glyoxal denaturation is somewhat surprising in comparison to other dsRNA viruses. Smith et al., (1981) performed similar experiments using the genomic RNA's from cytoplasmic polyhedrosis virus (from silkworms) and human reovirus. In that study, both genomic and mRNA's were treated with glyoxal and electrophoresed in 7M acid urea gels. In the case of both viruses, the genomic and mRNA's comigrated at the same positions with no separation between (+) or (-) strands. They could, however, attain separation of (+) and (-) strands by using 7M acid urea gels without glyoxal, an effect they attribute to differences in secondary structure. Either the neutral pH of the glyoxal gels used in this study creates a charge effect that would be neutralized at the low pH (3.5) of the acid urea gels, or there is some inherent difference in the nature of the dsRNA's of reoviruses and birnaviruses.

We have not performed an S1 analysis to determine the presence of genomic 5'- and 3'-termini, but our results here strongly suggest that we have cloned the entire sequence of dsRNA. The synthesis of ds cDNA should have

included the terminal ends of each segment of RNA as long as poly-A tails were added to full length molecules and not internal fragments. The second strand replacement synthesis should fail to copy only a few bases located internally (due to the priming by residual RNA) but once primed, continue to synthesize DNA in a 5'-3' direction all the way through the end of the oligo-dt primer present on the first strand of cDNA. Any internal sequences that were missed by this method would be recovered when two oppositely oriented clones overlap, as they have here for both segments A and B. Further data to support the full-length cloning of genomic RNA comes from the accompanying paper demonstrating the synthesis of all four viral proteins from in vitro synthesized ssRNA (Huang et al., 1985b).

The Sp serotype of IPNV was chosen for this study for several reasons:(1) it is one of the most highly virulent forms of the virus; (2) it grows to high titers in our laboratory; and (3) it has been shown to produce a tissue culture variant that is sensitive to a 6S component of normal rainbow trout serum (Dorsen, 1974, 1975). We intend to clone both the 6S sensitive and 6S resistant strains of Sp INPV in order to identify the nucleotide sequences which determine virulence. It is assumed that a virulence factor(s) resides with the outer capsid protein since it comprises over 60% of the viral protein (Dobos, 1977) and

elicits neutralizing antibody in rabbits (Stephens, pers. comm.). The stock of Sp IPNV used in this study is a 6S sensitive variant which was derived from repeated passage through CHSE-214 cells.

Having obained cDNA clones for the 6S sensitive strain of Sp, we have used 32P-nick-translated probes to test for similarities among the gemonic RNA's of VR299, Buhl, and the 6S resistant strain of Sp. The cDNA probes from segments A and B strongly hybridized only to RNA from the two strains of Sp (Figure III.8). These results support our hypothesis that there are only minor nucleotide differences, if any, that affect 6S serum sensitivity and virulence in strains of Sp IPNV. Significant is the fact that these cDNA probes did not cross-hybridize to either VR299 or Buhl. Eventhough these two strains are serologically distinct from both Sp and each other, we had expected some homology between the RNA sequences of the viral polymerase, and to some extent the remaining viral proteins. We are in the process of examining these hybridization patterns under less stringent conditions in order to detect lower levels of homology.

We have observed that the segment A dsRNA of the SpR strain migrates slightly higher than that of the SpS strain in TAE polyacrylamide gels (Figure III.7, lanes 4 and 5). If this difference represents a deletion in the

RNA sequence and not simply a charge effect, then the SpS variant may have a replicative advantage over the SpR strain that allows it to become the predominant strain after several passes in tissue culture. Its sensitivity to the 6S fraction of normal rainbow trout serum may develop from a change in the amino acid sequence of the major capsid protein (or other minor proteins). We are currently examining the nucleotide sequence of our cDNA clones and intend to make comparisons of the SpS and SpR strains at both the nucleotide and amino acid level.

Figure III.1. Strategy of the procedures used in the cDNA cloning of IPNV dsRNA.

Genomic RNA was first isolated from purified virus. Homopolymer tails of poly-adenylic acid were added to the 3'-ends of each strand of dsRNA using poly-A polymerase. First strand cDNA synthesis was performed using an oligo-dT primer and avian myeloblastosis virus reverse transcriptase. Second strand cDNA synthesis was carried out by the combination of RNaseH (specific for RNA/DNA hybrids), E. coli DNA polymerase I, and E. coli DNA ligase. Complementary homopolymer tails were added to the 3'-ends of the ds cDNA and plasmid vector pUC8 linearized with PstI. This allowed for reannealing of poly-dC tailed cDNA fragments to poly-dG tailed pUC8 DNA and subsequent transformation into competent cells of E. coli strain SC181.

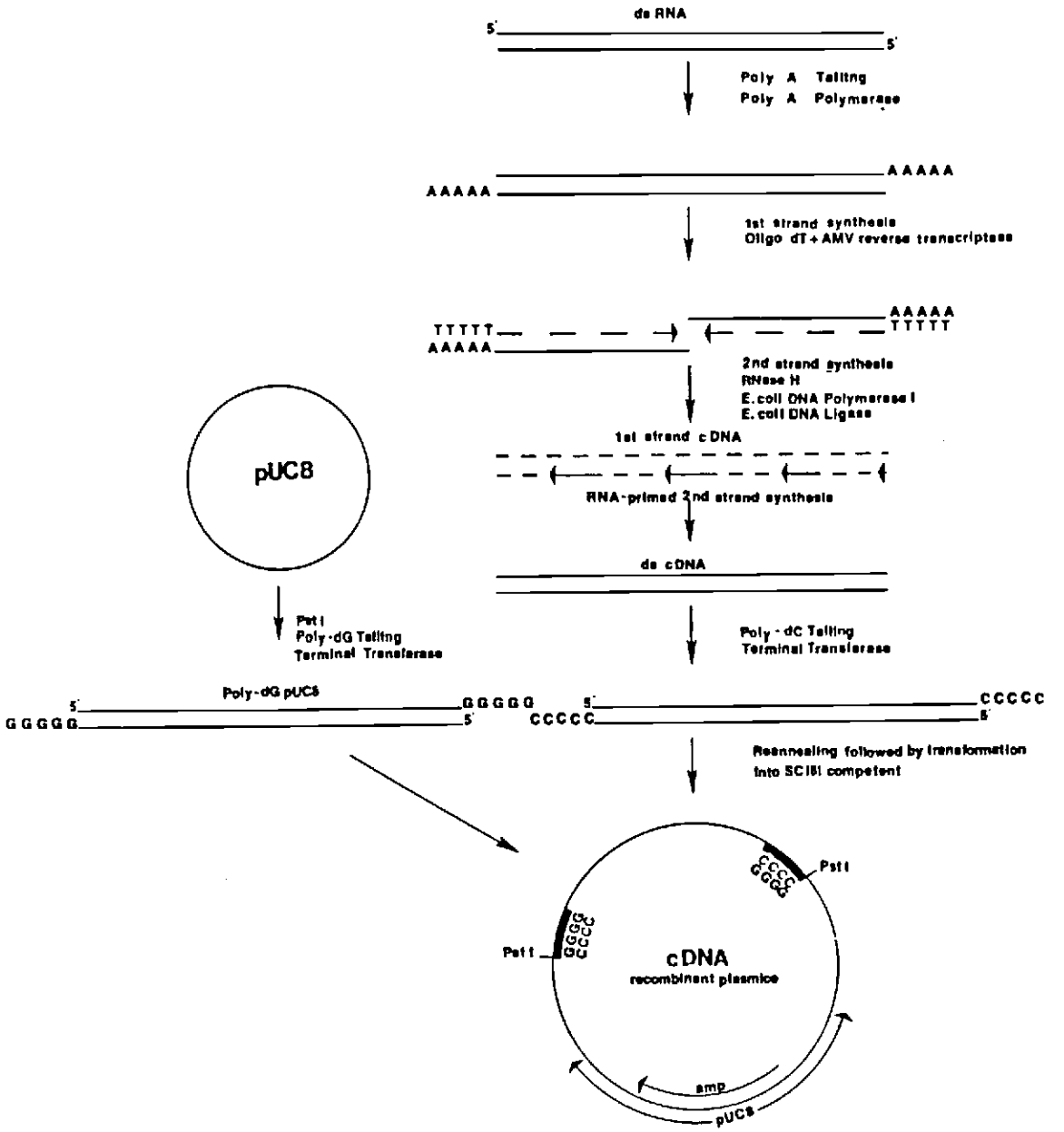

ds RNA
5'
5'
Poly A Tailing
Poly A Polymerase
AAAAA
AAAAA
1st strand synthesis
Oligo dT + AMV reverse transcriptase
TTTTT
AAAAA
AAAAA
TTTTT
2nd strand synthesis
RNase H
E.coli DNA Polymerase I
E.coli DNA Ligase
1st strand cDNA
RNA-primed 2nd strand synthesis
ds cDNA
pUC8
Pst I
Poly-dG Tailing
Terminal Transferase
Poly-dC Tailing
Terminal Transferase
Poly-dG pUC8
GGGGG
CCCCC
Reannealing followed by transformation
into SCI8i competent
Pst I
Pst I
cDNA
recombinant plasmice
amp
pUC8

Figure III.1

Figure III.2. Autoradiogram of first strand cDNA synthesis.

A sample of the 32P-labeled cDNA synthesis of viral RNA was electrophoresed in a 1% alkaline agarose gel. (1) P32-labeled PstI cut lambda DNA, (2) first-strand cDNA synthesis. Arrows indicate two bands presumed to represent full-length cDNA synthesis of viral segments A and B.

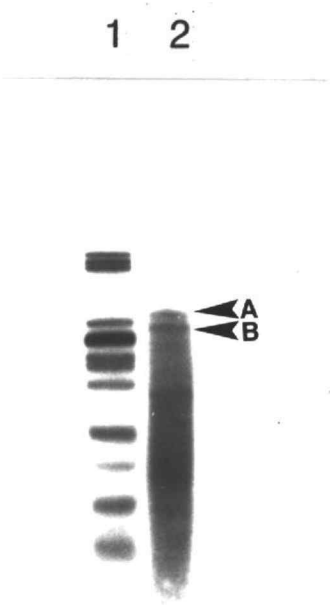
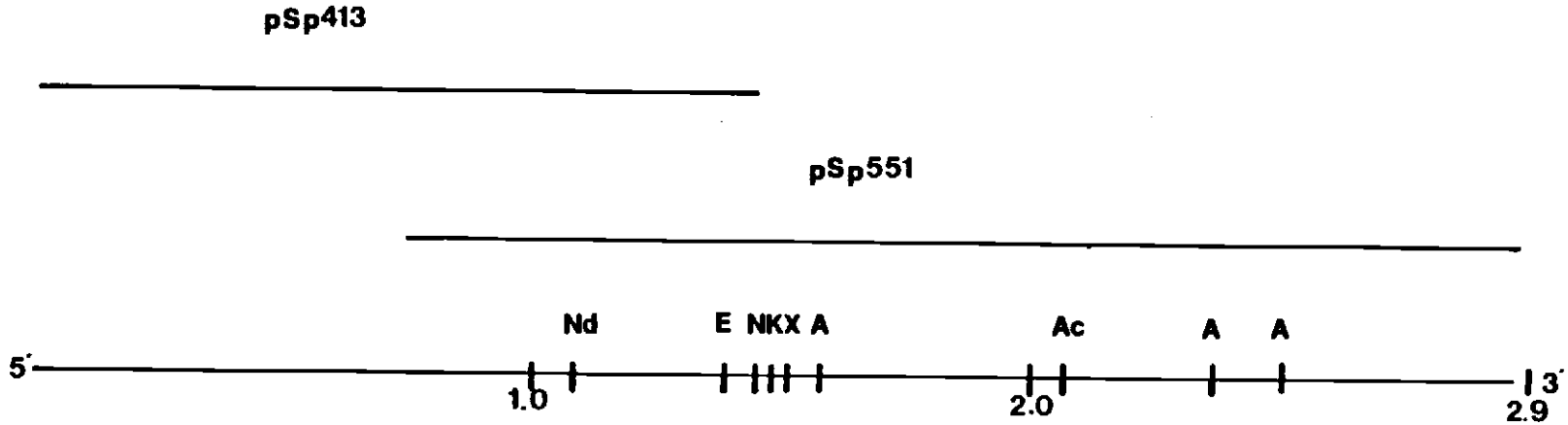


Figure III.2

Figure III.3. Partial restriction map of cDNA clones from genomic RNA segment A.

The two recombinant plasmids pSp413 and pSp551 contain overlapping sequences from segment A RNA. Each plasmid was digested with single and/or multiple enzymes and electrophoresed in 1.0% agarose or 4% NuSieve agarose gels (FMC). The gel was stained with ethidium bromide and the sizes of the individual DNA fragments were estimated from HindIII lambda DNA markers.

Figure III.3



PARTIAL RESTRICTION MAP OF Sp cDNA CLONES

SEGMENT A

A=Ava I Ac=Acc I E=EcoRI K=Kpn I N=Nco I
Nd=Nde I X=Xho I

Figure III.4. Partial restriction map of cDNA clones from genomic RNA segment B.

The two recombinant plasmids pSp234 and pSp424 contain overlapping inserts from segemnt B RNA. Each plasmid was digested with single and/or multiple enzymes and electrophoresed in 1.0% agarose or 4% NuSieve (FMC) agarose gels in Tris-acetate EDTA buffer. The gel was stained with ethidium bromide and the sizes of the individual DNA fragments estimated from HindIII lambda DNA markers.

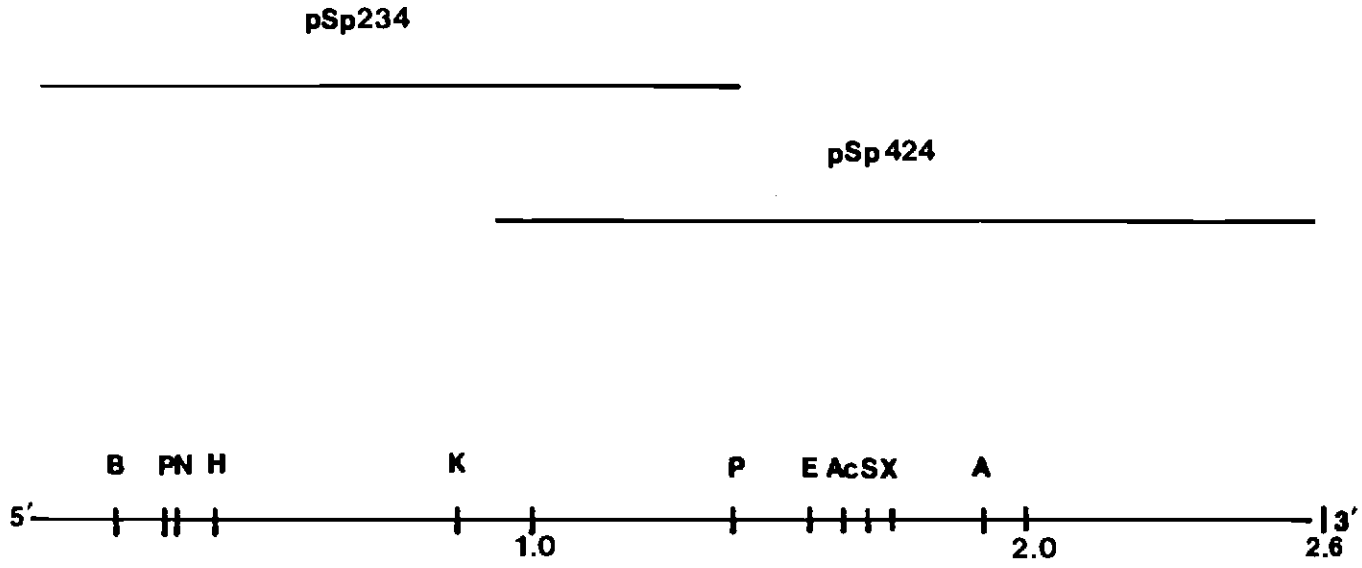


Figure III.4

PARTIAL RESTRICTION MAP OF Sp cDNA CLONES:
SEGMENT B

A=Ava I Ac=Acc I B=BamHI E=EcoRI H=Hind III
K=Kpn I N=Nco I P=Pst I S=Sst I X=Xho I

Figure III.5. Northern blot analysis of cDNA clones.

Two sets of cDNA inserts were defined by Southern blot analysis to be distinct from one another. The individual recombinant plasmids were labeled with 32P-nucleotides by nick-translation and hybridized to genomic RNA which had been denatured by glyoxal gel electrophoresis and transferred to nitrocellulose filters. A: northern blot analysis using 32P-probes of pSp551 (lane 2) and pSp413 (lane 3). Genomic dsRNA was labeled with pCp using T4 RNA ligase (lane 1). Both probes hybridized to segemnt A RNA only. B: a similar analysis using 32P-probes of pSp234 (lane 2) and pSp424 (lane 3). RNA markers were the same as III.5A (lane 1). Both of these probes hybridized to segment B only.

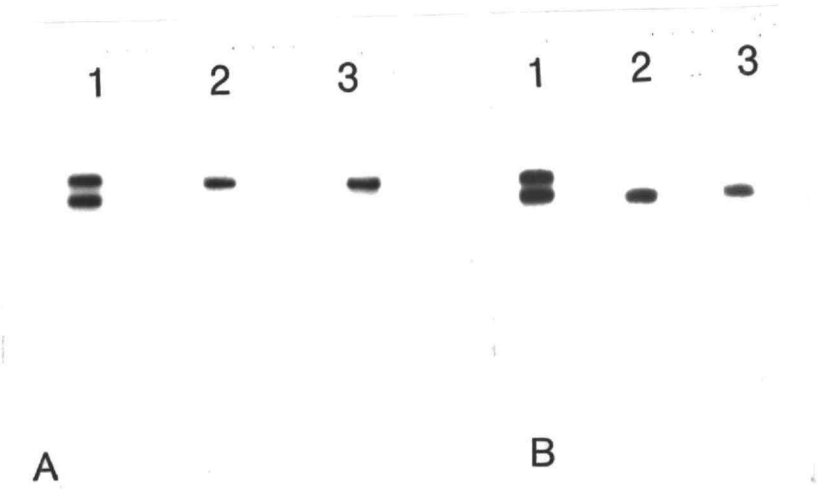


Figure III.5

Figure III.6. Autoradiogram of glyoxal denaturing gel comparing IPNV dsRNA and ssRNA originating from cloned viral sequences.

Complementary DNA sequences from segments A and B were subcloned into the T7 RNA polymerase vectors, pT71 and pT72. Three subclones were used to prepare ssRNA using T7 RNA polymerase: pT71/A and pT72/A, which contain cDNA from segment A in opposite reading orientations; and pT72/B, which contains cDNA from segment B. Each of these plasmids was linearized at a restriction site within the polylinker region immediately downstream from the 3'-insertion site of the cDNA. 3H-labeled ssRNA was then synthesized using T7 RNA polymerase which produced continuous run off copies of the cloned DNA sequences. Double- and single-stranded RNA's were added to solutions containing 1.8M glyoxal, 50% DMSO, and 10 mM phosphate buffer (pH7.0) and denatured at 65C for either one or three hours. The samples were added directly to a 1.2% agarose gel in 10mM phosphate buffer and electrophoresed for approximately four hours at 90mA. (1) IPNV dsRNA heated for 1 hr, (2) IPNV dsRNA heated for 3 hrs, (3) ssRNA (pT72/B) heated for 3 hrs, (4) ssRNA (pT72/A) heated for 3 hrs, (5) ssRNA (pT71/A) heated for 3 hrs, (6) ssRNA (pT72/B) heated for 1 hr, (7) ssRNA (pT72/A) heated for 1 hr, (8) ssRNA (pT71/A) heated for 1 hr.

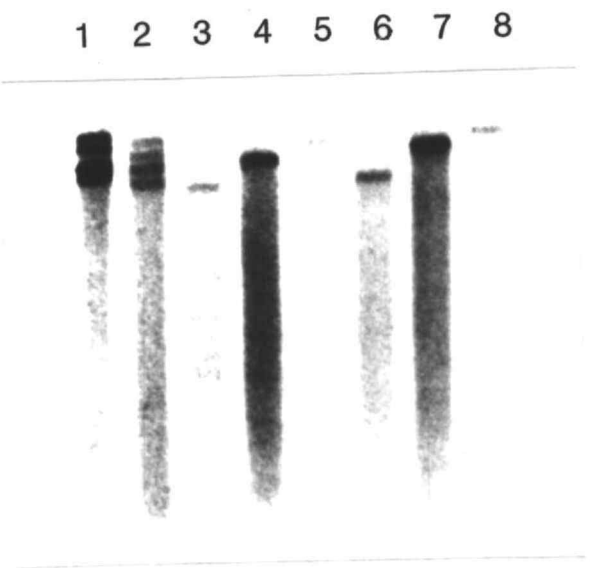


Figure III.6

Figure III.7. Non-denaturing polyacrylamide gel containing genomic RNA from 5 strains of IPNV.

Double-stranded RNA was isolated from purified virus and electrophoresed in a 5% polyacrylamide gel in Tris-acetate EDTA buffer at 100V for 20 hrs at 4C. The RNA was silver stained by the method of Allen (1980). (1) Ab, (2) VR299, (3) SpCS, (4) SpS, (5) SpR.

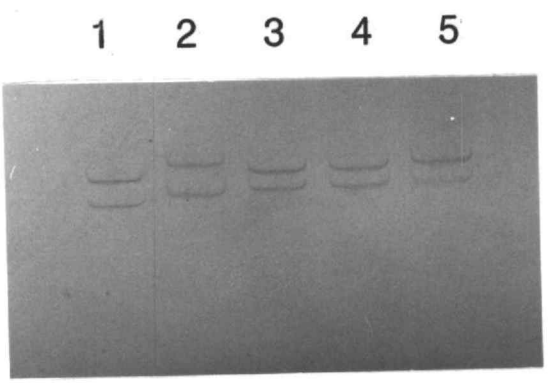


Figure III.7

Figure III.8. Northern blot analysis comparing the cross-hybridization patterns of Sp cDNA to the genomic RNA's of four strains of IPNV.

Genomic RNA's were isolated from purified virus and markers were prepared by labeleing the 3'-termini with pCp using T4 RNA ligase. Both labeled (60,000CPM) and unlabeled RNA's (700ng/well) were denatured for 1 hr at 65C in a solution containing 1.8M glyoxal, 50% DMSO, and 10 mM phosphate buffer (PB) (pH 7.0). Samples were loaded directly into a 1.2% agarose gel in PB and electrophoresed for approximately 4 hours. The RNA was transferred to nitrocellulose filters using 20X SSPE and hybridized with nick-translated probes prepared from either segment A (pT71/A) or segment B (pT72/B) cDNA. (1) pCp-labeled VR299, (2) pCp-labeled Ab, (3) pCp labeled SpCS. Lanes 4-7 and 8-11 represent duplicate filters: lanes 4-7 were hybridized with the probe from segment A, while lanes 8-11 received the probe from segment B. (4 and 10) SpCS, (5 and 11) SpR, (6 and 8) VR299, (7 and 9) Buhl.

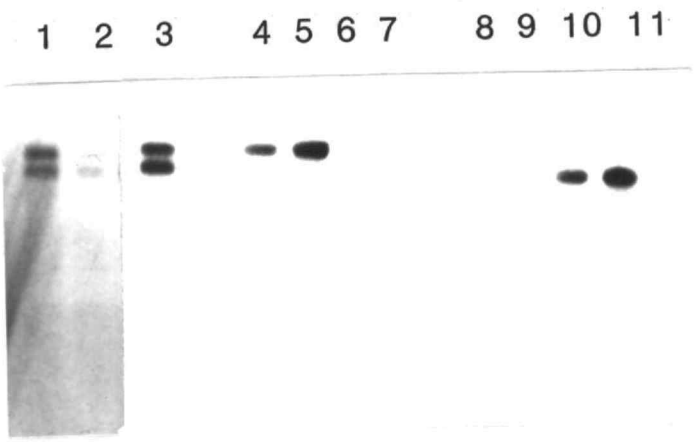


Figure III.8

Table 1. Comparison of the electrophoretic mobility of glyoxal denatured genomic RNA and ssRNA synthesized from viral cDNA by T7 RNA polymerase. Values represent distance of migration from the origin (mm). RNA samples were denatured at 65C for either 1 or 3 hours.

Sense strand of genomic RNA	dsRNA, 1 hr.	dsRNA, 3 hr.	ssRNA, 1 hr.	ssRNA, 3 hr.
Segment A (-)	69.7	70.7	70.7	71.7
Segment A (+)	71.5	72.5	72.5	74.0
Segment B (+)	75.5	77.0	77.0	77.6

IV. CELL-FREE TRANSLATION ANALYSIS OF IPNV cDNA

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is a primary viral pathogen of hatchery-reared rainbow and brook trout (for review, see Fryer and Pilcher, 1980; Huang et al., 1985a). It belongs to the proposed family Birnaviridae (Dobos, 1977, Fields, 1985), members of which contain two segemnts of double-stranded RNA. The coding assignments for the two segments have been determined by genetic reassortment (MacDonald and Dobos, 1981) and by analysis of the proteins synthesized *in vitro* from purified viral RNA (Mertens and Dobos, 1982). These two studies have determined that segment A RNA encodes three proteins while segment B encodes the putative viral RNA polymerase.

The three major serotypes of IPNV are VR299, Sp, and Ab. VR299 is the type strain for North America, and Sp and Ab are the predominant strains found in Europe. These serotypes can be distinguished by variations in the size of their RNA's as well as the size and relative proportions of the capsid proteins.

We have reported in the accompanying paper the cDNA cloning of a strain of Sp IPNV (SpCS) which is sensitive to a rainbow trout serum protein of 6S (Huang et al., 1985a). We confirm here that these clones contain the entire coding regions of the genomic RNA. The cloned viral

sequences of RNA segments A and B were subcloned into plasmid vectors, pT71 and pT72. These vectors are designed so that cloned DNA sequences may be transcribed into RNA from a T7 RNA polymerase promoter site. This technique yielded IPNV ssRNA that was active in a cell-free translation system. We describe here the proteins encoded by the cDNA clones and compare the in vitro translation products with those of purified virions and viral proteins synthesized within the first 8 hours of infection. In addition, we show that significant differences exist between the major capsid proteins of the 6S sensitive (SpCS) and 6S resistant (SpR) strains of Sp, and discuss these differences in terms of RNA coding capacity, virulence, and antigenicity.

METHODS

Virus stocks, growth, and purificaton. The strains of Sp IPNV used in this study are described in the accompanying paper (Huang et al., 1985a). Virus growth and purification are described elsewhere (Huang et al., 1985a). The SpCS strain was cultivated in CHSE-214 cells without the addition of normal rainbow trout serum, while the SpR and Buhl strains were passed in tissue culture in the presence of 1:150 normal rainbow trout serum.

35S-methionine labeling of viral proteins. Monolayers of CHSE-214 cells were infected with virus at a multiplicity of infection of between 0.05 and 0.1. At 40-42 hours post-infection, the media was removed and replaced with minimal essential media without serum or methionine, and supplemented with 70 uCi 35S-met. The infection was allowed to proceed to complete cytopathic effect (CPE) and the virus harvested as described elsewhere (Huang et al., 1985a).

Polyvalent antiserum to Sp IPNV. Intact virus was purified twice in equilibrium CsCl gradients (Huang et al, 1985) and dialyzed against 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM EDTA (TNE). One-half ml of virus (1 mg/ml)in TNE was mixed thoroughly with an equal volume of Freund's

complete adjuvant and injected subcutaneously at four locations around a rabbit's neck biweekly for a total of 6 weeks. At the end of 8 weeks, the rabbit was sacrificed and the blood withdrawn by cardiac puncture. The whole blood was stored at 4C overnight to permit clotting. The clot was removed by centrifugation at 5K RPM for 45 minutes in a Sorvall GSA rotor. Serum was collected and stored at -70C. Prior to use, the serum was preadsorbed twice on monolayers of CHSE-214 cells.

Reagents. All restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories. Calf intestinal phosphatase was purchased from Boehringer Mannheim Biochemicals. The pT7 plasmids and T7 RNA polymerase were purchased from U.S. Biochemicals. The preparation of IPNV cDNA clones is described elsewhere (Huang et al., 1985a). 35S-methionine and 32P-ATP were acquired from New England Nuclear, while 3H-ATP was purchased from ICN. Promega Biotec was the source of RNasin and the rabbit reticulocyte lysate *in vitro* translation kit used in this study.

Plasmid DNA purification. Details for small and large scale plasmid DNA isolation are described elsewhere (Huang et al., 1985a).

3H-labeled VSV mRNA. Baby hamster kidney cells (BHK-21) were infected with vesicular stomatitis virus in the presence of 3H-uridine. Cell monolayers were infected with a 1:20 dilution of stock virus for one hour at which time the virus was replaced with minimal essential media supplemented with 5% fetal bovine serum and 0.5 ug/ml actinomycin D. At 2.5 hours post-infection, 3H-uridine was added to a final concentration of 15 uCi/ml. When ca. 25% of the cells exhibited CPE, the cells were harvested and total RNA extracted by the guanidinium/cesium chloride method of Maniatis et al. (1982). Poly-adenylated RNA was purified by oligo-dT column chormatography (Maniatis et al., 1982) and served as markers in agarose gel electrophoresis.

Subcloning of cDNA segments A and B into pT7 vectors. Restriction maps of the cDNA fragments for RNA segments A and B are presented in the accompanying paper (Huang et al, 1985). The two overlapping fragments which cross-hybridized to IPNV segment A RNA were ligated together at the common EcoRI site and then subcloned into plasmid vectors, pT71 and pT72 (Fig. 1). Plasmids pSp413 and pSp551 were cleaved with PstI and BamHI, respectively. The free ends were phosphatased using 0.5 units calf intestinal phosphatase (CIP) in a final volume of 20 ul of 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, containing 10 ug

plasmid DNA. The reaction was incubated at 30C for 30 minutes afterwhich a second 0.5u of CIP was added. Following an additional 30 minutes, EDTA was added to 10 mM and the reaction heated at 68C for 45 minutes. The DNA was extracted once with phenol/chlorophorm:IAA and ethanol precipitated. Both DNA's were then cleaved with EcoRI and gel purified by electroelution using ISCO gel chambers. Individual DNA fragments were sliced out of a 1% agarose gel and electrophoresed into an ISCO chamber in Tris-borate buffer (Maniatis, et al., 1982) using 7 mA current for 20-60 minutes. The DNA's were phenol/chloroform extracted as above and ethanol precipitated. The two fragments were mixed at a 1:1 molar ratio and ligated with 1.5 units of T4 DNA ligase in 20 ul containing 66 mM Tris-HCl (pH 7.6), 7 mM MgCl2, 10 mM DTT, 0.4 mM ATP, and 0.2 mg/ml BSA. Plasmid vectors pT71 and pT72 (cut with BamHI and PstI) were each added to one-half the ligated segment A DNA in the presence of phenol/chloroform:IAA and the extraction continued as described earlier. After precipitation in ethanol, the mixed DNA's were ligated and transformed into fresh competent SC181 cells which were plated onto LB agar plates supplemented with 120 ug/ml ampicillin. Transformants were screened by the toothpick/alkaline lysis method (Kurath, 1985) and later verified to contain the appropriate orientation using restriction enzyme analysis. The resulting subclones were designated

pT71/A and pT72/A. All other subclones were prepared in a similar manner using electroelution to obtain the desired restriction fragments.

The two cDNA inserts from plasmids pSp234 and pSp424, which are related to segment B by Northern blot analysis, were ligated at the common KpnI site and inserted into pT72 (Fig 2). The insert from pSp424 was first subcloned into pT72 by digesting both of the DNA's with SalI and HindIII. The individual fragments were gel purified as described above, phenol/chloroform extracted, and precipitated in ethanol. Insert DNA was added to vector DNA at a molar ratio of at least 4:1, ligated, and transformed into SC181 cells. The resulting transformants contained pT72 plasmids containing the Sall/HindIII fragment of pSp424 and were designated pT72/424.

The pT72/424 plasmid was used as the recipient plasmid/vector for the Kpnl/HindIII fragment of pSp234. pSp234 was partially digested with HindIII and released from the remainder of the plasmid with Kpnl. The 900 bp fragment bordered on one side by Kpnl and containing both HindIII sites of pSp234 was gel purified and inserted into pT72/424 cut with KpnI and HindIII. This recombinant was designated pT72/B.

Synthesis of ssRNA. The conditions for the synthesis of ssRNA were those provided with the Genscribe Kit from U.S.

Biochemicals. The pT7 plasmids were linearized at the 3'-end of the viral inserts by an enzyme which cuts only within the polylinker region of the plasmid vector. These sites were no more than 13 bp downstream from the end of the poly-G/C tailed insert. Three micrograms of linearized plasmid DNA were resuspended in a final volume of 50 ul containing 15 mM MgC12, 20 mM Tris-HC1 (pH 8.0), 1 mM each of CTP, UTP, and GTP, 850 uM 3H-ATP (0.8 Ci/mM), 10 mM DTT, 500 ug/ml BSA, 25u T7 RNA polymerase, and 64u RNasin. The reaction was incubated at 37C for 75 minutes. Plasmid DNA was removed by the addition of 1 ug of DNase treated with proteinase K (Maniatis et al., 1982) and 37u RNasin followed by incubation at 37C for 10 minutes (Melton et al., 1984). The RNA was extracted as described above and ethanol precipitated in the preence of 0.7M sodium acetate. The RNA was ethanol precipitated again with 2% potassium acetate prior to *in vitro* translation.

RNA denaturing gels. Samples of 3H-labeled ssRNA were analyzed in glyoxal gels using 3H-labeled VSV mRNA as markers (above). Agarose gels were treated with ENHANCE (NEN) prior to autoradiography.

In vitro translation and SDS gel electrophoresis. Individual reactions were performed in 25 ul volumes. Each reaction contained 17.5 ul rabbit reticulocyte lysate, 0.5

ul of a 5 mM stock solution containing 19 amino acids less methionine, 2.5 ul 35S-methionine (Spec. act. = 1100 Ci/mM), 37u RNasin, and approximately 2 ug ssRNA in 3.5 ul water treated with diethypyrocarbonate. Double-stranded genomic RNA was denatured with methyl mercury (Azad et al., 1985) before addition to the translation mixture. The translation reactions were incubated at 30C for 90 minutes and sampled for TCA precipitable counts.

Samples from each translation reaction were added directly to a 10% polyacrylamide gel with 3% stacking gel, or immunoprecipitated with a polyvalent rabbit serum prepared against whole virus. Immunoprecipitated samples were diluted with 120 ul RIPA buffer (1% wt/vol sodium deoxycholate; 1% vol/vol triton X-100; 0.2% wt/vol SDS; 150 mM NaCl; 50 mM Tris-HCl, pH 7.4). To this was added 33ul of a 10% suspension of protein-A sepharose beads plus 1.6 ul of polyvalent anti-Sp rabbit serum. The entire mixture was rotated at 4C for 6 hrs after which the protein A-sepharose beads were pelleted and rinsed three times with 200 ul RIPA at 4C. After the third rinse, the protein A-sepharose beads were resuspended in 20 ul sample buffer and boiled for 2 minutes. The mixture was microfuged briefly to pellet the protein A-sepharose beads and the sample buffer was removed for SDS-PAGE analysis using a 10% polyacrylamide gel with 3% stacking gel (Laemmli, 1970). The gel was fixed in 7.5% acetic acid for

one hour and dried onto filter paper prior to exposure to Kodak XAR-5 film.

Pulse-labeling of Sp IPNV infected cells. CHSE-214 cell monolayers in 35mm diameter dishes were infected with 0.5 ml of Sp IPNV at an M.O.I. of ca. 100-200 for 1 hour at 18C. The virus was then replaced with 2.0 ml MEM plus 10% fetal bovine serum and incubated at 18C. At various times post-infection, the media was replaced with 2.0 ml methionine-free media for 1 hour. This media was then replaced with 0.5 ml methionine-free media supplemented with 50 uCi 35S-methionine (sp. act. = 1100 Ci/mM). After 1 hour, the media was removed and the cells rinsed twice with phosphate buffered saline. The cells were harvested with 100 ul lysing buffer (9.5 M urea, 5% beta-mercapto-ethanol, and 2% triton-X). Samples were counted for incorporated 35S-methionine and analyzed by SDS PAGE. Uninfected control cells were labeled and harvested in an identical manner.

RESULTS

Construction of pT7 subclones. Overlapping cDNA fragments, which are related to either RNA segment A or B by Nothern blot analysis, were ligated together at common restriction sites and subcloned into the T7 RNA polymerase plasmids, pT71 and/or pT72. The cDNA inserts from pSp551 and pSp413 (segment A) were joined at a single overlapping EcoRI site and subcloned into the PstI and BamHI sites of pT71 and pT72 (Figure IV.1). The inserts from pSp234 and pSp424 (segment B) were ligated at a common KpnI site and inserted into the HindIII and Sal I sites of pT72 (Figure IV.2). Each pT7 subclone contains a cDNA insert bordered by poly-dG/dC tails and Pstl sites. The T7 RNA polymerase promoter is located just outside the polylinker region of the pT7 plasmids and adds a number of nucleotides to the 5'-end of the single-stranded RNA produced by T7 RNA polymerase. For pT71/A, this represents 50 bases (b); for pT72/A, 53b; and for pT72/B, 20b.

Synthesis of ssRNA using T7 RNA polymerase. Each pT7 recombinant plasmid was cleaved at the 3'-end of the insert prior to ssRNA synthesis. The enzymes HindIII, BamHI, and SalI were used to linearize pT71/A, p72/A, and p72/B, respectively (Figure IV.3). The ssRNA's were labeled with 3H-ATP and analyzed by electrophoresis in

glyoxal gels (Figure IV.4). In each case, only one size class of RNA was produced that corresponds to repeated run off synthesis by T7 RNA polymerase.

In vitro translation products of single- and double-stranded RNA's. The ssRNA's produced from pT71/A, pT72/A, pT72/B, and the dsRNA from purified virus were added to cell-free translation reactions. The translation products of these reactions were compared by SDS PAGE in order to determine the coding capacity of the individually cloned segments. Samples were either added directly to the gel (Figure IV.5a) or immunoprecipitated with a polyvalent rabbit serum prepared against whole virus (Figure IV.5b). The major bands corresponding to full-length viral proteins are present in gels containing both the direct and immunoprecipitated samples. However, the banding patterns are more distinct for the unprecipitated proteins.

The major translation products from the dsRNA of purified Sp IPNV (Figure IV.5a and b,lane 1) are the viral RNA polymerse (A), the major capsid protein precursor (B1), the minor capsid protein (C), and the non-virion protein seen only in infected cells (D). These assignments were determined from a comparison of the translation products with the proteins found in purified virus (lane 2), as well as an analysis of the viral proteins

synthesized within the first 8 hours after infection (Figure IV.6). Infected CHSE-214 cells were pulse-labeled with 35S-methionine between 2 and 8 hours post-infection (PI). At approximately 6-7 hours PI (Figure IV.6, lane 5), three viral proteins become evident: the RNA polymerase (A), the major capsid precursor (B1), and the minor capsid precursor (C). The final viral protein (D) appears between 7 and 8 hours PI (Figure IV.6, lane 7). Purified Sp (Figure IV.6, lane 9) contains the RNA polymerase (A), the major capsid protein (B3), the minor capsid protein (C1), three other forms of the major capsid protein (B1, B2, and B4), and what appears to be the breakdown product of C1 (C2).

Single-stranded RNA from cloned segment A (Figure IV.5a and b, lane 3) produces three major proteins corresponding to the major capsid protein precursor (B1), the minor capsid protein (C1) and the non-virion protein (D). Single-stranded RNA from cloned segment B (Figure IV.5a, lane 4) directed synthesis of the viral RNA polymerase (A) plus a number of polypeptides presumed to be premature translation products. The distribution of proteins between these two RNA's is identical to that observed for the individually translated segments of genomic dsRNA of VR299 (Mertens and Dobos, 1982) and infectious bursal disease virus of chickens (Azad, 1985).

Comparison of the proteins of purified SpCS and SpR IPNV. The relative protein banding patterns of purified SpCS and SpR strains are identical with respect to the number, positioning, and extent of labeling of the proteins (Figure IV.7, lanes 1 and 2). However, the electrophoretic mobililty of both the major capsid protein and its precursor show significant differences between the two strains. The major capsid protein precursor and the first intermidiate form (bands B1 and B2) are both larger for the SpCS strain while the major form found in whole virions (band B3) is larger for the SpR strain.

DISCUSSION

By subcloning the cDNA from each segment of Sp IPNV into the T7 RNA polymerase plasmids, pT71 and pT72, we were able to synthesize full-length ssRNA's of each cloned segment (Figure IV.4). Full-length synthesis was extremely efficient when an excess of nucleotide precursors was used. Further modification of the RNA's - either by 5'-capping or 3'-poly-adenlyation - was not required in order to obtain translatable RNA. However, the translation efficiency of these RNA's was approximately 10-fold less than that of control mRNA from brome mosaic virus.

The cell-free translation products produced from the genomic dsRNA and the ssRNA's of cloned DNA were analyzed by immunoprecipitation and SDS PAGE. The four viral proteins that have been characterized for VR299 (Dobos, 1977) were shown to be encoded by both the genomic RNA and cDNA sequences of Sp IPNV. Three major classes of viral proteins are synthesized and differentially processed in VR299-infected cells. These classes represent the viral RNA polymerase (alpha), the major capsid protein (beta) and two minor proteins (gamma), one of which is found only in infected cells. The cell-free translation products of both the ssRNA's and the ds genomic RNA (Figure IV.5) form the type of banding patterns that would be expected from the synthesis of the four unprocessed precursor molecules.

Figure IV.5a&b, lane 1 contains the translation products of the ds RNA from purified SpCS virions. Four bands migrate at positions expected for the RNA polymerase (A), the major capsid precursor (B), the minor capsid precursor (C), and the non-virion protein (D). This banding pattern is identical to that reported by Mertens and Dobos (1982) for the translation of the purified dsRNA of VR299. Furthermore, translation of RNA from the individually cloned genomic segments (Figure IV.5a&b, lanes 3&4) indicates that the RNA polymerase is encoded by segment B and that the remaining three proteins reside on segment A. Thus, the genome of the Sp IPNV has the same coding arrangement as VR299, as well as the non-related birnavirus, infectious bursal disease virus (Azad et al., 1985).

All four viral proteins could be synthesized from the pT72/A and pT72/B constructions alone. It was therefore unnecessary to subclone segment B into pT71 for similar analysis. Thus, pT72/A and pT72/B represent (+) strands of each segment, while pT71/A represents the (-) strand of segment A. Comparisons between the pT7 ssRNA strands and the (+) and (-) strands of genomic RNA are reported elsewhere (Huang et al., 1985a). The cloned ssRNA's comigrate with the individual (+) and (-) strands of genomic RNA in glyoxal denaturing gels, and under these conditions the (+) strand migrates faster than the (-)

strand. Although highly unlikely, the possibility exists that there are viral protein sequences present in the pT71/B orientation of segment B.

We have commented elsewhere about the size difference between the segment A RNA's of 6S sensitive (SpCS) and 6S resistant (SpR) strains of Sp IPNV (Huang et al., 1985a). In particular, we discussed how a deletion in the RNA could result in a change in protein conformation which would make the virus susceptible to the 6S fraction of normal rainbow trout serum. When we compared the size of the proteins found in purified virus samples of the SpCS and SpR strains, we discovered differences in the relative migration patterns of both the major capsid protein as well as its precursor (Figure IV.6, lanes 2 and 1). The SpS strain produces a slightly larger precursor molecule than does the SpR strain; however, the fully processed protein - which has at least one intermdiate form (Dobos, 1977, and seen here) - is slightly smaller than that found in the SpR strain. It seems likely that this alteration in the major capsid protein is related to 6S sensitivity and thus virulence.

Figure IV.1. Subcloning of segment A cDNA into the T7 RNA polymerse vectors, pT71 and pT72.

A. The two overlapping inserts from pSp413 and pSp551 were ligated together at their common EcoRI site with T7 DNA ligase and separated from sequences of pUC8 by restriction enzymes PstI and BamHI.

B. T7 RNA polymerase vectors, pT71 and pT72, were both linearized by restriction enzymes PstI and BamHI. Segment A cDNA was ligated to each of the two plasmids with T4 DNA ligase and transformed into E. coli strain SC181. The resulting recombinants were labeled pT71/A and pT72/B and contain the cDNA from segment A in opposite reading orientations with respect to the promoter site for T7 RNA polymerase.

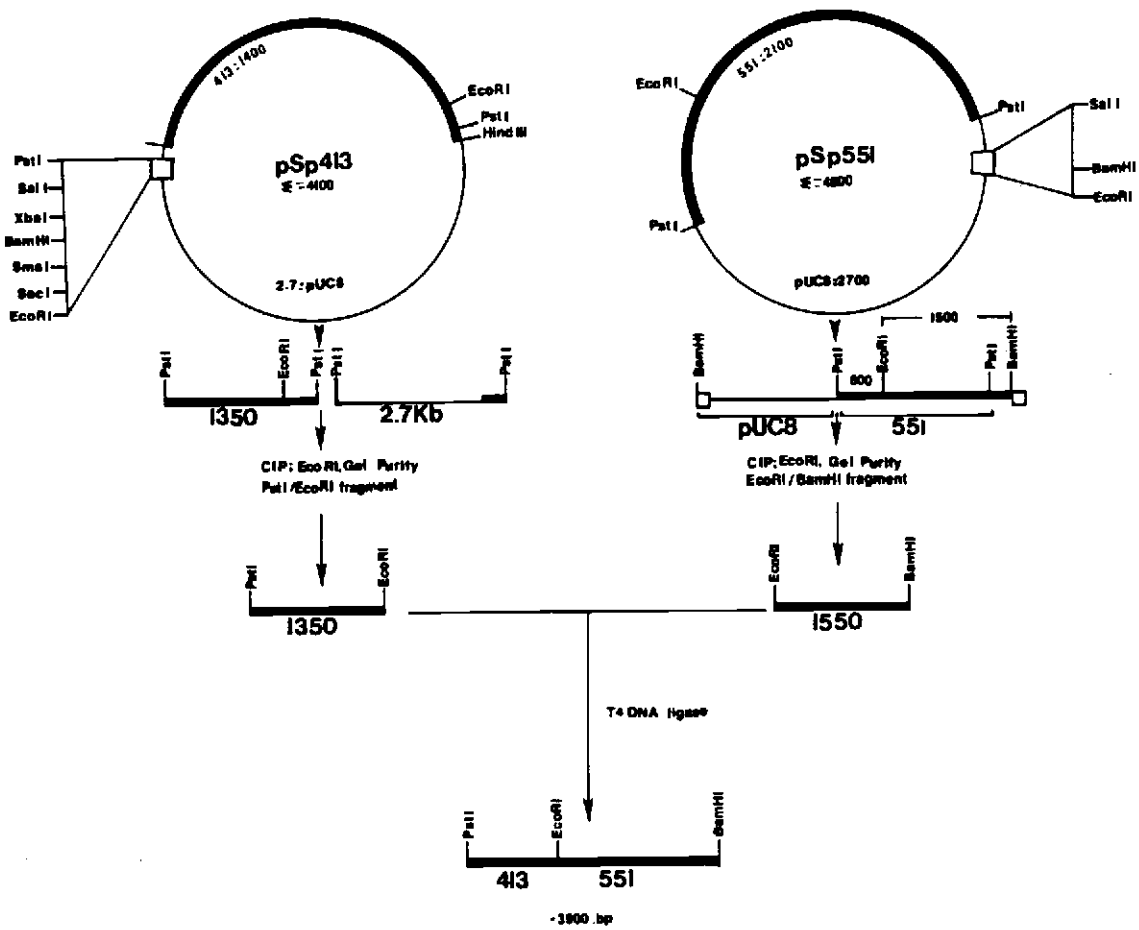

pSp413
pSp551
EcoRI
PstI
Hind III
Sal I
Xba I
BamHI
Sma I
Sac I
413:1400
2.7:pUC8
1350
2.7Kb
pUC8:2700
800
pUC8
551
CIP; EcoRI, Gel Purity
PstI/EcoRI fragment
EcoRI/BamHI fragment
1550
T4 DNA ligase
413
551
~3900 bp

Figure IV.1A

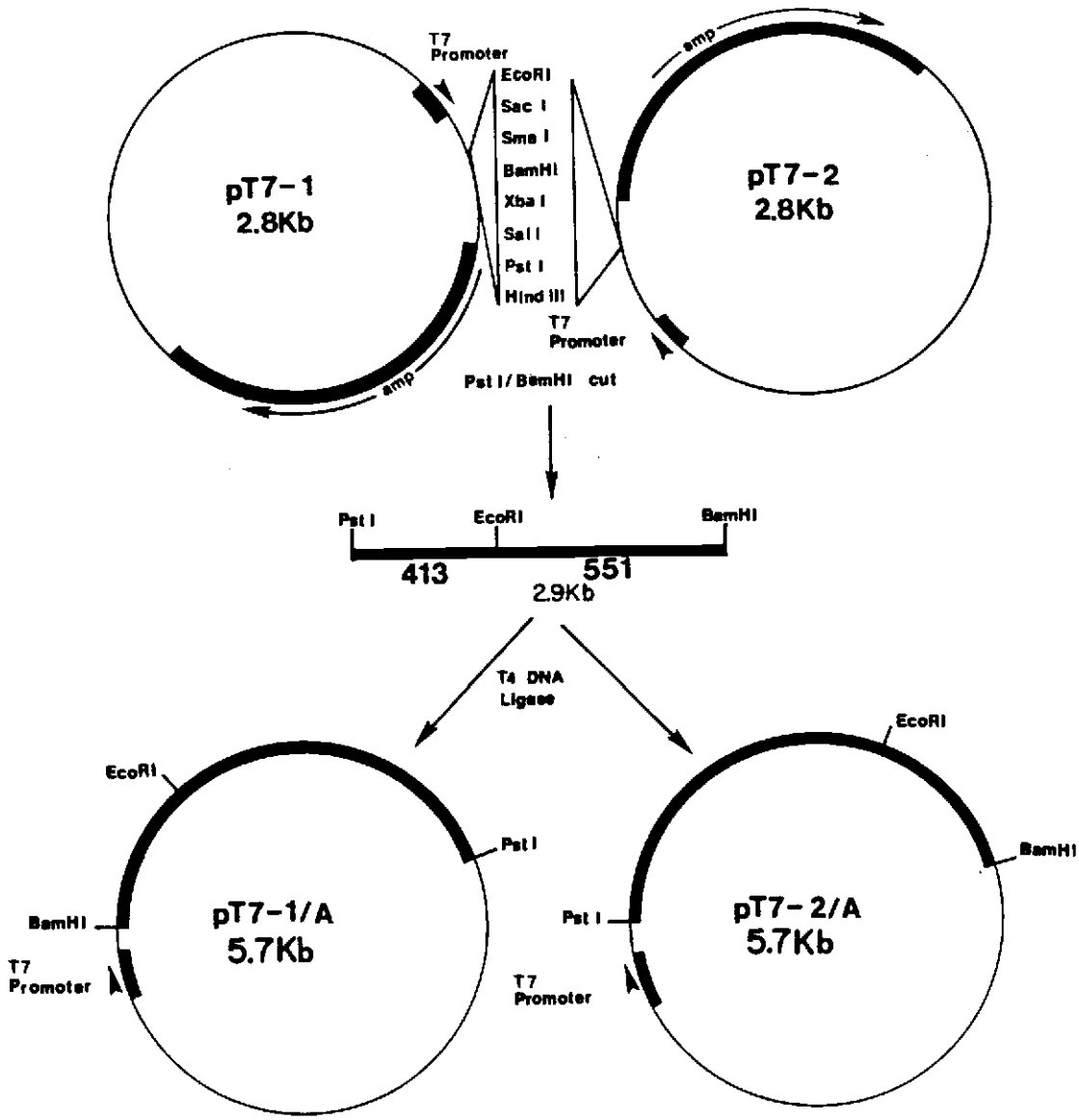

T7
Promoter
EcoRI
Sac I
Sma I
BamHI
Xba I
Sal I
Pst I
Hind III
T7
Promoter
amp
pT7-1
2.8Kb
pT7-2
2.8Kb
amp
Pst I/BamHI cut
Pst I
EcoRI
BamHI
413
551
2.9Kb
T4 DNA
Ligase
EcoRI
Pst I
BamHI
T7
Promoter
pT7-1/A
5.7Kb
EcoRI
BamHI
Pst I
T7
Promoter
pT7-2/A
5.7Kb

Figure IV.1B

Figure IV.2. Subcloning of segment B cDNA into the T7 RNA polymerase vector, pT72.

The two inserts from recombinant plasmids pSp234 and pSp424 overlap at a common KpnI site to form the continuous sequence of segment B. The HindIII/Sal I fragment of pSp424 was gel purified and ligated into the complementary sites of pT72. This recombinant was transformed into E. coli SC181 (designated pT72/424) and used to subclone the adjacent sequence from pSp234 bordered by HindIII and KpnI. This portion of pSp234 was partially digested with HindIII, followed by complete digestion with KpnI prior to subcloning into pT72/424. The final recombinant carrying segment B cDNA was designated pT72/B.

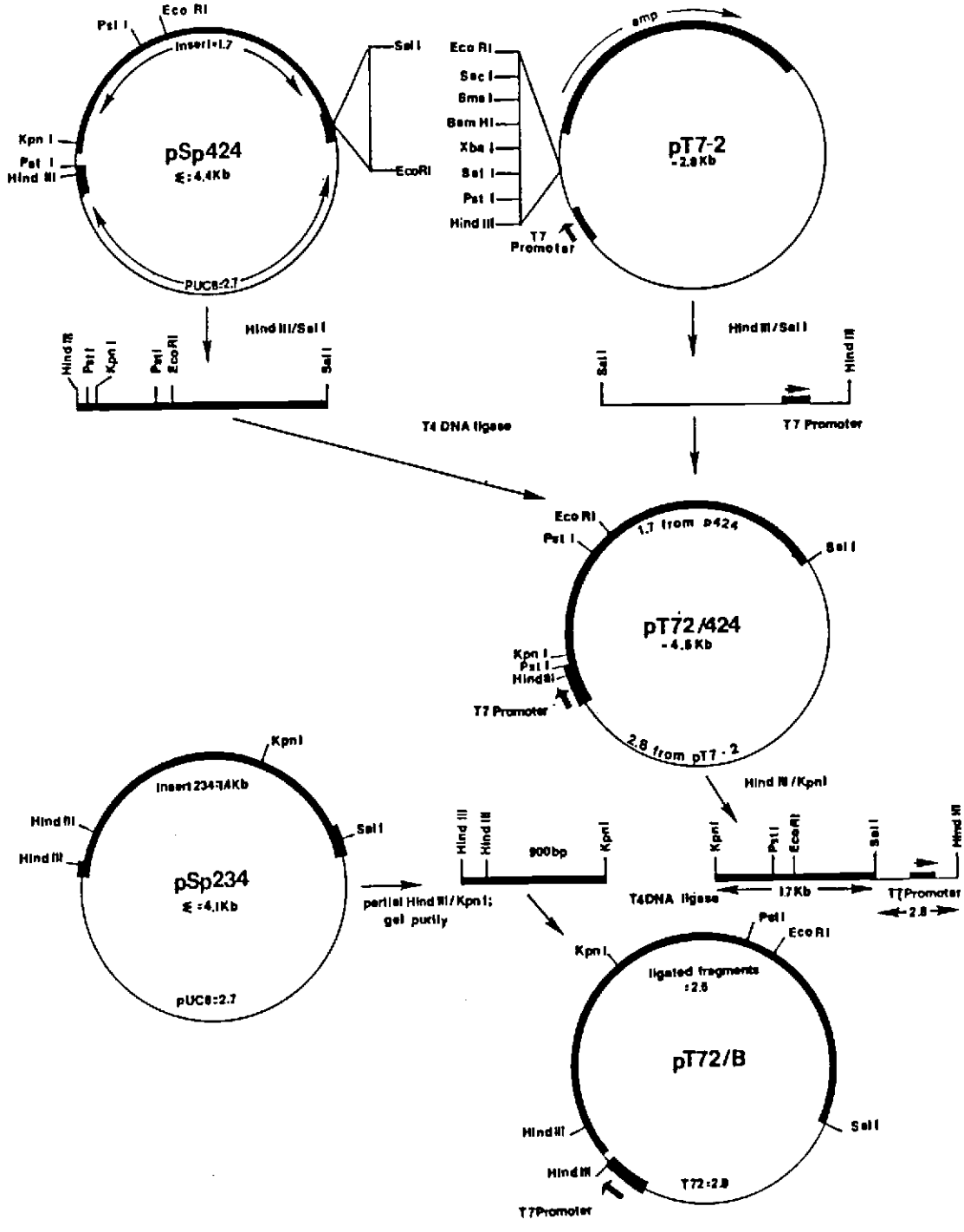

Eco RI
Psi I
Insert=1.7
Sal I
Kpn I
Pst I
Hind III
pSp424
=4.4Kb
EcoRI
PUC8=2.7
Eco RI
Sac I
Sma I
Bam HI
Xba I
Sal I
Pst I
Hind III
T7 Promoter
amp
pT7-2
=2.8Kb
Hind III/Sal I
Hind III
Pst I
Kpn I
Pst I
EcoRI
Sal I
Sal I
Hind III
T7 Promoter
T4 DNA ligase
Eco RI
Pst I
1.7 from p424
Sal I
pT72/424
=4.5Kb
Kpn I
Pst I
Hind III
T7 Promoter
2.8 from pT7-2
Kpnl
Insert 2.3+1.4Kb
Hind III
Hind III
Sal I
pSp234
=4.1Kb
pUC8=2.7
partial Hind III/Kpn I;
gel purify
Hind III
Hind III
900bp
Kpnl
Hind III /Kpnl
Kpnl
Pst I
EcoRI
Sal I
Hind III
T4DNA ligase
1.7Kb
T7Promoter
2.8
Kpnl
Pst I
Eco RI
ligated fragments
:2.8
pT72/B
Hind III
Hind III
T7Promoter
T72:2.8
Sal I

Figure IV.2

Figure IV.3. Linear maps of pT7 recombinant plasmids used to synthesize ssRNA.

Partial restriction maps for recombinant plasmids pT71/A, pT72/A, and pT72/B are presented which depict (i) the location of the restriction sites used to linearize the plasmids at the 3'-end of the viral insert, (ii) the relative position of the promoter site for T7 RNA polymerase, and (iii) the sense or anti-sense orientation of the ssRNA as determined from the analysis of cell-free translation products.

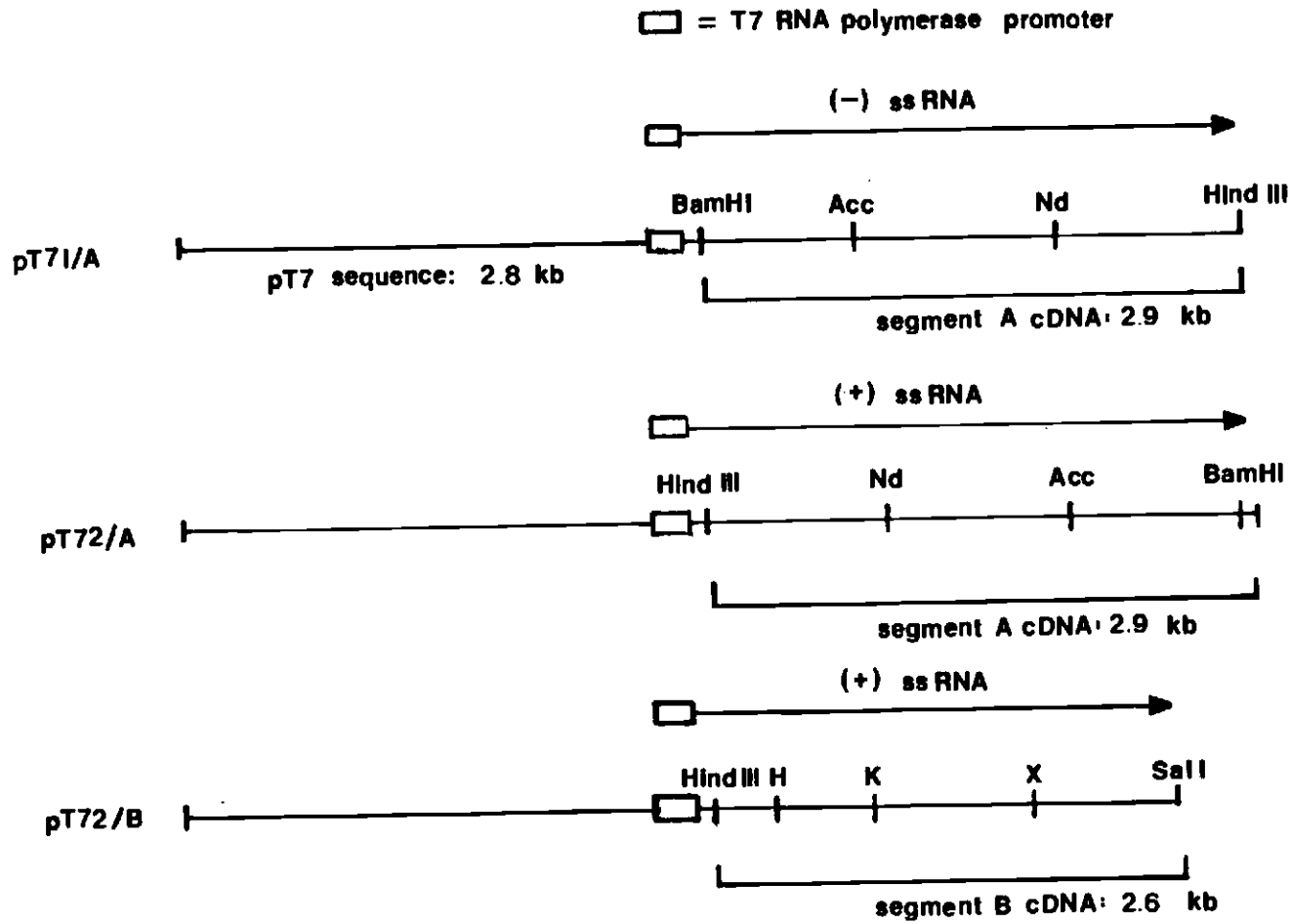

= T7 RNA polymerase promoter
(−) ss RNA
BamHI
Acc
Nd
Hind III
pT7I/A
pT7 sequence: 2.8 kb
segment A cDNA: 2.9 kb
(+) ss RNA
Hind III
Nd
Acc
BamHI
pT72/A
segment A cDNA: 2.9 kb
(+) ss RNA
Hind III H
K
X
Sal I
pT72/B
segment B cDNA: 2.6 kb

Figure IV.3

Figure IV.4. Autoradiogram of the ssRNA's synthesized by T7 RNA polymerase.

T7 RNA polymerase was used to synthesize 3H-labeled ssRNA from pT71/A, pT72/A, and pT72/B. The RNA's were denatured in solutions containing 1.8M glyoxal, 50% DMSO, and 10mM phosphate buffer (PB) (pH7.0) for 1 hour at 65C and electrophoresed in a 1.2% agarose gel for approximately 4 hours at 90mA. (1) pT71/A, (2) pT72/A, (3) pT72/B. VSV mRNA markers are indicated in the margin.

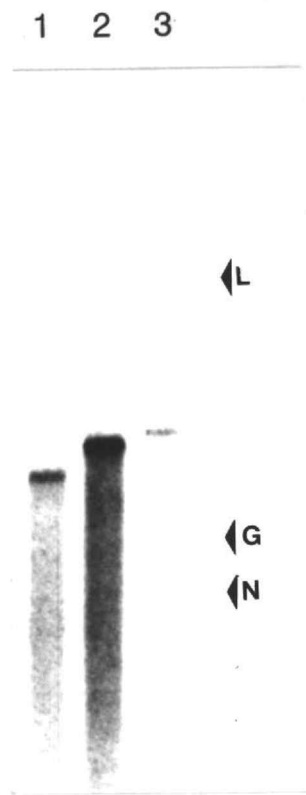


Figure IV.4

Figure IV.5. Electrophoretic analysis of the cell-free translation products of genomic dsRNA and ssRNA synthesized by T7 RNA polymerase.

Subsamples of 35S-methionine labeled cell-free translation reactions were added (A) directly to a 10% polyacrylamide gel with 3% stacking gel; or (B) after immunoprecipitation with polyvalent rabbit antiserum prepared against whole virus. (1) translation products from genomic dsRNA, (2) purified Sp IPNV, (3) translation products from segment A cDNA, (4) translation products from segment B cDNA, (5) endogenous proteins from the translation reaction. Letters indicate viral proteins: (A) RNA polymerase, (B1) major capsid protein precursor, (B2) first intermediate product, (B3) major capsid protein found in purified virus, (B4) third processed protein of B1, (C1) minor capsid protein, (C2) apparent breakdown product of C1, (D) non-structural viral protein seen only in infected cells.

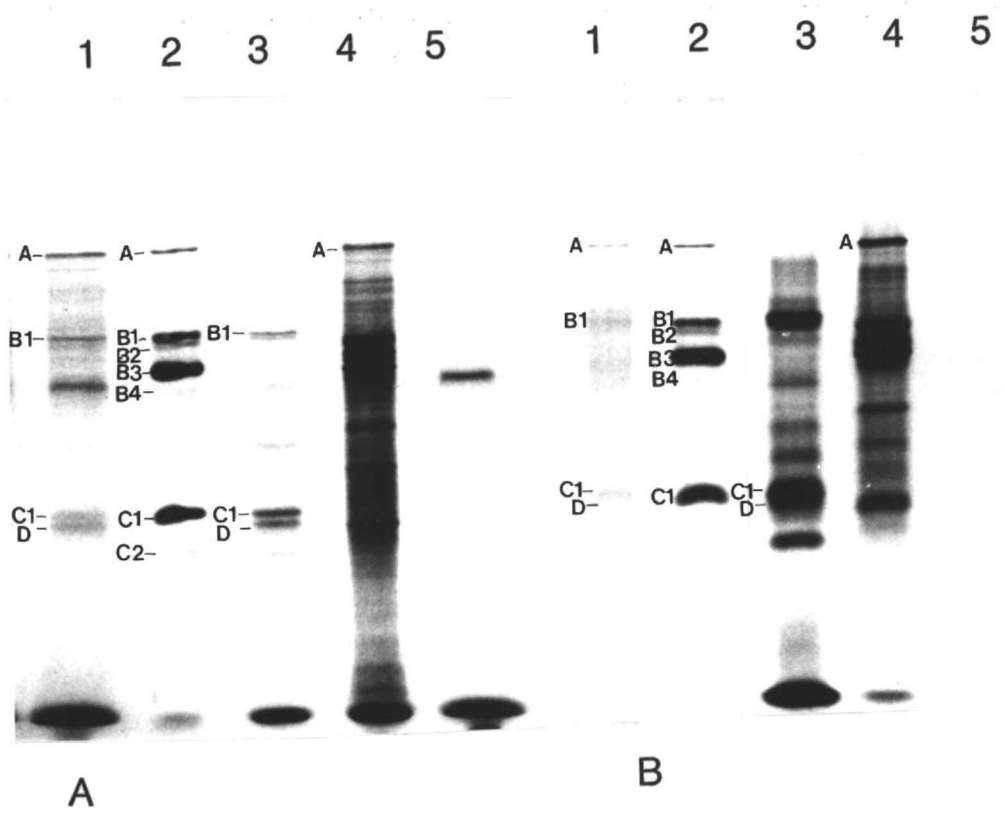


Figure IV.5

Figure IV.6. Electrophoretic analysis of 35S-methionine-labeled proteins from CHSE-214 cells infected with IPNV.

Monolayers of CHSE-214 cells in 35mm diameter dishes were infected at an M.O.I. of between 100-200 with IPNV. At various times post-infection, the cells were incubated in methionine-free media supplemented with 35S-met (100uCi/ml) for 1-2 hours. The cells were lysed and analyzed directly by SDS PAGE. Lanes (2), (4), (6), and (8) contain samples from uninfected cells; infected cells were labeled at (1) 2-3 hrs PI, (3) 3-4 hrs PI, (5) 5-7 hrs PI, and (7) 7-8 hrs PI. Purified virus is present in (9) SpCS. Viral protein bands: (A) RNA polymerase, (B) major capsid protein precursor, (C) minor capsid protein precursor, (D) non-virion protein seen only in infected cells.

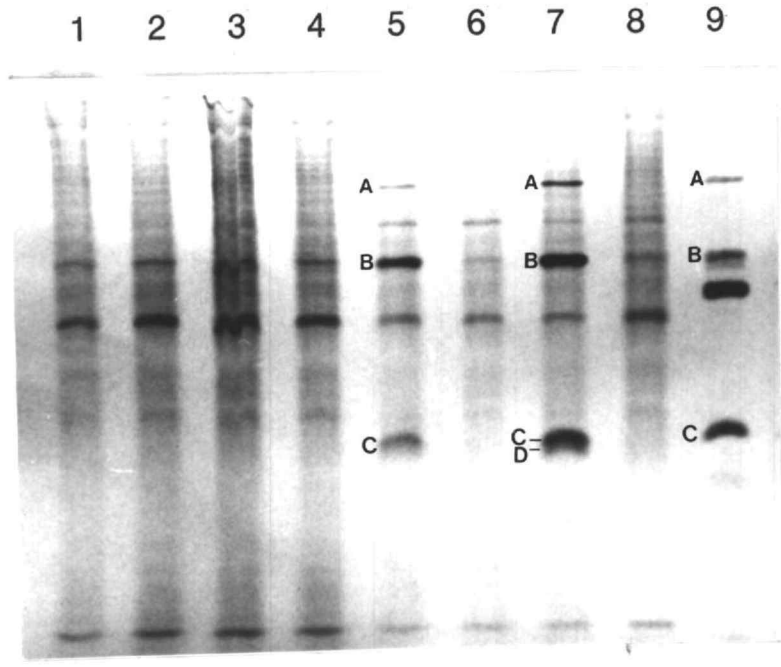


Figure IV.6

Figure IV.7. SDS PAGE comparing the protein banding patterns of SpCS and SpR strains of IPNV.

IPNV strains SpCS and SpR were labeled with 35S-methionine during infection of CHSE-214 cells. The viruses were purified by CsCl equilibrium centrifugation and electrophoresed in a 10% SDS PAGE gel with 3% stacking gel. (1) SpR, (2) SpCS. Letters indicate viral proteins: (B1) major capsid protein precursor, (B2) first intermediate form of B1, (B3) second intermediate form of B1 which is the predominant form found in purified virus.

Figure IV.7

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