AN ABSTRACT OF THE THESIS

Carla L. Mason for the degree of Doctor of Philosophy in Microbiology presented on September 30, 1992

Title: Molecular Characterization of the Proteinase and RNA-Dependent RNA Polymerase of Infectious Pancreatic Necrosis Virus, a Fish Birnavirus

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The A segment of infectious pancreatic necrosis virus (IPNV) is expressed as a polyprotein encoding three primary gene products, VP2, NS and VP3, from a large open reading frame. The nucleotide sequence for the A segment of the Sp isolate of IPNV was determined. The NS protein is the putative autocatalytic proteinase responsible for the cleavage of the polyprotein. The functional boundaries of the NS proteinase were mapped by plasmid deletion analysis and examined in an in vitro translation system. The NS proteolytic activity was determined to lie within the EcoRI and NsiI restriction sites. Characterization of the NS proteinase also was approached by use of proteinase inhibitors and site-directed mutagenesis of the putative catalytic and cleavage sites. Eight proteinase inhibitors, representative of all four proteinase classes, were tested and all failed to inhibit the NS enzyme. Mutagenesis of a putative aspartyl proteinase catalytic

motif, DTG, to VTG did not affect proteolytic processing. Additionally, the mutagenesis of the predicted N-terminal cleavage site did not alter processing, however, altered processing was observed when the predicted C-terminal cleavage site was mutated.

The major capsid protein, VP2, was mapped with polyclonal and monoclonal antisera. The VP2 gene was digested with Sau3A and subcloned into the pATH expression vector. The trpE-fusion proteins were characterized with polyclonal and monoclonal antisera. Two immunoreactive regions were identified with anti IPNV-Sp sera. A common immunoreactive region, B10, was reactive with antisera to three serotypes of IPNV as well as a neutralizing monoclonal antibody, AS-1. A serotype specific immunoreactive region, A43, also was identified, being recognized only by anti IPNV-Sp sera.

The B segment of IPNV encodes the putative RNA-dependent RNA polymerase (RdRp), VP1. The nucleotide sequence for the B segment of the Sp isolate was determined and the deduced amino acid sequences were compared to other polymerases. Concensus sequences associated with GTP-binding proteins and RdRps were identified in the VP1 sequence. However, unlike RdRps associated with single-stranded RNA viruses, the IPNV VP1 proteins lack the Gly-Asp-Asp motif characteristic of this enzyme family. Additionally, the VP1 protein was expressed in a bacterial system and polyclonal antisera was raised against the protein.

Molecular Characterization of the Proteinase and RNA-Dependent RNA Polymerase of Infectious Pancreatic Necrosis Virus, a Fish Birnavirus.

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Molecular Characterization of the Proteinase and RNA-dependent RNA Polymerase of Infectious Pancreatic Necrosis Virus: a Fish Birnavirus

I. INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) causes a serious disease to a number of salmonid and nonsalmonid species. IPNV is a birnavirus which consist of double-stranded RNA with a bipartite genome. The virion is an unenveloped icosahedron approximately 60 nm in diameter. The large genomic RNA, segment A, encodes three viral proteins (MacDonald and Dobos, 1981), the major capsid protein, VP2; a minor capsid component, VP3; and a non-structural protein, NS. The smaller genomic RNA, segment B, encodes the putative RNA-dependent RNA polymerase, VP1 (Cohen, 1975; Dobos, 1977; Dobos and Rowe, 1977). The gene order of the A segment was determined to be NH_2-pVP2-NS-VP3-COOH (Huang et al., 1986; Nagy et al., 1987; Azad et al., 1987). The VP1 protein has been mapped to segment B (MacDonald and Dobos, 1981).

The nucleotide sequence has been determined for the large segment of IPNV and infectious bursal disease virus (IBDV), another member of the birnavirus family, and both were found to contain a large open reading frame of approximately 2900 basepairs (Duncan and Dobos, 1986; Hudson et al., 1986). The open reading frame identified was large enough to code for all three

proteins. These results suggested that the A segment was either expressed as a polyprotein or the proteins were produced by the internal initiation of ribosomes.

Recent reports have shown that a polyprotein containing NH_2-VP2-NS-VP3-COOH is synthesized and that a proteolytic activity associated with the NS protein may be involved in processing this polyprotein to pVP2, NS and VP3 (Duncan et al., 1987; Jagadish et al., 1988). In addition, the IPNV and IBDV A segments have been expressed in Escherichia coli (Jagadish et al., 1988; Manning and Leong, 1990) and in both cases the polyprotein was processed to yield VP2, NS and VP3. The NS protein was shown to be required for proteolytic processing when a series of deletions were introduced into the A segment of the Sp serotype (Manning et al., 1990). When portions of the VP2 or VP3 proteins were deleted, proteolytic processing was not affected. However, successive deletions into the coding region of NS eliminated proteolytic processing and resulted in the production of a truncated polyprotein (Manning et al., 1990). These results suggested that the IPNV-NS protein was the proteolytic enzyme responsible for the processing of the A segment polyprotein.

The viral segment B encodes VP1, the putative RNA-dependent RNA polymerase (RdRp). The protein is approximately 90 kDa in size (MacDonald and Dobos, 1981; Nagy and Dobos, 1984; Azad et al., 1985). IPNV, IBDV and Drosophila X virus all possess genome-linked proteins (VPg) tightly associated with the ends of the genomic RNA (Persson and MacDonald, 1982; Revet and Delain,

1982; Muller and Nitschke, 1987). In the cases of IPNV and IBDV, this VPg has been shown to be VP1. The RdRp also may contain guanylyl and methyl transferase activities (Spies and Muller, 1990).

The segment B sequence was determined for IBVD and reported to have no homology between the predicted VP1 sequence and the sequences of putative ssRNA-dependent RNA polymerases (Morgan et al., 1988). The sequence was subsequently reanalyzed and reported to have homology with concensus sequence elements found in ssRdRps (Gorbalenya and Koonin, 1988; Kamer and Argos, 1984; Argos, 1988).

This thesis describes the determination of the nucleotide sequences for both the A and B genome segments of the Sp serotype of IPNV and the characterization of the NS proteinase by deletion analysis resulting in truncated polyproteins expressed in vitro. Site-directed mutagenesis studies are described that were conducted to determine the catalytic residues involved with proteolytic activity and those designed to determine the cleavage sites recognized by this enzyme. In addition, eight proteinase inhibitors effective at inhibiting enzymes from all four proteolytic classes (aspartyl, cysteine, metallo and serine proteinases) were utilized to determine which class, if any, the NS proteinase belongs.

The Sp isolate of IPNV is representative of the Sp serotype, the major virulent IPNV type in Europe. The sequence determination of the A segment was the first step in developing an

IPNV vaccine and understanding the basis for IPNV virulence. Neutralizing antibodies raised against the virus are directed against VP2, the major capsid protein of IPNV. This protein was subcloned and mapped with polyclonal and monoclonal antisera to define regions of the protein with common and serotype specific antigenic determinants.

This thesis also describes the genome segment B nucleotide sequence of the Sp serotype and the deduced amino acid sequence of the VP1 protein. Sequence analysis revealed the presence of extensive homology between IPNV and IBDV VP1 in the central regions of the proteins and the presence of several conserved domains associated with RdRps and GTP binding proteins. The results show that contrary to other putative RdRps, IPNV lacks the conserved Gly-Asp-Asp motif, the proposed catalytic site of this enzyme family. In addition, the VP1 protein was expressed in *Escherichia coli* as a *trpE* fusion protein and a polyclonal rabbit antisera was raised to produce a reagent against VP1 that could be used to confirm the identity of the VPg protein.

II. LITERATURE REVIEW

History

Infectious Pancreatic Necrosis Virus (IPNV) is the etiological agent of a disease which affects a variety of fish species worldwide. The disease was first described in 1941 (McGonigle) in young brook trout that exhibited "acute catarrhal enteritis". In 1953, a similar outbreak occurred in fingerling brook trout in West Virginia and a viral etiology was proposed. Histopathological studies by Wood et al. (1955) described the condition as "infectious pancreatic necrosis" (IPN), although no causative agent was identified. The infectious nature of the disease was demonstrated by transmitting IPN to naive fish with tissue homogenates of infected fish (Snieszko, 1959). The viral nature of IPN was first confirmed by Wolf et al. (1960) when cell-free tissue culture homogenates were used successfully to transmit the disease to brook trout fry. Additionally, salmonid cell lines propagated in tissue culture exhibited cytopathic effect when infected with cell-free homogenates.

The geographic distribution of IPNV is extensive. Isolation of the virus has been reported in Europe, North America and Eastern Asia. It appears to be ubiquitous in the aquatic environment. The isolation of IPNV has been reported in several salmonid species, including Atlantic salmon, as well as molluscs and crustaceans. Despite its wide distribution, the virus is primarily virulent to

salmonid species, although, an IPNV-like disease has also been reported in eels.

The early clinical signs of the IPN disease include unusually high mortalities among juvenile brook and rainbow trout, reaching as high as 90%. The infected fish display a characteristic corkscrew spiralling or whirling along the long axis of the body (Wolf and Quimby, 1969). The fish develop a darkened body, exopthalmia, abdominal swelling and hemorrhaging at the base of the ventral fins. Fecal casts are often present in infected fish due to the sloughing of cells from the intestine. Internally, the fish have pale livers, spleens and stomachs. Histological examination of the pancreatic cells revealed that they were necrotic with intracytoplasmic inclusions and pyknosis (Wolf, 1966; Pilcher and Fryer, 1980).

Serotyping

Classification of the IPNV isolates has become increasingly complex. Cross-neutralization studies using rabbit antisera have been used to compare a number of isolates (Wolf and Quimby, 1971; Hill, 1976; McDonald and Gower, 1981). Most of the viral isolates had some degree of cross-reactivity with one another however, differences did exist. Another study recognized only three serotypes: VR299, a North American strain, and two European isolates were classified into two distinct groups Sp, which was highly pathogenic for trout and Ab, a non-pathogenic group. Several other North American isolates including Buhl, Powder Mill and West Buxton (WB) were cross-reactive with both VR299 and Sp

strains. The most extensive study of the antigenic relationships of aquatic birnaviruses compared 175 virus isolates from 44 fish and shellfish species from eleven countries by reciprocal plaque reduction tests using polyclonal antisera (Hill and Way, 1983). From these results, it was proposed that there were two major serogroups: serogroup A containing nine serotypes which included 171 isolates from fish and serogroup B containing one serotype which included the mollusc isolates..

Due to the limitations of the cross-neutralization classification strategy, groups began to examine the antigenic relationships of these isolates with the use of monoclonal antibodies (Mabs) (Okamoto et al., 1983; Caswell-Reno et al., 1986; Lipipun et al., 1989). Results from the Mab analysis supported the earlier reports of the two serogroups and identified specific serotypes within serogroup A. Caswell-Reno et al. (1986) identified isolates within the three serotypes previously described, WB, Sp and Ab as well as characterizing six other virus as distinct and representing new serotypes, Hecht (He), Tellina (Te), Canada 1(C1), Canada 2 (C2), Canada 3 (C3), and Canada 4 (C4). The Jasper isolate from Canada examined by Mabs appeared to be closely related antigenically to the WB serotype (Caswell-Reno et al., 1989).

Molecular Biology

Early studies on IPNV described the virus as belonging to the Reovirus family. However, a closer examination of the virus and related viruses have classified them into a new group, namely the

Birnaviridae (Matthews, 1982). This group of viruses includes infectious bursal disease virus (IBDV), which causes a serious disease in fowl, and Drosophila X virus (DXV). Birnaviruses are characterized as containing two segments of double-stranded ribonucleic acid (dsRNA), enclosed in a non-enveloped icosahedral shell approximately 60 nm in diameter.

Nucleic Acid Composition

The nature of the nucleic acid genome of IPNV was first determined by examination of the effects of metabolic inhibitors. Inhibitors of deoxyribonucleic acid (DNA) synthesis had no effect on the replication of IPNV, however, inhibitors of RNA synthesis were able inhibit IPNV replication by as much as 90% (Malsberger and Cerini, 1963).

Early electron microscopy studies indicated that the diameter of the virus was 25-29 nm and had picornavirus-like morphology (Cerini and Malsberger, 1965). Moss and Gravell (1969) later performed negative staining on both purified virus and thin sections of infected cells, their results indicated that there was icosahedral symmetry and the virions were about 65 nm in diameter. This information led to the reclassification of IPNV as a reovirus.

In 1972, Kelly and Loh demonstrated that ^{32}P-labeled IPNV had an isopycnic sedimentation in CsCl at a density of 1.33 g/cm^3. Negatively stained preparations of purified virus revealed a non-

enveloped virion with icosahedral symmetry as was described earlier, this preparation lacked readily discernable inner capsids that are characteristic of Reoviridae. Additionally, ^{32}P- or ^{3}H-uridine labeled RNA isolated from purified virus was examined for its base composition. The dsRNA was found to be non-complementary, with a purine to pyrimidine ratio of 0.88 to 0.90 (Kelly and Loh, 1972). These results suggested that the dsRNA nature of IPNV differed from that of the reoviruses. In 1973, Cohen et al. demonstrated that IPNV has a buoyant density of 1.615 in $CsSO_4$ density gradients. This equals the values reported for the dsRNA of reoviruses. The melting profile of IPNV RNA resulted in a calculated Tm of 89°C. The conclusion from these experiments, along with more recent information obtained by Dobos in 1976, is that the genome of IPNV was composed of dsRNA. The RNase sensitivity of IPNV was investigated. RNase digestion resulted in the initial loss of about 15% of the RNA, but the remainder of the RNA was resistant to high concentrations of RNase. This result indicated that the RNA of IPNV might contain short tails of ssRNA. Finally, the dsRNA nature of IPNV was provided by direct visualization with an electron microscope. The IPNV RNA appeared as a uniform linear strand with a width equal to that of DNA (MacDonald and Yamamoto, 1977).

Additional evidence that IPNV did not belong to the Reoviridae was IPN virions contained only two segments of dsRNA, while reoviruses have been shown to contain ten dsRNA segments. When IPNV RNA was analyzed by gel electrophoresis, two distinct

bands were observed. Their molecular weights were estimated to be 2.3×10^6 and 2.5×10^6 (Dobos, 1976).

During the isolation and characterization of the viral RNA, it was observed that the RNA extracted from virions in the presence and absence of protease treatment behaved differently (Persson and MacDonald, 1982). RNA obtained by the dissociation of virions by 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol at 100°C exhibited a slower electrophoretic mobility, had a lower buoyant density and tended to aggregate, as compared to RNA isolated by proteinase digestion and phenol extraction. When RNA which had not been proteinase treated was subjected to sequential RNase III and RNase A digestion, a 110,000 dalton protein was released. Electron microscopy indicated that this protein was bound only to the ends of the viral RNA segments (Persson and MacDonald, 1982).

Many RNA and DNA viruses possess genome-linked proteins (VPg) which are virus-encoded polypeptides covalently linked to the 5' termini of virus nucleic acids (Wimmer, 1982). VPgs play a fundamental role in the replication of viral genomes by acting as a primer for the polymerase. It was predicted that the protein attached to the ends of the IPNV genomic RNA may be similar to the VPg proteins that have been identified for the picornaviruses, adenoviruses and other related viral families. Therefore, it was predicted that this protein may play a similar role of the VPg of these viruses, possibly serving as a primer for replication. A similar protein has been reported for IBDV (Muller and Nitschke,

1987) and DXV (Revet and Delain, 1982). For these viruses it was proposed that the protein bound to the ends of the genome may be the viral RNA-dependent-RNA polymerase, VP1.

Replication

Animal viruses with dsRNA genomes, such as the reoviruses, replicate via a particle associated transcriptase. Viral mRNA is synthesized from the negative strand of the genomic dsRNA. This mRNA can then be used as a template for the production of progeny in a conservative mode of replication. Although the exact mechanism for IPNV replication is not known, it is thought to proceed in a manner similar to the reoviruses.

An RNA polymerase activity is associated with birnavirus particles and is capable of catalyzing the synthesis of ssRNA (Cohen, 1975). When all four nucleosides were present, purified virions were capable of incorporating radiolabeled nucleotides into an RNase susceptible product, presumably ssRNA (Cohen, 1975). Polymerase activity was reported to increase with an increase in temperature from 10°C to 40°C.

In 1980, the nature of IPN virus-specific RNA synthesis was investigated. Pulse-labeling experiments with ^{3}H-uridine revealed that the rate of IPNV-specific RNA synthesis was maximal between 8 to 10 hours post-infection and was completely diminished by 12 to 14 hours. Three forms of RNA intermediates were detected; a putative transcription intermediate that co-migrated with genomic dsRNA in acrylamide gels after RNase treatment, a 24S genomic length mRNA which could be resolved

into two bands by polyacrylamide gel electrophoresis, and a 14S genomic dsRNA component (Somogyi and Dobos, 1980). The viral polymerase activity was further characterized by Mertens et al. (1982). In this study, the major reaction product was found to be a RNase resistant 14S RNA. A genome-length 24S ssRNA could be detected in small quantities if RNase inhibitors were added to the reaction. Attempts were made to determine if the synthesis of ssRNA occurred by a conservative mechanism, by which the nascent RNA strand is released as a ssRNA, or by a semi-conservative mechanism, by which the nascent RNA strand displaces one of the parental strands.

The ssRNA was labeled in the presence of actinomycin D and could be "chased" into dsRNA and hybridized to genomic dsRNA (Somogyi and Dobos, 1980). IPN virions were ^{3}H-labeled and the polymerase reaction was performed in the presence of unlabeled nucleotides. Most of the ^{3}H-labeled virion RNA remained as 14S genomic RNA, although some of the labeled RNA was found in the 24S ssRNA fraction (Mertens et al., 1982). These results indicated that the mechanism of virus-specific RNA synthesis in IPNV infected cells appeared to be similar to that of reoviruses, being a semi-conservative mode of replication.

RNA-dependent RNA polymerases

RNA-dependent RNA polymerases direct the synthesis of RNA from a RNA template. This includes the synthesis of mRNA and replication of plus-, minus-, and dsRNA viruses. These viruses must encode the RNA-dependent RNA polymerase and carry it into

the cell as an integral component of infection, since mammalian cells do not harbor such an enzyme.

The genomic nucleotide sequences for several plant and animal viruses has been determined in the last few years and the deduced sequences of RNA-dependent RNA polymerases have been compared. Amino acid similarities between these viruses resulted in the identification of several motifs that are conserved in these enzymes. A Gly-Asp-Asp motif present in all the putative RNA-dependent RNA polymerases suggested that this sequence was part of the active site or recognition site region (Kamer and Argos, 1984). Poch et al. (1989) identified four conserved motifs present in RNA-dependent polymerases. These were within a large domain of 120-200 amino acids and were present in polymerases encoded by retroviruses, viral and non-viral retrotransposons, plus-, minus-, and dsRNA viruses (Poch et al., 1989). The conserved Gly-Asp-Asp was preceded by two concensus sequences, VxDxSLYP and NSxYG and followed by the final conserved sequence GxxxxxxxK (Poch et al., 1989). The significance of these sequence similarities was strengthened by the presence of invariant amino acids between viruses from different groups, identical linear arrangement of the motifs and the comparable distances between the motifs (Poch et al., 1989).

The importance of the Gly-Asp-Asp sequence present in RNA-dependent RNA polymerases was examined in the poliovirus RNA polymerase using site-directed mutagenesis. Mutations were introduced to the Tyr-Gly-Asp-Asp conserved motif. Amino acid

changes of the Gly residue to Cys, Met, Pro or Val abolished enzyme activities, while an Ala or Ser substitution resulted in reduced enzymatic activities (Jablonski et al., 1991). These results provided evidence that the Tyr-Gly-Asp-Asp core element was important for enzymatic activity since single amino acid substitutions in this motif dramatically altered enzymatic activity (Jablonski et al., 1991).

Proteins

IPN viral proteins were first characterized by Cohen et al. (1973). Virus labelled with ^{14}C was separated on SDS-urea gels. Bands representing viral proteins were observed migrating at 80, 50 and 30 kilodaltons (kDa), which accounted for 3, 68 and 29% of the total virion protein, respectively.

In 1977, Chang et al. compared the protein banding patterns of ten IPNV isolates by Coommassie blue stained SDS-PAGE. They found all of the isolates examined to be nearly identical, proteins of 50, 30 and 27 kDa were observed. The large 80-100 kDa protein was not observed, presumably due to the lack of sensitivity with the Coommassie blue stain technique.

Virus-specific protein synthesis of cells infected with IPNV was examined by Dobos in 1977. Time-course of protein synthesis was measured by pulse-label experiments with the ^{35}S-methionine labeled products analyzed by SDS-PAGE and autoradiography. The protein products observed were of three size classes, 90-100 kDa, designated α proteins; 59, 56, 54 and 50 kDa, designated β proteins; and 32, 30 and 28 kDa proteins, designated γ

proteins. The pulse-chase experiments also revealed that the α proteins were not precursors to the β and γ proteins, since the label could not be chased from the α protein to the smaller viral products. Additional experiments aimed at determining the relationship of the viral proteins were unsuccessful. The use of amino acid analogues and protease inhibitors did not reveal a common precursor for the viral proteins. Processing was observed in the β protein family. In pulse-chase experiments, label could be chased from the 59 and 56 kDa proteins, the first to appear in the infective cycle, to the 54 and 50 kDa forms, the 50 kDa form was the major form of the protein found in purified virus. Another finding of this study was that the frequency of translation was inversely proportional to the molecular weights; the smaller proteins were produced more frequently than the larger proteins. Since only two mRNA species had been detected, this observation was of interest. The data suggested that four viral proteins were produced at different rates from only two mRNAs.

In 1977, Dobos and Rowe localized the relative position of the various proteins to the genome with peptide mapping experiments. Proteins were labeled with ^{35}S-methionine and separated by SDS-PAGE. Viral bands were excised from the gels and two-dimentional tryptic digests were performed. Comparison of the peptide maps indicated that the 90, 59, 29 and 27 kDa proteins were distinct from each other and were, therefore, primary gene products. Peptide maps confirmed the earlier prediction of the three protein classes α, β and γ. The smallest

protein (26 kDa) was not observed in the virion and was termed nonstructural (NS). The α, β and γ proteins have been more recently designated VP1, VP2 and VP3, for virion protein.

The genome of IPNV consists of two dsRNA molecules that encode the four viral proteins. This suggested that several proteins must somehow arise from a single RNA segment. In order to determine the proteins encoded by each RNA segment, recombinant (hybrid) viruses were selected (MacDonald and Dobos, 1981). Reassortment studies with two temperature sensitive isolates that differed significantly in both the apparent molecular weights of proteins and RNA were utilized in these experiments. Mixed infection experiments and the selection for wild-type revertants resulted in the identification of only two assortment groups. It was determined from these experiments that the larger genomic RNA, segment A, encoded three smaller gene products, 50, 30 and 28 kDa, while it was determined that the smaller genomic RNA, segment B, codes for a 90 kDa polypeptide which is a minor, internal component of the virion, designated VP1. VP1 was presumed to be the viral RNA dependent RNA polymerase.

The mechanism by which IPNV synthesized three proteins from a single RNA remained unknown. Several different methods of gene expression could account for a virus producing several proteins from a single genetic element. Subgenomic mRNA production, polyprotein gene expression or internal translational initiation were three possible mechanisms that needed to be investigated.

Protein Expression

There are four known gene products produced from the two genomic dsRNAs of the birnaviruses. Investigators wanted to determine how these proteins were expressed. The nucleotide sequence of a cDNA of the large dsRNA, segment A, of IPNV (Jasper strain) revealed that it contained a large single open reading frame (ORF) (Duncan and Dobos, 1986). The ORF contained 2,916 bp. The cDNA clones of the Sp serotype for both the A and B segments of IPNV (Sp strain) have been constructed (Huang et al., 1986). A physical map of the genome was determined using plasmid DNA containing deletions in the A segment coding regions. The mRNAs synthesized in vitro were translated in rabbit reticulocyte lysates and the resulting protein products were immunoprecipitated and compared with native viral proteins. The gene order of the A segment was found to be 5'-VP2-NS-VP3-3' (Huang et al., 1986; Nagy et al., 1987). Since segment A encodes the viral structural proteins from a single ORF, protein expression was thought to arise by cotranslational proteolytic processing of a large polyprotein, similar to polio virus. This was supported by the fact that only a single species of mRNA can be detected in an infected cell (Somogyi et al., 1980). Viral protein expression of IPNV also could be explained by the internal initiation of protein synthesis.

Hybrid arrested translation experiments revealed that the initiation of translation could take place at an internal, in-frame, AUG codon. This internal initiation resulted in the production of both NS and VP3 (Nagy et al., 1987). The gene order of the A

segment was determined to be 5'-pVP2-NS-VP3-3'. A similar gene arrangement was found on the large RNA segment of IBDV and DXV (Nagy et al., 1987; Nagy and Dobos, 1984; Hudson et al., 1986). In addition, deletion analysis was used to define the processing sites of the proteins (Azad et al., 1987; Duncan and Dobos, 1986; Manning et al., 1990).

Proteinases

The replication and maturation of many plant and animal viruses are dependent on the action of virally-encoded proteolytic enzymes. Polyprotein expression followed by the proteolytic processing by a virally-encoded proteinase is found primarily in RNA viruses and retroviruses. Proteolytic processing as a means of viral gene expression was first described for the picornaviruses by Summer et al. (1968). More recently, this method of protein expression has been described for retroviruses. The picornaviruses, ssRNA viruses of positive sense, contain a large ORF. When the polyprotein is expressed several virally-encoded proteinases are responsible for processing it to its structural and nonstructural proteins.

These viruses express all or portions of their genome as high molecular weight polyproteins which are then processed by cellular- or viral-derived proteinases. These reactions involve controlled processing at a limited number of sites throughout the polyprotein. Proteolytic processing can occur as an intramolecular or intermolecular event, the intramolecular event acts in _cis_ while the nascent polypeptide chain is being synthesized. The

latter produces an active proteinase that is free from the polyprotein and able to act in trans on their target molecules. These proteinases are highly specific and are capable of cleaving a large polyprotein at defined amino acid pairs.

Proteases catalyzing the cleavage event can be virally-encoded or occur in the host prior to infection. There is a difference in the location in the cell of polyproteins cleaved by virus-encoded and host-encoded proteinases. Polyproteins that reside in the cytoplasm are cleaved by virally encoded enzymes. (Rice and Strauss, 1981). Proteolytic processing can regulate viral expression in several ways: 1) some cleavage events only occur under certain conditions; 2) different cleavage products may have significantly different half lives (Wellnick and van Kammen, 1988). Proteolytic enzymes are classified based on the nature of their catalytic residues which are usually determined from the enzymes susceptibility to a number of proteinase inhibitors. Virtually all proteolytic enzymes can be classified in one of four groups based on the reactive groups of their catalytic site. The four classes of proteinases are aspartic, cysteine, metallo and serine (Kay and Dunn, 1990).

Aspartic proteinases

Aspartic proteinases contain catalytic active sites composed of two aspartic acid residues. Recently, the proteinase of HIV-1 has been identified as a member of the aspartic class (Pearl and Taylor, 1987). Aspartic proteinases contain two domains harboring a conserved Asp-Thr-Gly that contribute to the active

site. The finding that aspartic acid proteinases contain these two domains suggested that they may have evolved through gene duplication (Hellen et al., 1989).

Retroviral proteinases contain a single Asp-Thr(Ser)-Gly domain and, therefore, may function as a dimer (Pearl and Taylor, 1987). The Asp-Thr-Gly motif in conjunction with a conserved Ile-Ile-Gly motif some 60 residues downstream of the active site led to the model for retroviral proteinases (Pearl and Taylor, 1987; Kay and Dunn, 1990). Based on the sequence homology they were predicted to belong to the aspartic proteinase class. Inhibition studies with pepstatin-A, a known inhibitor of aspartic proteinases, was shown to inhibit retroviral proteolytic processing (Katoh et al., 1989). However, pepstatin-A is a poor inhibitor of the HIV-1 proteinase (Richards et al., 1989). Therefore, early studies concluded that HIV-1 did not carry an aspartic proteinase. However, the compounds H261 (tBoc-His-Pro-Phe-His-Leu[CHOH-CH2]Val-Ile-His) and isovaleryl-pepstatin (Iva-Val-Val-Sta-Ala-Sta) were strong inhibitors of the HIV-1 proteinase (Richards et al., 1989). It was concluded that the binding pocket of the Hiv-1 proteinase was smaller than cellular aspartic proteinases and that the pepstatin-A was unable to fit into yhe binding cavity, while the smaller aspartyl inhibitors H261 and isovarely-pepstatin-A were effective in binding the proteinase. The HIV-2 proteinase was more strongly inhibited by acetyl-pepstatin (Ac-Val-Val-Sta-Ala-Sta), an inhibitor of the classical aspartic proteinases (Richards et al., 1989).

Confirmation of the role of Asp-Thr-Gly in the active site of retroviral proteinases came from mutational analysis. A complete loss of proteolytic activity was observed when the active site Asp residue was altered (Kay and Dunn, 1990). The HIV-1 Asp-Thr-Gly sequence was modified and mutations of the Asp residues to Ala or Thr were introduced, it resulted in a loss of processing. Additionally, computer modeling of the proteinase sequence predicted a viral proteinase with a folding pattern, for the central core of the molecule, nearly identical to that of the archetypal aspartic proteinases (Richards et al., 1989), so a number of pepstatin derivatives were examined for their effect on processing.

Cysteine proteinases

Cysteine proteinases contain an active site thiol group. In the case of RNA viral proteinases the cysteine proteinases are related to the trypsin-like family of serine proteinases. Animal picornaviruses and plant como- and potyviruses form a related group of serine-like cysteine proteinases (Bazan and Fletterick, 1988). In this class of enzymes, the active site serine residue of trypsin is replaced by cysteine in the viral proteinases. The animal and plant viruses containing these serine-like cysteine proteinases all contain positive strand RNA genomes. These are expressed as a polyprotein which is preferentially cleaved at Gln-Gly and Tyr-Gly pairs to release mature proteins (Bazan and Fletterick, 1988). The 3C and 2A proteinases of picornaviruses have been identified as members of the cysteine class by inhibitor

studies and by site-specific mutagenesis experiments (Bazan and Fletterick, 1988; Hellen et al., 1989).

Serine proteinases

Serine proteinases are subdivided into two families based on the catalytic site present in the enzyme. Crystallographic studies of serine proteinases has revealed a characteristic tertiary folding pattern of the trypsin-like enzymes (Bazan and Fletterick, 1990). The serine proteinases are characterized by the catalytic triad His, Asp and Ser which create the active site crevice and are closely related to the cysteine proteinases. The serine residue of the catalytic triad was observed to be in a similar geometric arrangement to that of the papain-like cysteine proteinases and site-directed mutagenesis were performed to substitute the catalytic residues of these enzymes. A Ser to Cys mutation in the serine proteinase active site severely reduced activity due to the intrusion of the larger sulfur atom of the cysteine residue (Bazan and Fletterick, 1990). The structural changes observed between the serine and cysteine proteinases could represent compensating changes to the geometry of the active site. Serine proteinases were identified in enveloped RNA viruses after the discovery of viral cysteine proteinases. The flavi- and pestiviruses were identified as serine proteinases because of the presence of a Ser residue in the active site and the binding pocket formed exhibited structural differences with the cysteine proteinases.

Metallo proteinases

Metalloproteinases contain a Zn atom that is present in the catalytic site of the enzyme. These residues coordinate divalent cations for catalytic activity. Two families of the cellular metallo proteinases have been described based on the catalytic residues present in the enzyme. The catalytic site of these enzymes is Zn, Tyr and Glu or Zn, Glu and His. There have been no reports of viral metallo proteinase enzymes.

Viral proteinases

Viral-encoded proteinases can be divided into three distinct groups. A large group of picornavirus 3C-related proteinases exists including the proteinases of the como-, poty- and nepoviruses (Wellink and van Kammen, 1988). The proteinases encoded by these viruses show significant amino acid similarities with each other. A second group of virus-encoded proteinases is formed by the retroviruses. These proteins share amnio acid similarity with each other, but not with other enzymes. These enzymes belong to the aspartyl proteinase group, as described above. The third group of virus-encoded proteinases includes enzymes from the the alpha- and flaviviruses. These proteinases are responsible for processing the non-structural proteins of these viruses. The proteinases from the alpha- and flaviviruses have not been characterized, but since they show no similarity to known viral or cellular proteinases, it was proposed that these enzymes may constitute a new group within one of the four main families of proteinases (Wellink and van Kammen, 1988).

III. Characterization of the Proteinase, NS, Responsible for the Autocatalytic Processing of the Large Segment of Infectious Pancreatic Necrosis Virus

ABSTRACT

The large genomic RNA, segment A, of infectious pancreatic necrosis virus (IPNV) is expressed as a polyprotein which is processed to yield the structural proteins VP2, major capsid protein, and VP3, minor capsid protein, by the putative autocatalytic proteinase NS. Molecular and biochemical analysis of NS was performed to identify the proteolytic class and functional properties of this enzyme. A cDNA encoding the entire IPNV-Sp A segment was expressed in a cell-free transcription and translation system. Deletion mapping localized the NS coding region within the A segment, between the EcoRI and NsiI sites of IPNV-Sp. Site-directed mutagenesis was used to define the catalytic regions of the NS proteinase and its cleavage sites. Amino acid similarity between NS and aspartyl proteinases suggested that a Asp-Thr-Gly triad, the catalytic center of aspartyl proteinases, was a good target for site-directed mutagenesis. Conversion of this sequence to Val-Thr-Gly had no effect on proteolysis. Mutagenesis of potential cleavage sites indicated that the conversion of Tyr-Leu to Pro-Gly altered, but did not completely inhibited, proteolytic processing at the C-terminus of NS. Traditional methods of classifying the enzyme by sensitivity to proteinase inhibitors failed to give any insight as to the nature of the NS proteinase. These findings suggest that NS may represent a novel class of viral proteinases.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) causes an acute contagious disease in juvenile salmonids (Pilcher and Fryer, 1980) and outbreaks of the disease may be economically devastating to a hatchery. The virus has also been isolated from eels and marine molluscs, but any association between IPNV and disease in these animals has not been observed. IPNV is a member of the Birnaviridae family which also includes infectious bursal disease virus (IBDV) of fowl and Drosophila X virus. This family of viruses is characterized by a bisegmented genome consisting of double-stranded RNA enclosed in a non-enveloped virion. The large segment, A, contains a large open reading frame which is expressed as a polyprotein (NH2-pVP2-NS-VP3-COOH) that is processed to yield the structural proteins pVP2 (63 kDa) and VP3 (31 kDa), as well as the non-structural protein NS (28.5 kDa) (Huang et al., 1986; Nagy et al., 1987). The smaller segment, B, encodes the viral RNA-dependent RNA polymerase, VP1 (90 kDa) (Duncan et al., 1991).

Proteolytic processing has been found to regulate viral expression for many plant and animal viruses. Virtually all proteolytic enzymes can be classified in one of four groups by their sensitivity to different classes of inhibitors as aspartic, cysteine, metallo and serine proteinases (Kay and Dunn, 1990). For IPNV, NS was identified as the viral proteinase which acted autocatalytically to produce VP2 NS and VP3 from a polyprotein.

This processing was shown to occur *in vitro* (Duncan et al., 1987; Manning et al., 1990) and in *Escherichia coli* (Manning et al., 1990). Evidence for NS function was derived from deletion analysis in which the boundaries of VP2, NS and VP3 were approximated. A deletion into the NS catalytic site resulted in a VP2-truncated NS (VP2-NS') polyprotein (Manning et al., 1990).

A comparison of the NS amino acid sequence with other proteinases has revealed no significant similarity between these enzymes. Although there were limited regions of possible similarity, the classification of NS by sequence comparison was not possible. Thus, the sensitivity of the NS proteinase to proteinase inhibitors was tested by *in vitro* translation assays. Eight inhibitors were tested and all failed to inhibit processing. In addition, site-directed mutagenesis at canonical aspartyl proteinase and cysteine proteinase sites did not alter the proteinase activity. These findings have led us to propose that NS is a novel viral proteinase.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from Bethesda Research Laboratories (BRL), Boehringer-Mannheim and Promega. The T7 RNA polymerase, T4 DNA ligase and RNAsin were obtained from Boehringer-Mannheim Corporation, Bethesda Research Laboratories (BRL) and Promega. The source for SeaKem GTG and Sea Plaque (low gelling temperature) agarose was FMC. Hyperfilm (Amersham) and XAR film (Kodak) were used for autoradiography.

Virus

The Sp serotype of IPNV was used in this study. IPNV-Sp was first isolated in Denmark (Jorgensen, 1969). This new virus isolate was highly pathogenic to rainbow trout. The isolate of IPNV-Sp used in this study was obtained from R. P. Hedrick, University of California, Davis. Virus was propagated in CHSE-214 cells, originally derived from Chinook salmon embryos (Fryer et al, 1965), at 16C.

Plasmid Constructs

The construction of cDNA clones for IPNV-Sp pT7-2/A and recombinant plasmid pGEM A- has been described (Huang et al., 1986; Manning et al., 1990). These recombinant plasmids encode the entire A segment of IPNV-Sp. The pGEM A-5'EcoRI was constructed by the deletion of the majority of VP2 from the 5' end of the A segment to the EcoRI site (Fig. III.1). The pSELECT-A clone

was constructed by linearization of the pSELECT phagemid (Promega) with the restriction enzymes BamHI and HindIII. The cDNA clone of the A segment was isolated from the plasmid pGEM A- with BamHI and HindIII. The phagemid was then used to synthesize single-stranded DNA (ssDNA) for site-directed mutagenesis experiments.

In vitro Transcription and Translation

Plasmids containing wild type or mutated DNA sequences were linearized with restriction enzymes BamHI (pT7-2/A) or Hind III (pGem A-, pGem A- 5'EcoRI) and transcribed with T7 RNA polymerase, as per manufacturer instructions. Deletions from the 3' end of the A segment were introduced using restriction enzymes within the A segment coding region (Fig. III.1). The RNA transcripts were translated in a rabbit reticulocyte lysate (Promega) containing 50 μCi ^{35}S methionine (Amersham), ^{14}C`leucine (NEN-Dupont) or ^{35}S methionine/^{14}C leucine. The reaction volume was 50 μl. Translation reactions were incubated for 60 minutes unless indicated otherwise. Co-translation reactions were performed as described, with two RNA templates to examine "*trans*" proteolytic activity. Translation reactions were terminated by the addition of Laemmli sample buffer. The protein products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were fixed in 30% methanol, 10% glacial acetic acid, and autoradiographs of the radioactive gels were prepared by standard methods.

Proteinase Inhibitor Studies

Proteinase inhibitors were purchased from Boehringer-Mannheim and Sigma Biochemicals. The aspartyl proteinase inhibitor H261 was kindly provided by Dr. John Kay, University of Cardiff, Wales, U.K. The inhibitor TPCK was solubilized in ethanol, therefore translation reactions were performed using ethanol as a control. All the other inhibitors were soluble in water. The effect of each inhibitor was determined by incubating translation reactions containing ^{35}S methionine in the presence of the inhibitor. Varying concentrations of the inhibitors were added directly to in vitro translation reactions, at the start of the reaction or at 15 min after the initiation of the reaction. The translation reactions were programmed with full-length mRNA. Full-length mRNA transcripts without the addition of inhibitors were used as controls. The pH of the translation reaction was adjusted to pH 7.0 and pH 6.5 with HEPES (pH 6.0), to examine the ability of pepstatin-A or H261 to inhibit proteolysis. The inhibitors, concentrations used and results are shown in Table III.1. Reactions were stopped by the addition of Laemmli sample buffer and the reaction products were examined by SDS-PAGE and autoradiography.

Site-directed Mutagenesis

Site-directed mutagenesis was performed on the cDNA insert obtained from pT7-2/A. This cDNA, containing the entire A segment, was subcloned into the pSELECT vector (Altered Sites in vitro Mutagenesis System, Promega), which resulted in pSelect/A.

Amino acid alignments between Sp, Jasper and N1 were used to target site specific mutagenesis (Fig. III.2). A single-stranded form the pSelect/A DNA was synthesized using the helper phage M13KO7 or R408. Five different oligonucleotides were used to introduce specific amino acid substitutions into the pSelect/A target DNA (Fig. III.4). Oligonucleotides containing the desired mutation were hybridized along with an ampicillin repair oligonucleotide to the single-stranded template, double-stranded DNA was synthesized. Recombinant colonies were screened by colony blots using the mutagenic oligonucleotide as a probe. The oligonucleotides were labeled with digoxigenin (Boehringer-Mannheim). Mutations introduced were identified by restriction analysis and confirmed by nucleotide sequence analysis (Sanger et al., 1977).

Polymerase chain reaction (PCR) site-directed mutagenesis was also performed. Single- or double-stranded DNA was used as a template. Mutagenic oligonucleotides were used as an upstream primer, non-mutagenic oligonucleotides were used as a downstream primer. The amplified product, which contained the desired mutation, was then used again as a primer and a larger portion of the cDNA was amplified (Fig. III.5). Mutated DNA was then digested with NcoI and NsiI and subcloned back into the parent plasmid pGem A- containing wildtype A segment sequences. The mutant recombinants were screened for processing activity by an *in vitro* transcription and translation assay.

Radioimmune Precipitation

Samples from in vitro translation reactions were immunoprecipitated with polyclonal antisera specific for either whole Sp virus, VP2 or VP3. The precipitations were performed in a volume of 60 µl containing 35 µl of a 10% protein-A sepharose suspension, 20 µl of a 1:20 dilution of polyclonal rabbit antisera, 5µl ^{35}S-methionine labeled translation products. Reactions were incubated at 37C for 4 h with intermittent gentle rocking and then left at 4C overnight. Samples were washed four times with 200 µl of radioimmune precipitation assay (RIPA) buffer (0.1% SDS, 0.2% sodium deoxycholate, 150 mM NaCl, 10 mM Tris pH 7.4, 0.5% NP40). Laemmli sample buffer was added and samples were analyzed by SDS-PAGE and autoradiography.

RESULTS

Deletion Mapping of NS

The domain of the NS proteinase was mapped by specific deletions in plasmids encoding NS and examination of the products produced in an in vitro translation system. The results of these studies are summarized in Fig. III.1. The plasmid pGEM A-, encoding the entire A segment, was used as a template. When RNA, encoding the full-length A segment, was used to program translation reactions and the products analyzed by SDS-PAGE and autoradiography, the resulting products were VP2 (63 kDa), 38 kDa protein, VP3(31 kDa) and NS(28.5 kDa) (Fig. III.B, lane 1). Deletions from the 5' end of the A segment to the EcoRI site (pGEM A-5'EcoRI) resulted in the production of the 38 kDa protein, VP3 and NS (Fig. III.1B, lane 2). Deletions to the XbaI site (pGEM A-5'XbaI) resulted in the production of only the 38 kDa protein (Fig. III.1B, lane 5). Deletions proceeding from the 3' end of the A segment to the NsiI site (pGEM A-3'NsiI) resulted in the production of VP2 and NS (Fig. III.1B, lane 3). Deletions to the AccI site (pGEM A-3'AccI) resulted in the production of a truncated polyprotein (tpp-AccI) (Fig. 1B, lane 4) (Manning et al., 1990). Thus, the functional boundaries of the NS protein appeared to lie within the EcoRI and NsiI sites (Fig. III.1). The observed size of the NS protein (28.5 kDa) would conform to the region encoded by the EcoRI-NsiI DNA fragment. The C-terminus of NS has been localized between the AccI and NsiI sites, a region spanning 186 base pairs. Deletions 3' to the NsiI site result in the correct processing of the polyprotein. The larger

molecules (Fig. 111.1b, lane 3) found indicate that the processing was not as efficient as the wild-type molecule, but cleavage did occur at the VP2-NS junction. However, deletions to the AccI site result in the production of a truncated polyprotein. These results suggest that an active site domain is present between the AccI and NsiI sites or that NS proteolysis is dependent on the folding of domains in this region.

Inhibitor Studies

In an effort to determine the proteolytic class of the NS protein a number of proteinase inhibitors were utilized. Compounds known to inhibit proteinases from several classes, including aspartyl-, cysteine, metallo- and serine were used in this study. Eight inhibitors including serine proteinase inhibitors, TLCK-HCL and TPCK; metallo inhibitor EDTA; aspartic inhibitors pepstatin-A and H261; cysteine inhibitors iodoacetimide, N-ethylmaleimide and zinc sulfate were used in an in vitro translation assay for the NS proteinase. The results are shown in Table III.1. Although none of the inhibitors examined in this study altered proteolytic processing, sequence similarities observed between NS and retroviral aspartyl proteinases indicated that NS may be related to the aspartyl proteinases. Therefore, the putative catalytic site was targeted by site-directed mutagenesis.

Catalytic Site of NS Examined by Site-directed Mutagenesis

Computer alignment of the NS sequence with that of other known proteinases demonstrated little homology with NS. However, there were some sequence similarities with both the aspartyl- and the serine-like cysteine proteinases (Fig. III.3A and 3B). The putative NS proteinase contained a sequence Asp_{575}-Thr_{576}-Gly_{577} (Box I) which corresponded to the catalytic site of aspartyl proteinases (Fig. III.3A). Although this sequence alone doesn't classify the protein as an aspartyl proteinase, NS also contained other sequence similarities with aspartyl enzymes. Sequences in box II form a loop or a "flap " sequence which may form the binding site cavity (Fig. III.3A) (Kay and Dunn, 1990). Finally, a hydrophobic-hydrophobic-Gly, box III, is necessary in retroviral proteinases for proper folding (Fig. III.3A). In the case of NS, this is a Leu-Ile-Gly near the C-terminus of the proteinase. It was demonstrated that the Asp residue was critical for the HIV-1 aspartyl proteinase activityby site-directed mutagenesis. The conversion of the Asp residue to Ala or Thr resulted in the loss of proteolytic activity (Richards et al., 1989). Therefore, if NS was, indeed, an aspartyl proteinase, then the canonical Asp-Thr-Gly active site should be inactivated upon conversion to Val-Thr-Gly. The Asp residue was converted to a Val by PCR mutagenesis (see Materials and Methods) (Fig. III.5). The altered region was then subcloned into a wild-type cDNA to produce the clone, RNA transcribed from this clone was translated _in vitro_ and the products synthesized were examined by SDS-PAGE. The mutation of the Asp to Val did not affect the proteolytic processing of NS

(Fig. III.6, lane 2). The Val conversion did not alter processing of NS, and the A/D-V_{584} clone produced VP2 (63 kDa), 38 kDa, VP3 (31 kDa) and NS (28.5 kDa). The mutation was verified by nucleotide sequence analysis of the A/D-V_{584} clone (Fig. III.7).

Another series of alignments, indicated that there might be some similarity between NS and the serine-like cysteine proteinase family (Fig. III.3B). The catalytic residues Asp_{585}, Cys_{569} and His_{579} could be aligned with NS and there was comparable spacing between these sites in NS. Mutagenesis of the catalytic Cys to a Ser, similar to experiments with poliovirus, did not result in reduced activity (data not shown).

Cleavage Site of NS Examined by Site-directed Mutagenesis

Since the foregoing experiments did not identify a catalytic active site, an attempt was made to determine the cleavage sites recognized by NS. Numerous failed attempts to obtain micro protein sequence data with radiolabeled gel purified NS and VP3 and the inability of other researchers to obtain N-terminal protein sequence data from purifed virion proteins (Duncan et al., 1987), suggested that these proteins may be N-terminally blocked. We then examined the alignments between the sequences available for birnaviruses (Duncan et al., 1987; Hudson et al., 1987; Haverstein et al., 1991 and Mason et al., manuscript in preparation). Alignments at the predicted borders of NS revealed a high degree of similarity and potential cleavage sites. A Tyr_{591}-His_{592} conserved between the Sp and N1 isolates of IPNV and a Tyr-Leu

found in the Jasper isolate were predicted to be the processing site at the N-terminus of NS on the basis of size and similarity. At the C-terminus there was a conserved Tyr_{725}-Leu_{726} for all three IPNV isolates (Fig. III.2) as well as for IBDV. These were targeted in site-directed mutagenesis and converted to Pro-Gly. The N-terminal Tyr-His to Pro-Gly had no affect on proteolytic processing, yielding VP2 (63 kDa), 38 kDa, VP3 (31 kDa) and NS (28.5 kDa) (Fig. III.8, lane 1). However, conversion of the C-terminal Tyr-Leu to Pro-Gly resulted in altered processing. The Pro-Gly mutation resulted in a reduced ability for NS to process the NS-VP3 junction. In vitro translation of RNA from the pGEM A-5'EcoRI/YL-PG resulted in the production of an NS-VP3 fusion as well as VP3 and NS (Fig. III.8, lane 2). In vitro translation of RNA from the pGEM double mutant (DM) resulted in the production of VP2 and an NS-VP3 fusion protein (Fig. III.8, lane 3). The identity of the NS-VP3 fusion was confirmed by radioimmune precipitation (Fig. III.9). These results suggest that the Tyr-Leu is either the processing site of the NS-VP3 junction or that the Pro-Gly introduced secondary structure that was able to interfere with normal processing. Although processing was not entirely eliminated, it was reduced and high levels of the NS-VP3 fusion protein were detected.

In addition to the mutagenesis of potential active and cleavage sites, Duncan and Dobos (1987) reported a single amino acid difference between two Jasper isolates, within the NS coding region, that resulted in the detection of the full-length

polyprotein. Normally, proteolytic processing occurs rapidly and the polyprotein is not detected. The single amino acid change Gly_{691} was an Arg in the "slow-processing" mutant. This mutation was introduced into the Sp isolate by site-directed mutagenesis. Subsequent analysis of this mutant showed that the polyprotein processing proceeded normally (Fig. III.6, lane 3) and suggested that this amino acid substitution was not responsible for the "slow-processing" of the Jasper isolate.

Trans-processing Activity of NS

We have examined the ability of NS to act in *trans* to process another molecule. The tppAccI polyprotein was used as a target molecule and co-translated with RNA synthesized from the full-length A segment, pGem A-, and a clone in which VP2 was deleted, pGem A-5'EcoRI. Translation reactions were incubated for 60 min, 4 h and 16 h. Products were then analyzed by SDS/PAGE (Fig. III.10). The tppAccI polyprotein product did not decrease over time, nor did VP2 appear in the reactions co-translated with RNA synthesized from the pGEM A-5'EcoRI template, containing no VP2. Co-translation of the full-length A segment RNA and the tppAccI polyprotein, even in reactions carried out overnight, failed to demonstrate the ability for NS to act in *trans*. Therefore, NS appears to act only in *cis* by an autocatalytic mechanism.

DISCUSSION

Many plant and animal viruses regulate protein expression by virally-encoded proteinases. The genomes of RNA viruses are often expressed as a polyprotein which must be proteolytically processed to yield mature viral proteins. Proteinases have been classified into four groups based on the prominent catalytic groups which constitute the active site (Kay and Dunn, 1990). Aspartic proteinases, including the retroviral enzymes, contain an Asp-Thr-Gly sequence required for catalytic activity (Kay and Dunn, 1990). Mutagenesis of the Asp residue resulted in the complete loss of proteolytic activity. In addition, these enzymes are inhibited by compounds which characterized them as belonging to the aspartic proteinase class. Cysteine proteinases contain a catalytic triad of Cys, His and Asn residues. Recently, cysteine proteinases related to the trypsin-like serine proteinases has been described (Bazan and Fletterick, 1988). This enzyme family contains a catalytic triad composed of His, Asp and Cys residues, where the Cys replaced the location of the Ser residue in serine proteinases. Mutational analysis of the tobacco etch virus has confirmed the importance of the Cys residue, as well as inhibitor studies which demonstrated that these enzymes are sensitive to cysteine proteinase inhibitors (Dougherty et al., 1989).

We have approached identifying the proteolytic nature of NS using deletion analysis and site specific mutagenesis. The NS proteolytic activity has been localized through plasmid deletion analysis to a region spanning approximately 740 bp. These

deletions localized a domain of NS necessary for proteolytic processing either by the removal of the proteolytic active site or by the deletion of amino acid residues critical for the native folding of the polypeptide allowing proper presentation of the processing sites.

Site-specific mutations were introduced and proteinase inhibitors were used in an attempt to identify the active site residues and the proteolytic processing site of NS. Putative cleavage sites were identified on the basis of conserved amino acid residues and the predicted molecular weights of the IPNV proteins. A putative cleavage site at the N-terminus of NS was identified as Tyr_{591}-His_{592} and was converted to Pro-Gly through site directed mutagenesis. This conversion failed to alter the proteolytic processing of the polyprotein, as these mutants processed normally in an in vitro transcription and translation system. Another mutation was introduced at the predicted C-terminus of NS, a conserved Tyr_{725}-Leu_{726} was converted to Pro-Gly. In this case, the Pro-Gly introduced at the C-terminus resulted in only two bands being produced in vitro, a band comigrating with VP2 and a band migrating slightly slower than VP2. A similar event was observed when this Pro-Gly was introduced to the clone lacking VP2. However, with this clone VP3 and NS were detected. The Pro-Gly introduced at the C-terminus of NS eliminated processing between NS and VP3, resulting in a NS-VP3 fusion. The presence of VP3 and NS with the VP2-deleted clone could have resulted from a reduced stringency of secondary

structure. The absence of VP2 may not have allowed proper folding of the molecule and NS was able to process its target site between NS and VP3 more efficiently but not completely. Deletions 3' to the NsiI site result in the correct processing of the polyprotein. The larger molecules found indicate that the processing was not as efficient as the wild-type molecule, but cleavage did occur at the VP2-NS junction. The absence of the VP3 protein may have disrupted the proper secondary structure influencing the fidelity of the NS proteinase.

Traditional methods of characterizing viral proteinases have not affected the processing of the NS protein. Inhibitors of proteinases were used to identify the porteolytic class of NS. All eight inhibitors examined had no affect on the processing event. The insensitivity of NS to proteinase inhibitors does not eliminate it from being related to one of the four proteinase classes. HIV-1 contains an aspartyl proteinase, however, pepstatin, an aspartyly proteinase inhibitor was a poor inhibitor of HIV-1 (Richards et al., 1998). Thus, on the basis of inhibitors alone it is not possible to classify the NS proteinase. Although amino acid alignments indicated that NS may be a aspartic or cysteine proteinase, the results of mutagenesis and inhibitor studies indicate that NS may represent a novel class of viral proteinases.

Since the A segment polyprotein has not been detected _in vivo_ or _in vitro_, NS must process the polypeptide rapidly. Mounting evidence suggests NS processing occurs during protein synthesis or shortly after completion, since time course studies in which

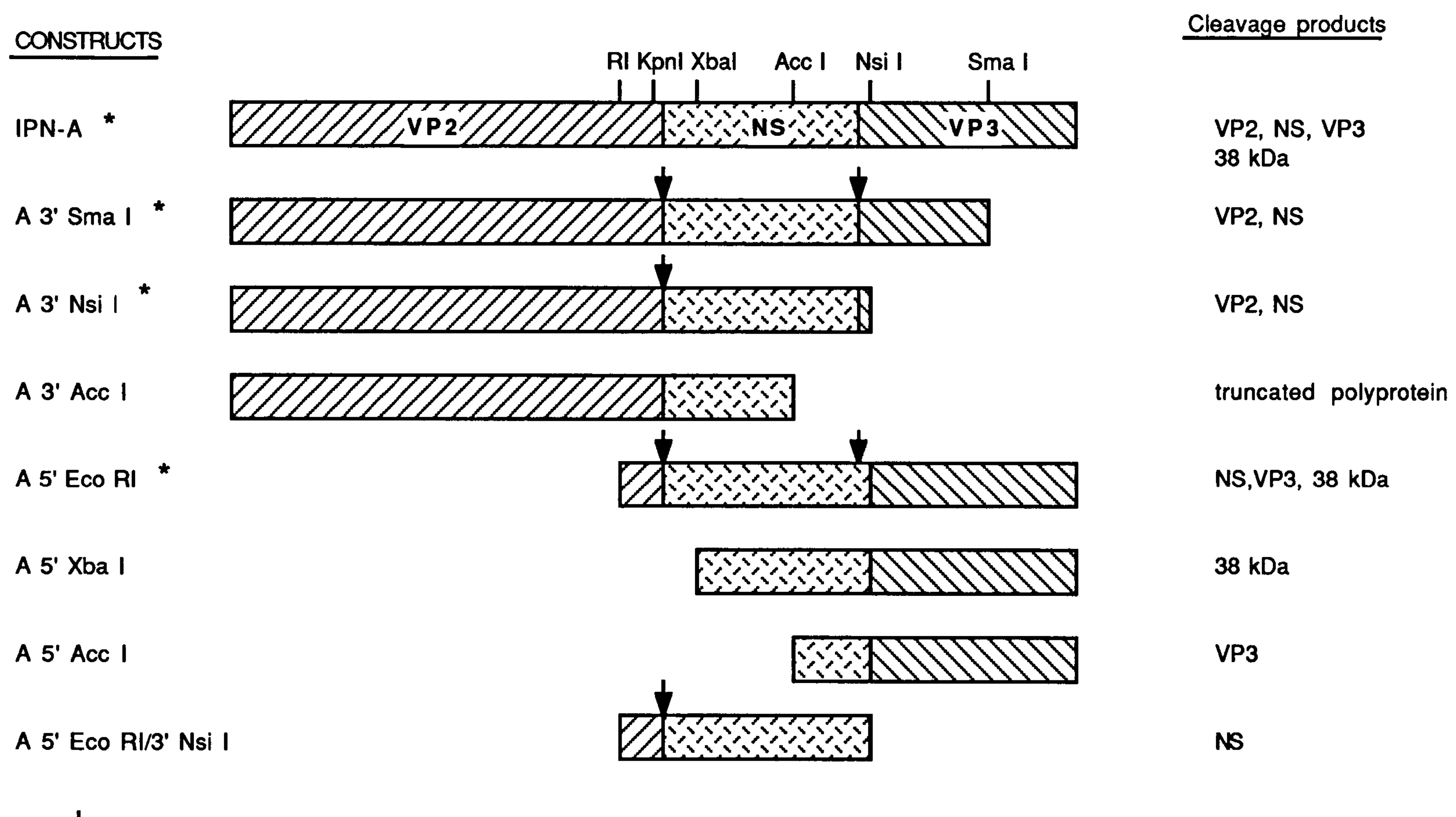
samples were removed from in vitro translation reactions demonstrated that as soon as protein products were detectable NS had processed the polyprotein (data not shown). We present evidence that NS is an autocatalytic proteinase. Previously, it was reported that antibodies specific for IPNV were unable to inhibit the proteolytic processing of the A segment. It was suggested that polyprotein processing may occur rapidly prior to antibody binding (Manning et al.,1990). Additionally, the presence of the 38 kDa protein in vivo and in vitro suggests that NS is unable to act in trans. The 38 kDa protein appears to be produced from internal ribosomal initiation downstream of the 5' end of NS from an in-frame ATG codon (Manning et al., 1990). The 38 kDa protein therefore carries a cleavage site for NS at the NS-VP3 junction. However, due to the existence of this protein the cleavage site of the 38 kDa does not appear to be utilized.

Further characterization of the proteinase is required to determine its cleavage and catalytic sites, however, the information obtained in this study indicates that the NS protein is a novel proteinase. The unique properties of this enzyme make it an ideal target for antiviral therapy.The inability to inactivate the proteinase by the use of inhibitors or through site-directed mutagenesis and additional evidence that the NS proteinase acts only in cis indicate that NS is a novel proteolytic enzyme, possibly representing an undefined class of viral proteinases.

In this and previous reports (Manning et al., 1990), we have demonstrated the production of VP3 and NS, despite the deletion of

the 5' initiation codon in the VP2 gene. The presence of the 38 kDa protein in both in vitro translations and the infected cell (Manning and Leong, 1990), raised the question of how the 38 kDa is formed. Since the A segment is expressed as a polyprotein and proteolytically processed, internal translation initiation has been proposed as a mean of expressing the 38 kDa protein. The initiation site for the synthesis of NS, VP3 and the 38 kDA has not been determined. However, we have examined the nucleotide sequence surrounding Met_{603} and Met_{630}, where the 38 kDa protein would be initiated based on its size. The context of Met_{630} is favorable and contains a Kozak concensus sequence (Kozak, 1986) ANNAUGG. We have examined the secondary structure of these potential initiation codons and the regions contain hairpin and loop structures that could initiate protein synthesis.

Fig. III.1. Plasmid transcription vectors used to produce RNA transcripts containing various deletions of the A segment. A.) A schematic representation of the gene arrangement of the A segment is shown here. Restriction sites used are indicated in the top portion of the figure; RI for EcoRI, KpnI, XbaI, AccI, NsiI and SmaI. The estimated gene regions for VP2, NS and VP3 are shown. The plasmid vectors pT7-2/A and pGem A- (Huang et al., 1986; Manning et al., 1990), were used to construct progressive deletions from the 3' and 5' ends of the A segment at the designated restriction sites. The coding regions contained by the respective constructs are indicate by the filled boxes. A summary of the resulting cleavage products observed by SDS-PAGE are listed to the right of each construct. B.) *In vitro* translation products resulting from the plasmid deletions performed on the A segment. Translation products were separated by SDS-PAGE and autoradiography. Arrows indicate the resulting products VP2 (63 kDa), VP3 (31 kDa), NS (28.5 kDa), tpp (80 kDa) and the 38 kDa polypeptides. Lane 1, full-length A segment; lane 2, 5'EcoRI deletion; lane 3, 3' NsiI deletion; lane 4, 3' AccI deletion, 80 kDa tpp indicated; lane 5, 5' XbaI deletion.



↓ = PROTEOLYTIC CLEAVAGE

* = CONSTRUCTS WICH PRODUCE PROTEOLYTICALLY ACTIVE PROTEINS

B.

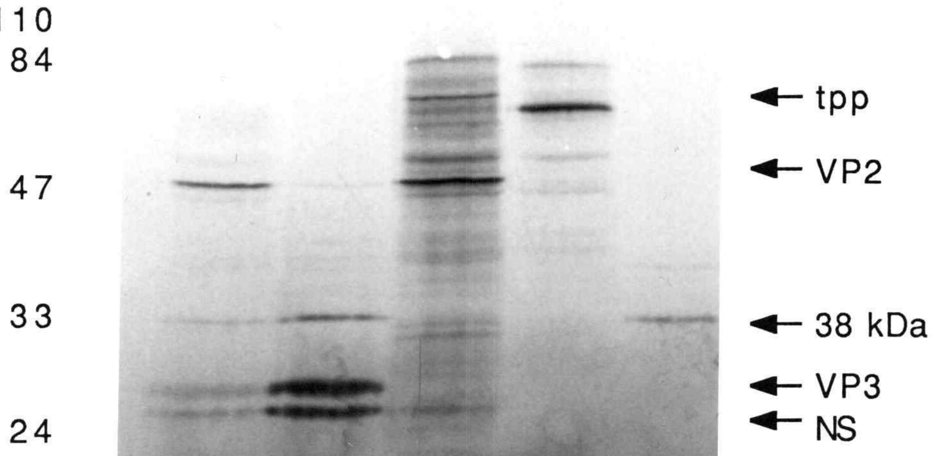


Fig. III.2. Deduced amino acid alignments for the IPNV NS proteins are represented by the single amino acid code. The IPNV Sp, Jasper (Ja) and N1 are shown. Regions spanning the putative VP2-NS and NS-VP3 borders are indicated. Restriction sites for the Sp isolate are shown.

```
                                                  VP2-NS border
                                                      KpnI
Sp  AWGWRDIVRGIRKIAAPVLSTLFPMAAPLIGTADQFIGDLTKTNAAGGRYHSMAAGGRYKDVL
JA  AWGWRDIVRGIRKVAAPVLSTLFPMAAPLIGAADQFIGDLTKTNSAGGRYLSHAAGGRYHDVM
N1  AWGWRDIVRGIRKVAAPVLSTLFPMAAPLIGMADQFIGDLTKTNAAGGRYHSMAAGGRHKDVL

Con AWGWRDIVRGIRK*AAPVLSTLFPMAAPLIG*ADQFIGDLTKTN*AGGRY*S*AAGGR**DV*


Sp  ESWASGGPDGKFSRALKNRLESANYEEVELPPPSKGVIVPVVHTVKSAPGEAFGSLAIIIRGE
JA  DSWASGSEAGSYSKHLKTRLESNNYEEVELPKPTKGVIFPVVHTVESAPGEAFGSLVVVIPGA
N1  ESWASGGPDGKFSRALKNRLESANYEEVELPPPSKGVIVPVVHTVKSAPGEAFGSLAIIIPGE

Con *SWASG***G**S**LK*RLES*NYEEVELP*P*KGVI*PVVHTV*SAPGEAFGSL***I*G*

        XbaI
Sp  YPSFLDANQQVLSHFANDTGSVWGIGEDIPFEGDNMCYTALPLKEIKRNGNIVVEKIFAGPIM
JA  YPELLDPNQQVLSYFKNDTGCVWGIGEDIPFEGDDMCYTALPLKEIKRNGNIVVEKIFAGPAM
N1  YPELLDANQQVLSHFANDTGSVWGIGEDIPFEGDNMCYTALPLKEIKRNGNIVVEKIFAGPIM

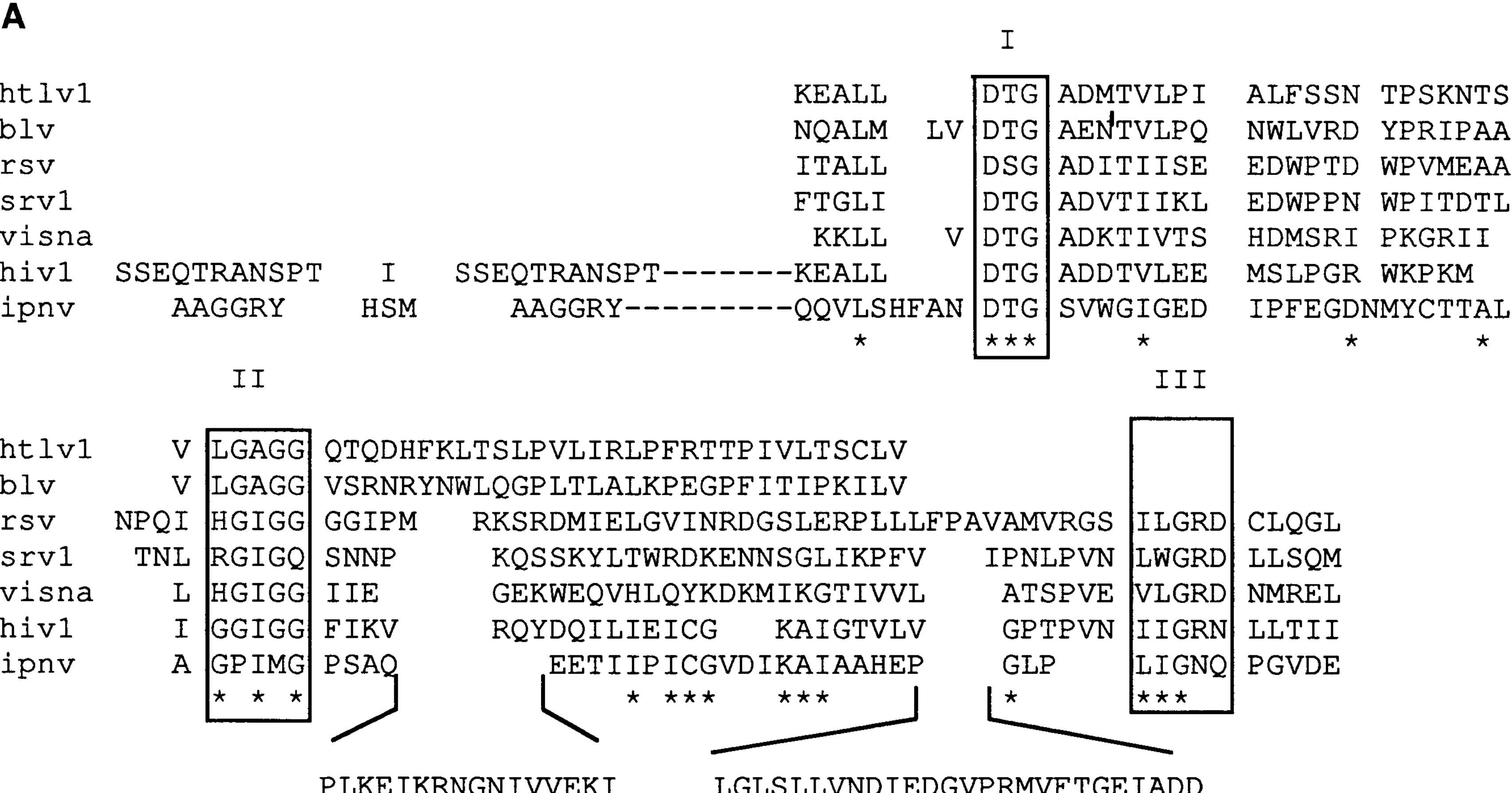
Con YP**LD*NQQVLS*F*NDTG*VWGIGEDIPFEGD*MCYTALPLKEIKRNGNIVVEKIFAGP*M

                                          AccI
Sp  GPSAQLGLSLLVNDIEDGVPRMVFTGEIADDEETIIPICGVDIKAIAAHEQGLPLIGNQPGVD
JA  GPSSQLALSLLVNDIDEGIPRMVFTGEIADDEETVIPICGVDIKAIAAHEHGLPLIGCQPGVD
N1  GPSAQLGLSLLVNDIEDGVPRMVFTGEIADDEETIIPICGVDIKAIAAHEPGLPLIGNQPGVD

Con GPS*QL*LSLLVNDI**G*PRMVFTGEIADDEET*IPOCGVDIKAIAAHE*GLPLIG*QPGVD
                                     NS-VP3 border
                                         NsiI
Sp  EEVRNTSLAAHLIQTGTLPVQRAKGSNKRIKYLGELMASNASGMDEELQRLLNATMARAKEVQ
JA  EMVANTSLASHLIQSGALPVQKAQGACRRIKYLGQLMRTTASGMDAELQGLLQATMARAKEVK
N1  EEVRNTSLAAHLIQTGTLPVQRAKGSNKRIKYLGELMASNASGMDEELQRLLNATMARAKEVQ

Con E*V*NTSLA*HLIQ*G*LPVQ*A*G***RIKYLG*LM***ASGMD*ELQ*LL*ATMARAKEV*
```

Fig. III.3. Amino acid alignment of the NS proteinase with aspartyl and cysteine proteinases. A.) Comparison of aspartyl proteinases of retroviruses with IPNV NS proteinase. Amino acid alignments of several retroviral proteinases with the catalytic triad DTG in box I. Box II contains the "flap" sequence necessary to aid in dimerization. Box III contains the hydrophobic-hydrophobic-Gly sequence important in proper folding. Asterisks indicated conserved amino acids between the aspartyl proteinases and IPNV-NS. B.) Comparison of serine-like cysteine proteinases. Amino acid alignments between several cysteine proteinases. Similarities between the birnavirus NS and poty- and picornaviruses are boxed. Numbering represents the IPNV-Sp NS amino acid numbering.


A
I
htlv1 KEALL DTG ADMTVLPI ALFSSN TPSKNTS
blv NQALM LV DTG AENTVLPQ NWLVRD YPRIPAA
rsv ITALL DSG ADITIISE EDWPTD WPVMEAA
srv1 FTGLI DTG ADVTIIKL EDWPPN WPITDTL
visna KKLL V DTG ADKTIVTS HDMSRI PKGRII
hiv1 SSEQTRANSPT I SSEQTRANSPT-------KEALL DTG ADDTVLEE MSLPGR WKPKM
ipnv AAGGRY HSM AAGGRY---------QQVLSHFAN DTG SVWGIGED IPFEGDNMYCTTAL
* *** * * *
II
III
htlv1 V LGAGG QTQDHFKLTSLPVLIRLPFRTTPIVLTSCLV
blv V LGAGG VSRNRYNWLQGPLTLALKPEGPFITIPKILV
rsv NPQI HGIGG GGIPM RKSRDMIELGVINRDGSLERPLLLFPAVAMVRGS ILGRD CLQGL
srv1 TNL RGIGQ SNNP KQSSKYLTWRDKENNSGLIKPFV IPNLPVN LWGRD LLSQM
visna L HGIGG IIE GEKWEQVHLQYKDKMIKGTIVVL ATSPVE VLGRD NMREL
hiv1 I GGIGG FIKV RQYDQILIEICG KAIGTVLV GPTPVN IIGRN LLTII
ipnv A GPIMG PSAQ EETIIPICGVDIKAIAAHEP GLP LIGNQ PGVDE
* * * * *** *** * ***
PLKEIKRNGNIVVEKI LGLSLLVNDIEDGVPRMVFTGEIADD

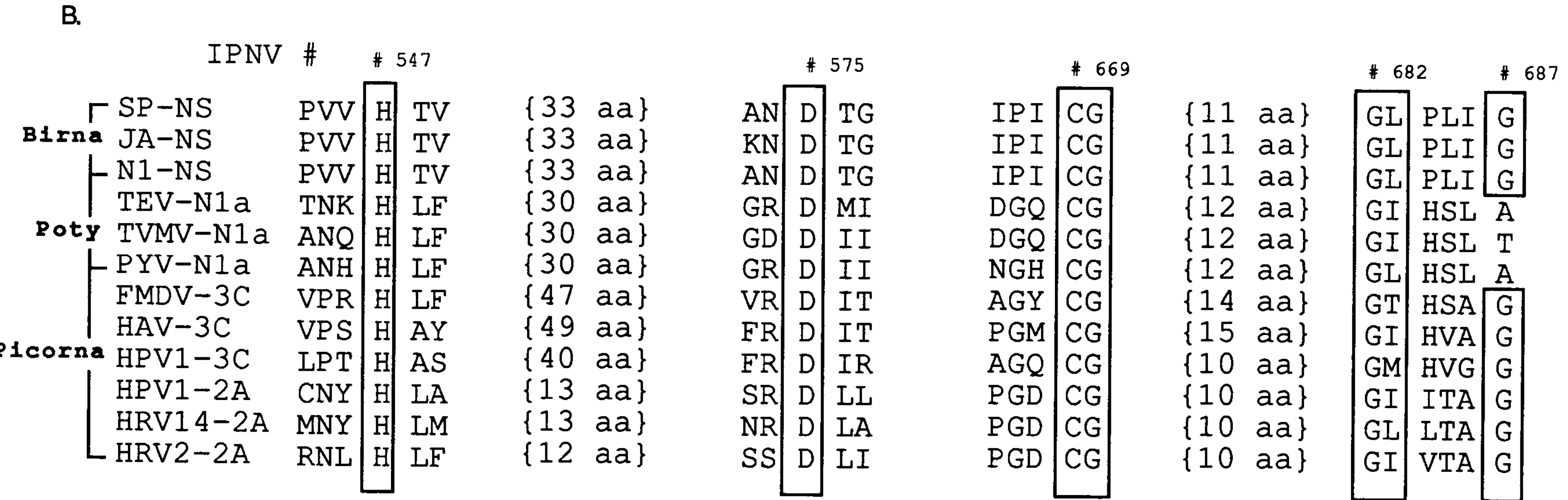
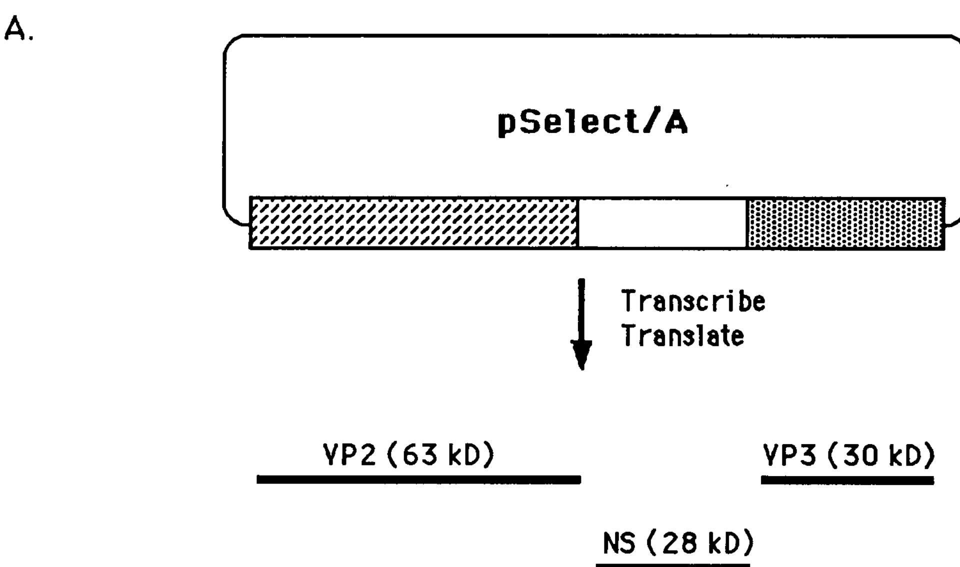

B.
IPNV #
547
575
669
682
687
Birna
Poty
Picorna
SP-NS PVV H TV {33 aa} AN D TG IPI CG {11 aa} GL PLI G
JA-NS PVV H TV {33 aa} KN D TG IPI CG {11 aa} GL PLI G
N1-NS PVV H TV {33 aa} AN D TG IPI CG {11 aa} GL PLI G
TEV-N1a TNK H LF {30 aa} GR D MI DGQ CG {12 aa} GI HSL A
TVMV-N1a ANQ H LF {30 aa} GD D II DGQ CG {12 aa} GI HSL T
PYV-N1a ANH H LF {30 aa} GR D II NGH CG {12 aa} GL HSL A
FMDV-3C VPR H LF {47 aa} VR D IT AGY CG {14 aa} GT HSA G
HAV-3C VPS H AY {49 aa} FR D IT PGM CG {15 aa} GI HVA G
HPV1-3C LPT H AS {40 aa} FR D IR AGQ CG {10 aa} GM HVG G
HPV1-2A CNY H LA {13 aa} SR D LL PGD CG {10 aa} GI ITA G
HRV14-2A MNY H LM {13 aa} NR D LA PGD CG {10 aa} GL LTA G
HRV2-2A RNL H LF {12 aa} SS D LI PGD CG {10 aa} GI VTA G

Fig. III.4. Schematic of IPNV mutagenesis vector and amino acid substitutions at the putative catalytic and cleavage sites. A) A schematic of the pSelect/A plasmid containing the entire coding region of the A segment. When pSelect/A was transcribed and translated in vitro the resulting products were VP2 (63 kDa), 38 kDa, VP3 (31 kDa) and NS (28.5 kDa) as depicted by the bars. B) The bar is a schematic of the NS protein. The borders of VP2/NS and NS/VP3 are shown above. Amino acids, represented by single letter code, are shown for the Sp isolate. The arrows mark the five mutations introduced by site specific mutagenesis. From the N-terminal of the protein: Tyr-His (YH) was converted to Pro-Gly (PG), Asp (D) to Val (V), Cys (C) to Ser (S), Gly (G) to Arg (R) and Tyr-Leu (YL) to Pro-Gly (PG).

A.



B.

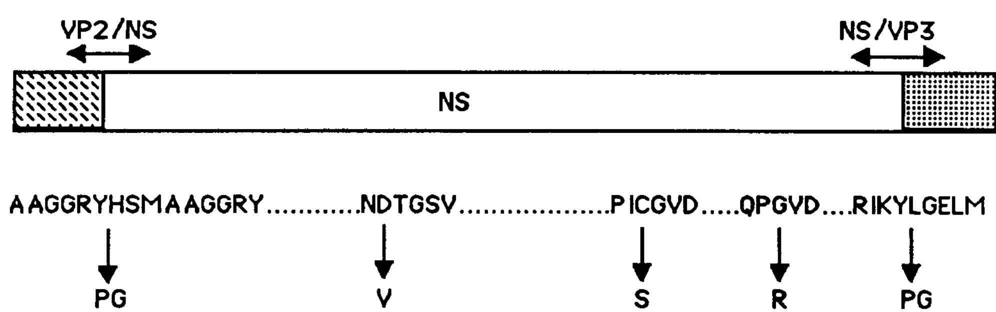
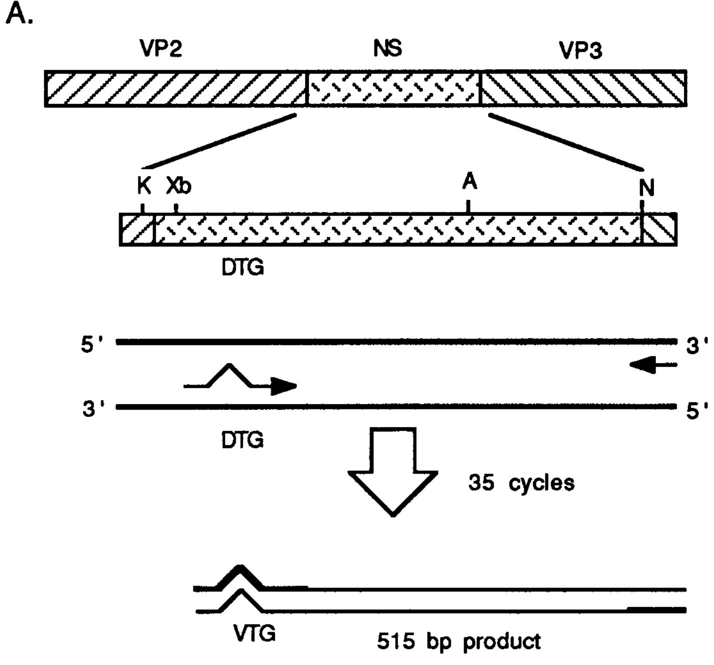


Fig. III.5. Schematic of the Polymerase chain reaction (PCR) site-directed mutagenesis. A.) A mutagenic oligonucleotide containing an Asp to Val mutation was used as an upstream primer and a non-mutagenic oligonucleotide was used as a downstream primer. A 515 bap fragment was isolated after 35 PCR cycles. B.) The 515 bp product from A, containing the Asp to Val mutation was purified and used as a downstream primer in a second round of PCR. A non-mutageneic upstream primer was used to amplify an 1184 bp product. This fragment was purified and digested with the restriction enzymes XbaI and NsiI which was then subcloned into wild-type A segment sequences.


A.
VP2
NS
VP3
K
Xb
A
N
DTG
5'
3'
3'
5'
DTG
35 cycles
VTG
515 bp product

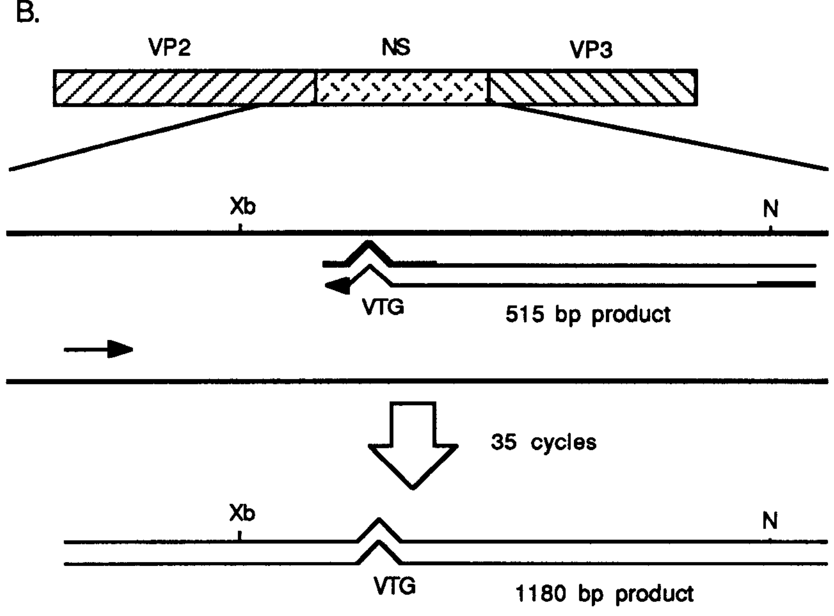

B.
VP2
NS
VP3
Xb
N
VTG
515 bp product
35 cycles
Xb
N
VTG
1180 bp product

Fig. III.6. In vitro translation products of the mutations introduced into the A segment. Translation products were separated by SDS-PAGE and visualized by autoradiography. Lane 1, full-length wild-type A segment; lane 2, mutation Asp-Val_{584} containing the VP2 gene in the negative orientation; lane 3, mutation Asp-Val_{584}, proper orientation; lane 4, mutation Gly-Arg_{691}. Arrows indicate VP2 (63 kDa), 38 kDa, VP3 (31 kDa) and NS (28.5 kDa). Molecular weight standards are shown.

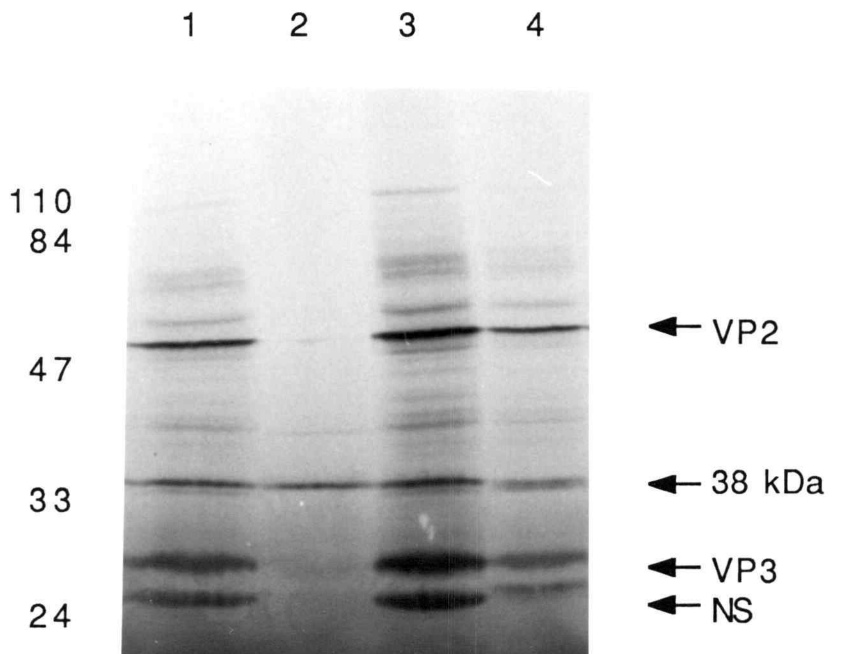
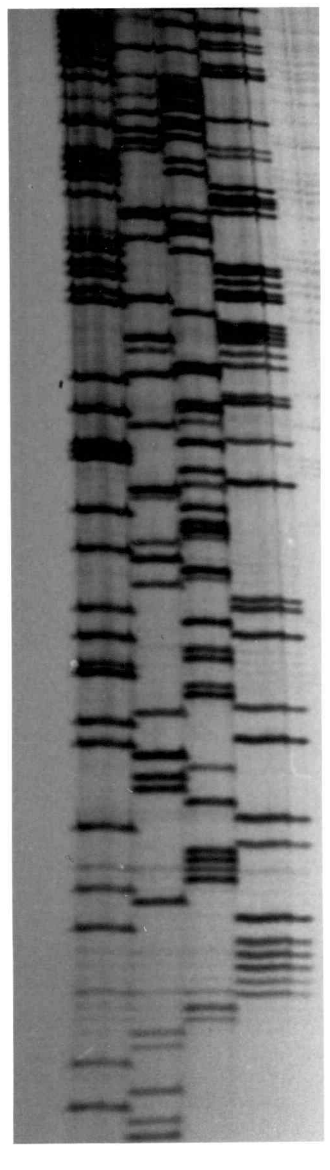

1
2
3
4
110
84
47
33
24
VP2
38 kDa
VP3
NS

Fig. III.7. Nucleotide sequence determination of Asp to Val mutagenesis. Nucleotide sequence spanning the Asp-Val$_{584}$ mutation is shown. Lanes are labeled A, T, C or G. The nucleotide changes introduced are indicated.

A T C G



C
T
G
C

D → V mutation

Fig. III.8. In vitro translation products of the mutations introduced at putative cleavage sites. Translation products were separated by SDS-PAGE and visualized by autoradiography. Lane 1, full-length A segment containing a Tyr_{591}-His_{592}; lane 2, 5' EcoRI deletion containing a Tyr_{725}-Leu_{726} to Pro-Gly; lane 3, double mutation in which both Tyr_{591}-His_{592} and Tyr_{725}-Leu_{726} were converted to Pro-Gly. Arrows indicate VP2 (63 kDa), 38 kDa, VP3 (31 kDa), NS (28.5 kDa) and the NS-VP3 fusion. Molecular weight standards are shown.

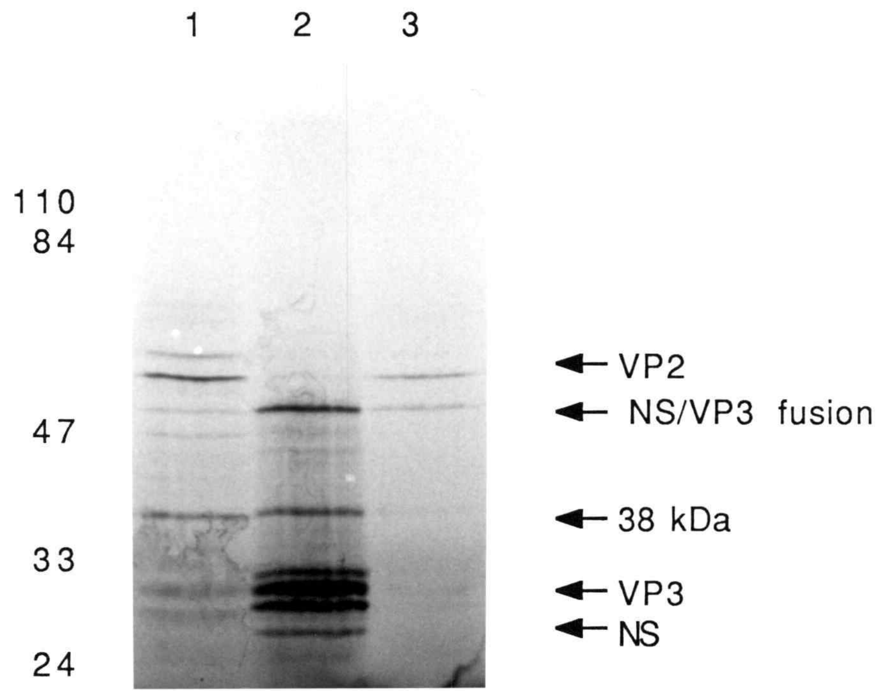

1
2
3
110
84
47
33
24
VP2
NS/VP3 fusion
38 kDa
VP3
NS

Fig. III.9. Radioimmune precipitation of *in vitro* translation products of the cleavage site mutants. Samples were precipitated with anti-VP2 or anti-VP3. Lanes 1-3 were precipitated with anti-VP2; lane 1, wild-type A segment; lane 2, 5'EcoRI deleted A segment; lane 3, double mutant Tyr_{591}-His_{592} and Tyr_{725}-Leu_{726} were converted to Pro-Gly; lanes 4-6 were precipitated with anti-VP3; lane 4 full-length wild-type A segment; lane 5, double mutant; lane 6, 5'EcoRI deleted A segment; lanes 7 and 8 contain non-precipitated samples; lane 7 wild type A segment; lane 8, double mutant. Arrows indicate VP2 (63 kDa), 38 kDa, VP3 (31 kDa), NS (28.5 kDa) and the NS-VP3 fusion. Molecular weight standards are shown.

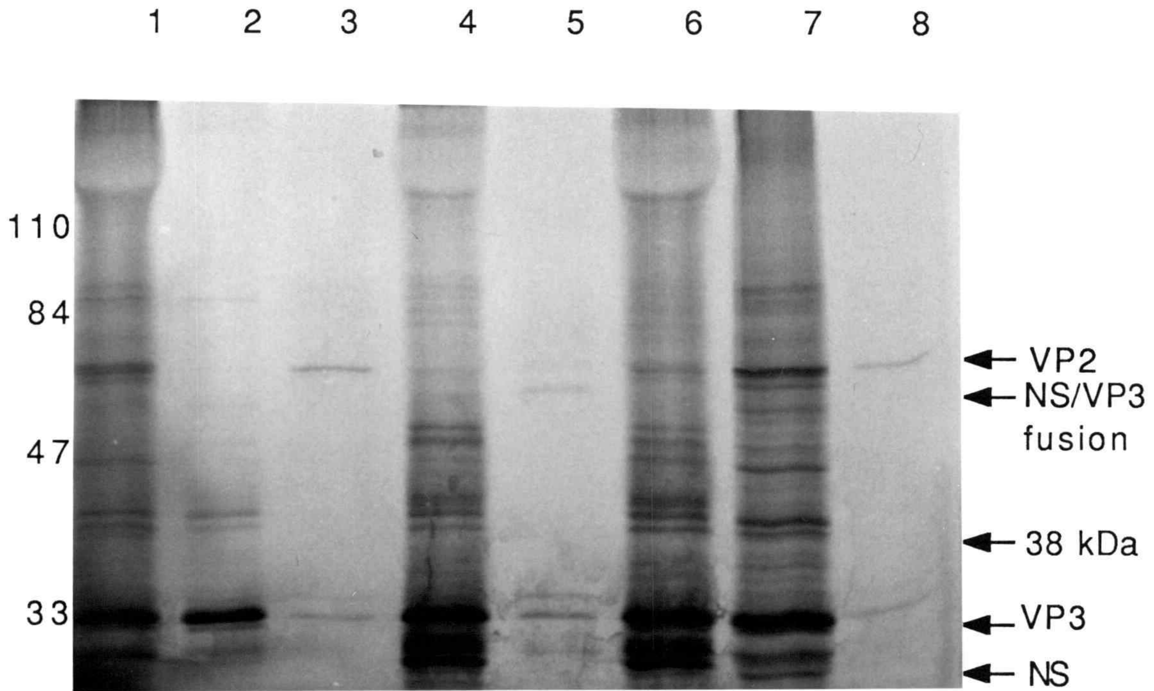

1
2
3
4
5
6
7
8
110
84
47
33
VP2
NS/VP3 fusion
38 kDa
VP3
NS

Fig. III.10. Examination of trans activity of the viral proteinase by co-translation of full-length and deleted IPNV RNA. Lanes 1, 2 and 3 were incubated 60 min. Lane 1, full-length A segment, lane 2, 5'EcoRI deleted A segment, lane 3, tpp. Lane 4 and 5 containing tpp RNA incubated alone for 4 h or 16 h, respectively. Lanes 6, 7 and 8 are co-translation reactions in which tpp RNA was co-translated with full-length A segment RNA for 60 minutes, four hours and 16 hours, respectively. Lanes 9, 10 and 11 are co-translation reaction in which tpp RNA was co-translated with 5'EcoRI deleted A segment RNA for 60 min, 4 h and 16 h, respectively.

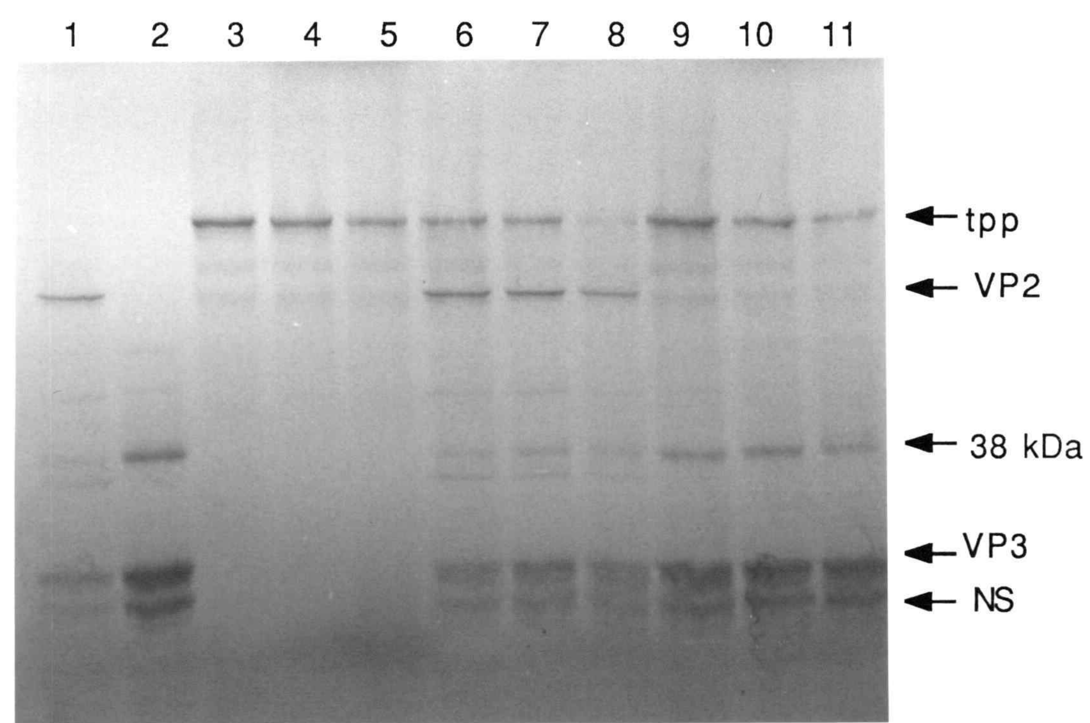

1
2
3
4
5
6
7
8
9
10
11
tpp
VP2
38 kDa
VP3
NS

Table III.1 Effect of Proteinase Inhibitors on the IPNV-NS Proteinase

Inhibitor	Proteinase Class Specificity	Inhibitor Concentrations Tested	Inhibition
TLCK-HCl	Ser	37 ug/ml	-
TPCK	Ser	70 ug/ml	-
EDTA	Metallo	100 uM	-
Pepstatin-A	Aspartic	0.7 ug/ml	-
H261	Aspartic	0.5, 1, 2 uM	-
Iodoacetamide	Cys	1, 2, 5, 10 uM	-
N-Ethylmaleimide	Cys	1, 2, 5, 10 uM	-
ZnS04	Cys	1, 5, 10, 20 uM	-

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IV. Nucleotide Sequence of Infectious Pancreatic Necrosis Virus (IPNV) SP and Identification of a Conserved Antigenic Domain in the Major Capsid Protein

ABSTRACT

The nucleotide sequence of a cDNA clone for the large RNA segment of the genome of infectious pancreatic necrosis virus (IPNV) was determined for an Sp isolate of that virus. The A segment was found to encode three viral genes, VP2 (major capsid protein), NS (nonstructural autocatalytic proteinase) and VP3 (minor capsid protein) in a single open reading frame of 2,916 nucleotides. A comparison of the deduced amino acid sequences of three isolates of IPNV, Sp, Jasper and N1, revealed a high degree of similarity. The Sp and N1 isolates shared an amino acid similarity of 97%, while that of Sp and Jasper was 76%. Regions of the VP2 gene were subcloned and expressed in Escherichia coli as trpE fusion proteins. Two unique clones were identified which differed in their reactivity to polyclonal and monoclonal antisera. The fusion protein expressed by pB10, encoding amino acids 99 to 206 of VP2, was recognized by antisera to the three major serotypes of IPNV. The B10 fusion protein was also precipitated by the broadly reactive neutralizing monoclonal antibody AS-1. The fusion protein expressed by pA43, which contained an adjacent portion of VP2 from amino acid 207 to 314, was only recognized by anti-Sp sera. The sequence comparisons between the IPNV isolates revealed a high degree of similarity in the N- and C-terminal sequences of the major capsid protein VP2. These results suggest that the pB10 region of VP2 carried universal antigenic determinants and the pA43 region in the central portion of VP2 was diverse and contained serotype specific determinants.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) causes an acute contagious disease in juvenile salmonids and outbreaks of the disease may be economically devastating to a hatchery. IPNV has also been isolated from eels and marine molluscs, thus it is a very serious pathogen for aquaculture. Currently, there are no prophylactic or therapeutic treatments for the control of the disease and a vaccine is needed by the industry. As a first step in developing such a vaccine, the number of serotypes and an epitope map of the major capsid protein, VP2, must be determined.

The most extensive study of the antigenic relationships of aquatic birnaviruses compared 175 virus isolates from 44 fish and shellfish species from eleven countries by reciprocal plaque reduction tests using polyclonal sera (Hill and Way, 1983). From these results, it was proposed that there were two major serogroups: one containing nine serotypes which included 171 isolates from fish and the other containing one serotype which included those viruses isolated from molluscs. These viruses also contain common immunoreactive determinant(s). Other studies have recognized only three major serotypes characterized by the virus isolates: VR299, a North American strain; Sp, a European strain which is pathogenic for trout; and AB, a European strain which is nonpathogenic for trout (Wolf, 1988). Several serotypes of IPNV have also been characterized for their immunological and biochemical properties with monoclonal antibodies (Caswell-Reno et al., 1986; Lipipun et al., 1989). However, a careful epitope map

and the physical location of the neutralizing epitopes on VP2 has not been determined. Thus, an effort was made to characterize the immunoreactive regions of the major capsid protein of the Sp serotype of IPNV also to unravel some of the mechanisms that biologically distinguish the different IPNV isolates.

IPNV is a birnavirus with a genome of two segments of double-stranded RNA. Genomic RNA is encapsidated in a single-shelled icosahedral capsid, 60 nm in diameter, with no envelope. Other members of the birnavirus family include infectious bursal disease virus (IBDV), which causes a contagious disease of fowl, and Drosophila X virus (DXV).

The nucleotide sequence of the IPNV A segment had been determined for two IPNV serotypes (Duncan and Dobos, 1986; Havarstein et al., 1990). Both sequences revealed an open reading frame (ORF) encoding a 100 kDa polyprotein which was found to be rapidly processed into the major viral structural protein, VP2, a minor capsid component, VP3 and a nonstructural protein, NS. The NS protein had the properties of an autocatalytic proteinase responsible for the polyprotein cleavage (Duncan et al., 1987; Manning et al., 1990). The gene order of the A segment was NH_2-pVP2-NS-VP3-COOH (Huang et al., 1986; Duncan et al., 1987; Nagy et al., 1987).

The Sp isolate of IPNV is representative of the Sp serotype, the major virulent IPNV type in Europe. A sequence determination of this strain was the first step in developing an IPNV vaccine and understanding the basis for IPNV virulence. The sequence was

determined from cDNA clones prepared to the Sp strain in plasmid vectors (Huang et al., 1986). Sequence analysis of the A segment revealed a coding region of 2,916 base pairs (bp) in a large ORF. Nucleotide and deduced amino acid sequences of the Sp isolate were compared with that of the Jasper (Canadian) and N1 (Norwegian) isolates (Duncan et al., 1987; Havarstein et al., 1990) as well as IBDV (Hudson et al., 1986) and significant similarities were observed. In addition, regions of the VP2 gene were expressed in *Escherichia coli* as *trpE* fusion proteins (Manning and Leong, 1990) and reacted with polyclonal and monoclonal antisera prepared to different serotypes of IPNV. These studies defined regions of the VP2 protein with serotype specific and common antigenic determinants.

MATERIALS AND METHODS

Nucleotide Sequence Analysis

A cDNA clone of the entire IPNV-Sp A segment was constructed from overlapping cDNA clones as described (Huang et al., 1986). Subclones were generated using various restriction endonucleases into both M13 mp18 and mp19. Additional subclones were obtained using the restriction endonuclease Sau3AI. These fragments were ligated into the BamHI site of M13 cloning vectors in a shotgun manner. Nucleotide sequence information was determined using the dideoxy-chain-termination method (Sanger et al., 1977). In most cases, overlapping clones were used and sequence was obtained for both strands. Oligonucleotide primers, complimentary to nucleotide positions 228-242, 430-477, 816-830, 2240-2254 and 2785-2798 were used to confirm some sequence and to obtain overlapping sequences. In addition, direct plasmid sequencing was performed on some clones (Wang et al., 1988). The clones generated and the sequencing strategy employed are diagrammed in Fig. IV.1. Oligonucleotide primers were synthesized by the Center for Gene Research, Oregon State University. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer-Mannheim and Promega. The Sequenase enzyme and nucleotides from U.S. Biochemicals were used in sequence analysis. Sequence analyses (IntelliGenetics, Inc.) were performed at, Oregon State University.

Cloning and Bacterial Expression

The VP2 gene was excised from the plasmid pUC19/A+SDK (Manning and Leong, 1990) and digested with the restriction endonuclease Sau3AI, which generated seven fragments. These fragments were inserted in-frame with the trpE gene in one of three pATH vectors, pATH1, pATH2 or pATH3, which put the resulting trpE-VP2 fusion protein under the control of the tryptophan operator and promoter (Dieckmann and Tzagaloff, 1985). Recombinants expressing an immunoreactive portion of the VP2 gene were detected by colony immunoblot with anti-IPNV-Sp sera. Briefly, colonies were replica plated onto nitrocellulose. Colony lifts were then incubated on Luria Broth (LB) plates containing ampicillin (120 µg/ml) overnight. The lifts were then transferred to LB plates containing ampicillin (120 µg/ml) and indoleacrylic acid (IAA) (15 µg/ml). Bacterial colonies were lysed and reacted with polyclonal antisera to IPNV-Sp. Two positive colonies, pB10 and pA43, were selected for further study.

Western Blot Analysis

Escherichia coli cells containing the recombinant plasmids, pB10 or pA43, were grown to mid-log phase in LB before induction with IAA (15 µg/ml). Cultures were then grown to stationary phase and harvested by centrifugation. Cells were lysed and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). Proteins were transferred to nitrocellulose and reacted with antisera to IPNV-Sp and antisera raised against the heterologous

IPNV strains, IPNV-Buhl and IPNV-EVE as described (Gilmore et al., 1988).

Radioimmune Precipitation

Escherichia coli cells containing the recombinant plasmids, pB10 or pA43, were grown to mid-log phase in LB containing ampicillin (120 μg/ml). Cells were collected by centrifugation and washed three times with 2X minimal media salts (Na_2HPO_4, 12 g; KH_2PO_4, 6 g; NaCl, 1 g; NH_4Cl, 2 g; 1M $MgSO_4$, 4 ml; 20% glucose, 20 ml and 1M $CaCl_2$, 0.2 ml per liter). The cell pellets were then resuspended in minimal media lacking methionine and cysteine. Cultures were incubated at 37°C for 1 h at which time 100 μCi of ^{35}S methionine was added. The cultures were then induced with IAA (10 μg/ml) and incubated for an additional 30 min. The labeled proteins in the cultures were chased with unlabeled methionine and cysteine and the cultures were allowed to incubate for 4 to 16h Cells were harvested and the pellets washed three times with 2X minimal media salts. Pellets were resuspended in radioimmune precipitation assay (RIPA) buffer (0.1% SDS, 0.2% sodium deoxycholate, 150 mM NaCl, 10 mM Tris, pH 7.4, 0.5% NP40). The precipitations were performed in a volume of 60 μl containing 35 μl of a 10% protein-A sepharose suspension, 20 μl monoclonal antibody or 1:20 dilution of polyclonal rabbit antisera, 5 μl ^{35}S-methionine labeled fusion protein. Reactions were incubated at 37°C for 4 h with intermittent gentle rocking and then left at 4C overnight. Samples were washed four times with 200 μl RIPA

buffer, Laemmli sample buffer was added and samples were analyzed by SDS-PAGE and autoradiography.

Monoclonal Antibodies

Monoclonal antibodies (Mabs) and hybridoma cell lines were prepared as described (Caswell-Reno et al, 1989). Hybridomas for the Mab W3 and AS-1 were propagated in modified minimal essential medium (MEM) containing 10% fetal bovine serum. The Mab W3 was prepared against the West Buxton isolate of IPNV and was specific for VP2. This Mab was cross-reactive to a number of isolates of IPNV in both the VR299 and Sp serotype and also possessed neutralizing activity (Caswell-Reno et al., 1986). The Mab AS-1 was prepared against the AS (Atlantic salmon) isolate of IPNV and was specific for VP2. This Mab was cross-reactive with all of greater than 70 aquatic birnavirus isolates tested thus far and appeared to recognize a group-reactive determinant (Caswell-Reno et al., 1989).

RESULTS

Nucleotide and Amino Acid Sequence Analysis

We have determined the nucleotide sequence of the large segment of Sp isolate of IPNV. The IPNV-Sp A segment consists of a coding region of 2,916 basepairs (bp) containing a single ORF (Fig. IV.2) identical in length to those described for IPNV-Jasper and IPNV-N1 (Duncan and Dobos, 1986; Havarstein et al., 1990). The 5' and 3' non-coding regions were not contained in the cDNA clone used. Comparison of the nucleotide and deduced amino acid sequences of the IPNV-Sp, -Jasper and -N1 isolates revealed a high degree of similarity. The IPNV-Sp and IPNV-N1 isolates share an overall amino acid similarity of 97%, while that of Sp and Jasper was 76% (Table IV.I). Additionally, amino acid differences observed were often conservative replacements. Comparison of the VP2 sequences revealed a high degree of similarity with IPNV-Sp and IPNV-Jasper being 88.8% similar and IPNV-Sp and IPNV-N1 being 95.8% similar (Table IV.1). The amino acid sequence was highly conserved among the IPNV-Sp and IPNV-Jasper isolates between the amino acid residues 1-210 and residues 334-492. When IPNV-Sp and IPNV-Jasper were examined at the amino acid level, the region spanning the pB10 sequences revealed only three amino acid differences. In a comparison between IPNV-Sp and IPNV-N1 in the pB10 region, only two amino acid changes were observed. In contrast, 27 amino acid changes were observed between IPNV-Sp and IPNV-Jasper within the pA43 region. Only five amino acid changes were found between IPNV-Sp and IPNV-N1

within pA43 (Fig. IV.3) and this reflects the similar serotype relationship between IPNV-Sp and IPNV-N1 as reported by Christie et al. (1988).

Cloning and Bacterial Expression

The VP2 gene was excised from the parent plasmid pUC19A+SDK and digested with the restriction endonuclease Sau3AI. The seven Sau3A fragments generated were subcloned into the pATH expression vectors. Recombinant colonies were induced with IAA as described in Materials and Methods. Two distinct immunoreactive regions of VP2 were identified by colony immunoblots using anti-IPNV-Sp sera. The clones were designated B10 and A43. The B10 clone was comprised of 323 nucleotides encoding 107 amino acids and was localized through sequence analysis to the N-terminal portion of VP2, amino acid residues 99-206. The A43 clone was comprised of two neighboring Sau3AI fragments of 297 and 27 nucleotides, encoding 107 amino acids, most likely originating from a partial cleavage product and was mapped immediately adjacent to B10 to residues 207-314 of VP2 (Fig. IV.3).

Western blot Analysis and Radioimmune Precipitations

Polyclonal rabbit antisera prepared to different serotypes of IPNV has been shown to cross-react with heterologous VP2 proteins in Western immunoblots (Barrie and Leong, unpublished data). Thus, there are conserved linear epitopes among IPNV strains. In this study, two distinct immunoreactive regions of VP2

were identified and characterized by Western immunoblot and radioimmune precipitation. In Western immunoblots the pB10 region, which expressed a 47 kDa fusion protein, was found to be cross-reactive to antisera raised against three serotypes of IPNV, being recognized by antisera against viruses from the WB, AB and Sp serotypes (Figs. IV.4 and IV.5). This is consistent with the nucleotide sequence data which identified amino acids 1-209 of VP2 as being highly conserved between the IPNV-Sp, -Jasper and -N1 isolates. The second immunoreactive region of VP2, A43, was localized within amino acids 207-314 (Fig. IV.3). This region has a high degree of heterology when compared to IPNV-Jasper or IPNV-N1. The pA43 region, which expressed a 52 kDa fusion protein, appeared to exhibit strain specific antigenicity, being recognized only by anti-IPNV-Sp sera (Fig. IV.4).

In addition, B10 was immunoprecipitated by the Mab AS-1 (Fig. IV.6, lane 2). The A43 portion was not recognized by the Mab AS-1 (Fig. IV.6, lane 3). The pATH induced sample, that did not contain IPNV sequences, exhibited a non-specific affinity for the Mab AS-1, with minor amounts of pATH being brouhgt down in the reaction (Fig. IV.6, lane 4). The Mab AS-1 reagent was shown to recognize a conformational dependent epitope present on all three serotypes (WB, Sp and Ab), as well as six isolates representing newly proposed serotypes (Caswell-Reno et al., 1989; Lipipun et al., 1989). The epitopes recognized by Mab AS-1 appear to be highly conserved. Another neutralizing Mab, W3, displayed an extensive degree of cross-reactivity with the birnaviruses. This

indicated that the epitope recognized by W3 was widely dispersed (Caswell-Reno et al., 1986). However, the B10 portion of VP2 localized to amino acids 99-206, which displayed a high degree of similarity between IPNV isolates was not recognized by W3.

Although both pB10 and pA43 contained inserts encoding 107 amino acids, striking differences in the observed migration of the fusion proteins produced by each plasmid were noted. Careful analysis of the 3' terminal sequence of both plasmids by direct plasmid sequencing (Wang et al., 1988) indicated that the translational termination codon, TAG, was present immediately at the end of both VP2 cDNA inserts. The calculated pI for both fusion proteins was 6.6 and there was no dramatic difference in the amino acid composition. Thus far, the only possible explanation for the slower migration of the pA43 fusion protein might be found in the series of four prolines at the amino terminus of this insert (Fig. IV.3).

DISCUSSION

The nucleotide sequence for the IPNV-Sp A segment was determined and single large ORF of 2,916 basepairs was observed. The nucleotide sequence of the A segment of IPNV-Sp was compared to the sequences of IPNV-Jasper and IPNV-N1. An overall similarity of 76% and 97%, respectively, was observed. When the amino acid sequences were examined, Sp and Jasper had a similarity of 83.7%, while Sp and N1 were very closely related having a similarity of 96.8%. The similarity of the VP2 proteins was also significant at 88.8% between Sp and Jasper and 95.8% between Sp and N1. The cDNA clone derived for the IPNV-Sp isolate contained the entire coding region, however, it lacked the 5' and 3' non-coding sequences. A second small ORF has been reported for IPNV and had the potential of encoding a 17 kDa polypeptide (Havarstein et al., 1990). The initiation codon of this small ORF was located upstream of the initiation codon for the large ORF. Unfortunately, the sequences 5' of the large ORF initiation codon were lacking in the cDNA cloned for the IPNV-Sp isolate. Amino acid sequences that corresponded to those identified for the small ORF were present in the IPNV-Sp sequence and shared a similarity of 94.7% when compared to IPNV-N1. These results are consistent with the apparent relatedness of IPNV-Sp and IPNV-N1. The IPNV-N1 and IPNV-Jasper had an amino acid similarity of 63.5% for the small ORF. Although a protein product has been detected, its function is unknown.

The VP2 protein contains the antigenic regions responsible for inducing neutralizing antibodies and serotype specificity. We have mapped segments of VP2 with polyclonal and monoclonal antisera and found that the N-terminal portion of VP2, amino acid residues 99-206 of VP2, carried by B10, contained a nonlinear epitope that was common to three serotypes and six newly proposed serotypes. This region of 107 amino acids carried a common immunoprotective portion of VP2, while the region containing amino acid residues 207-314 maps to a serotype specific portion of VP2, as it was only recognized by homologous anti-IPNV-Sp sera. Examination of the amino acid sequence comparison between IPNV-Sp, -Jasper and -N1 revealed that VP2 was highly conserved from amino acids 1-210 between all three isolates and the region spanning 211-333 displayed heterogeneity. These results are in agreement with a comparison of the deduced amino acid sequence of IPNV-N1 and IPNV-Jasper capsid proteins by Havarstein et. al. (1990) which revealed heterogeneity from amino acid 206-350 and it was proposed that the region contained serotype-specific epitope(s), which was confirmed with this study. This region constituted a variable region when compared with both IPNV-Jasper and IBDV. The variable region of VP2 corresponded to a segment of IBDV-VP2 recognized by a neutralizing monoclonal antibody. It was suggested that amino acids 183-337 of VP2 carried serotype specific epitopes for both IPNV and IBDV (Havarstein et al., 1990).

The similarities between the protein products of the IPNV serotypes was a significant observation, since these isolates are serologically distinct. During an outbreak at a hatchery, IPNV can cause high levels of mortality in young fry. Current methods of control include the complete destruction of infected stocks. Analysis of the sequences between various isolates can be useful in vaccine development. Fish have been shown to produce high titers of neutralizing antisera to IPNV (Kelly and Nielsen, 1990). The antibodies induced in the fish were IgM-like and were found to be cross-reactive withheterologous strains of IPNV (Kelly and Nielsen, 1990). This report identified a portion of the major capsid protein, VP2, which contained a serotype specific epitope(s). These regions, based on their reactivity with polyclonal and monoclonal antisera, are important in the immune response in the development of protective immunity. The identification of the common and serotype specific epitope(s) will augment vaccine development. In particular, the conserved regions of VP2, identified in this study, can be used in the development of subunit vaccines to control IPNV outbreaks.

Fig. IV.1. Physical map and sequencing strategy for a cDNA of IPNV-Sp A segment. A restriction map of the A segment is shown, subclones generated for sequencing are represented by bars below the restriction map. Arrows indicate the regions sequenced and the direction of the sequencing.

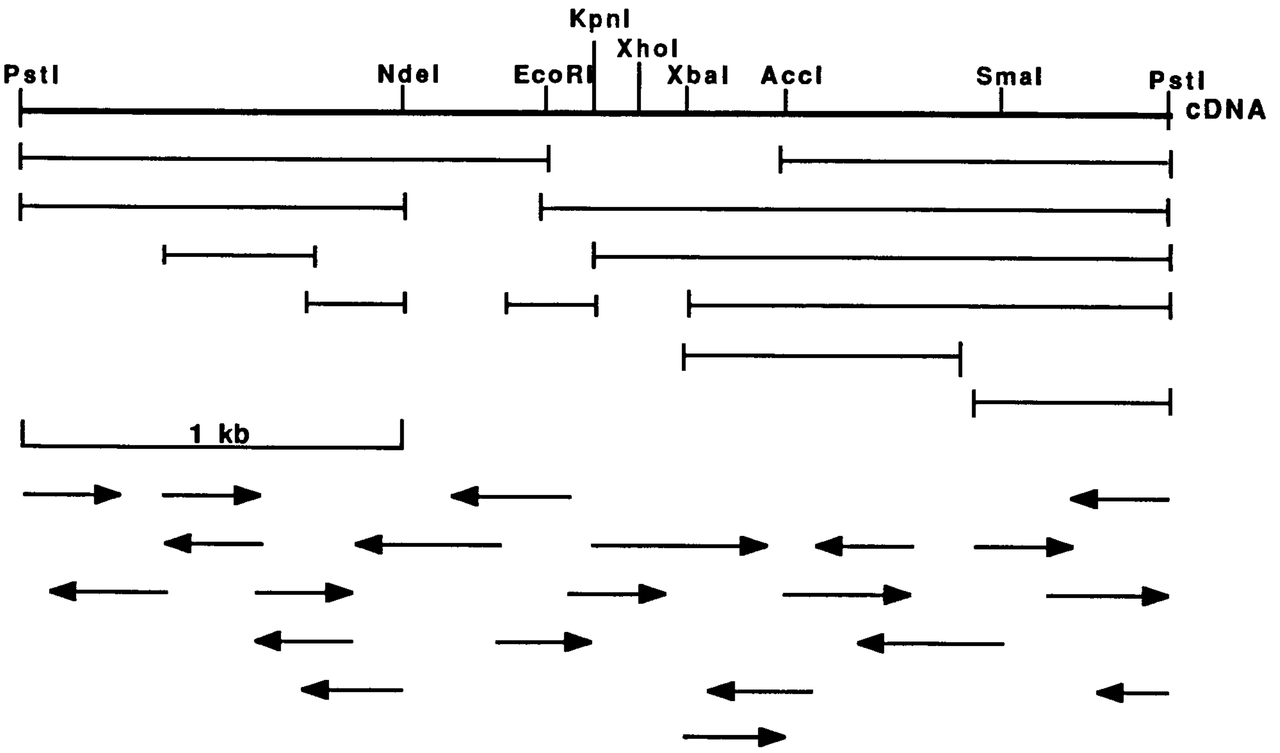
KpnI
XhoI
PstI
NdeI
EcoRI
XbaI
AccI
SmaI
PstI
cDNA
1 kb

Fig. IV.2. Nucleotide and deduced amino acid sequence for the large open reading frame of the IPNV-Sp segment A.

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1    ATG AAC ACA AAC AAG GCA ACC GCA ACT TAC TTG AAA TCC ATT     42
      M   N   T   N   K   A   T   A   T   Y   L   K   S   I

43   ATG CTT CCA GAG ACT GGA CCA GCA AGC ATC CCG GAC GAC ATA     84
      M   L   P   E   T   G   P   A   S   I   P   D   D   I

85   ACG GAG AGA CAC ATC TTA AAA CCA GAG ACC TCG TCA TAC AAC    126
      T   E   R   H   I   L   K   P   E   T   S   S   Y   N

127  TTA GAG GTC CCC GAA TCA GGA AGT GGC ATT CTT GTT TGT TTC    168
      L   E   V   P   E   S   G   S   G   I   L   V   C   F

169  CCT GGG GCA CCA GGC TCA CGG ATC GGT GCA CAC TAC AGA TGG    210
      P   G   A   P   G   S   R   I   G   A   H   Y   R   W

211  AAT GCG AAC CAG ACG GGG CTG GAG TTC GAC CAG TGC TGG AGA    252
      N   A   N   Q   T   G   L   E   F   D   Q   C   W   R

253  GCG TCG CAG GAC CTG AAG AAA GCC TTC AAC TAC GGG AGG CTG    294
      A   S   Q   D   L   K   K   A   F   N   Y   G   R   L

295  ATC TCA AGG AAA TAC GAC ATT CAA AGC TCC ACA CTA CCG GCC    336
      I   S   R   K   Y   D   I   Q   S   S   T   L   P   A

337  GGT CTC TAT GCT CTG AAC GGG ACG CTC AAC GCT GCC ACC TTT    378
      G   L   Y   A   L   N   G   T   L   N   A   A   T   F

379  GAA GGC AGT CTG TCT GAG GTG GAG AGC CTG ACC TAC AAT AGC    420
      E   G   S   L   S   E   V   E   S   L   T   Y   N   S

421  CTG ATG TCC CTA ACT ACG AAC CCC CAG GAC AAA GCC AAC AAC    462
      L   M   S   L   T   T   N   P   Q   D   K   A   N   N

463  CAG CTG GTG ACC AAA GGA GTC ACC GTC CTG AAT CTA CCA ACA    504
      Q   L   V   T   K   G   V   T   V   L   N   L   P   T

505  GGG TTC GAC AAA CCA TAC GTC CGC CTA GAG GAC GAG ACA CCC    546
      G   F   D   K   P   Y   V   R   L   E   D   E   T   P

547  CAG GGT CTC CAG TCA ATG AAC GGG GCC AGG ATG AGG TGC ACA    588
      Q   G   L   Q   S   M   N   G   A   R   M   R   C   T

589  GCT GCA ATT GCA CCA CGG AGG TAC GAG ATC GAC CTC CCA TCC    630
      A   A   I   A   P   R   R   Y   E   I   D   L   P   S

631  CAA AGC CTA CCC CCC GTT CCT GCG ACA GGA ACC CTG ACC ACT    672
      Q   S   L   P   P   V   P   A   T   G   T   L   T   T

673  CTC TAC GAG GGA AAC GCC GAC ATC GTC AAC TCC ACA ACA GTG    714
      L   Y   E   G   N   A   D   I   V   N   S   T   T   V

715  ACG GGA GAC ATA AAC TTC AGT CTG GCA GAA CAA CCC GCA AAC    756
      T   G   D   I   N   F   S   L   A   E   Q   P   A   N

757  GAG ACC AGG TTC GAC TTC CAG CTG GAC TTG ATG GGC CTT GAC    798
      E   T   R   F   D   F   Q   L   D   L   M   G   L   D

799  AAT GAC GTC CCA GTG GTC ACA GTG GTC AGC TCC GTG CTG GCC    840
      N   D   V   P   V   V   T   V   V   S   S   V   L   A

841  ACA AAC GAC AAC TAC AGA GGA GTC TCA GCC AAG ATG ACC CAG    882
      T   N   D   N   Y   R   G   V   S   A   K   M   T   Q

883  TCC ATC CCG ACC GAG AAC ATT ACC AAG CCG ATC ACC AGG GTC    924
      S   I   P   T   E   N   I   T   K   P   I   T   R   V

925  AAG CTG TCA TAC AAG ATC AAC CAG CAG ACA GCA ATC GGC AAT    966
      K   L   S   Y   K   I   N   Q   Q   T   A   I   G   N

967  GTC GCC ACC CTG GGC ACA ATG GGT CCA GCA TCC GTC TCC TTT   1008
      V   A   T   L   G   T   M   G   P   A   S   V   S   F
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1009  TCA TCG GGG AAC GGA AAT GTC CCC GGC GTG CTC AGA CCA ATC   1050
       S   S   G   N   G   N   V   P   G   V   L   R   P   I

1051  ACA CTG GTG GCA TAT GAG AAG ATG ACA CCG CTG TCC ATC CTG   1092
       T   L   V   A   Y   E   K   M   T   P   L   S   I   L

1093  ACC GTA GCT GGA GTG TCC AAC TAC GAG CTG ATC CCA AAC CCA   1134
       T   V   A   G   V   S   N   Y   E   L   I   P   N   P

1135  GAA CTC CTC AAG AAC ATG GTG ACA CGC TAT GGC AAG TAC GAC   1176
       E   L   L   K   N   M   V   T   R   Y   G   K   Y   D

1177  CCC GAA GGT CTC AAC TAT GCC AAG ATG ATC CTG TCC CAC AGG   1218
       P   E   G   L   N   Y   A   K   M   I   L   S   H   R

1219  GAA GAG CTG GAC ATC AGG ACA GTG TGG AGG ACA GAG GAG TAC   1260
       E   E   L   D   I   R   T   V   W   R   T   E   E   Y

1261  AAG GAG AGG ACC AGA GTC TTC AAC GAA ATC ACA GAG AAG ACC   1302
       K   E   R   T   R   V   F   N   E   I   T   E   K   T

1303  AGT GAC CTG CCC ACG TCA AAG GCA TGG GGC TGG AGA GAC ATA   1344
       S   D   L   P   T   S   K   A   W   G   W   R   D   I

1345  GTC AGA GGA ATT CGA AAA ATC GCA GCT CCT GTA CTG TCC ACG   1386
       V   R   G   I   R   K   I   A   A   P   V   L   S   T

1387  CTG TTT CCA ATG GCA GCA CCA CTC ATA GGA ACG GCA GAC CAA   1428
       L   F   P   M   A   A   P   L   I   G   T   A   D   Q

1429  TTC ATT GGA GAT CTC ACC AAG ACC AAC GCA GCA GGC GGA AGG   1470
       F   I   G   D   L   T   K   T   N   A   A   G   G   R

1471  TAC CAC TCC ATG GCC GCA GGA GGG CGC TAC AAA GAC GTG CTC   1512
       Y   H   S   M   A   A   G   G   R   Y   K   D   V   L

1513  GAG TCC TGG GCA AGC GGA GGG CCC GAC GGA AAA TTC TCC CGA   1554
       E   S   W   A   S   G   G   P   D   G   K   F   S   R

1555  GCC CTC AAG AAC AGG CTG GAG TCC GCG AAC TAC GAG GAA GTC   1596
       A   L   K   N   R   L   E   S   A   N   Y   E   E   V

1597  GAG CTT CCA CCC CCC TCA AAA GGA GTC ATC GTC CCT GTG GTG   1638
       E   L   P   P   P   S   K   G   V   I   V   P   V   V

1639  CAC ACA GTC AAG AGT GCA CCA GGC GAG GCA TTC GGG TCC CTG   1680
       H   T   V   K   S   A   P   G   E   A   F   G   S   L

1681  GCA ATT ATA ATT CGG GGG GAG TAC CCG AGC TTT CTA GAT GCC   1722
       A   I   I   I   R   G   E   Y   P   S   F   L   D   A

1723  AAC CAG CAG GTC CTA TCC CAC TTC GCA AAC GAC ACC GGG AGC   1764
       N   Q   Q   V   L   S   H   F   A   N   D   T   G   S

1765  GTG TGG GGC ATA GGA GAG GAC ATA CCC TTC GAG GGA GAC AAC   1806
       V   W   G   I   G   E   D   I   P   F   E   G   D   N

1807  ATG TGC TAC ACT GCA CTC CCA CTC AAG GAG ATC AAG AGA AAC   1848
       M   C   Y   T   A   L   P   L   K   E   I   K   R   N

1849  GGG AAC ATA GTA GTC GAG AAG ATC TTT GCT GGG CCA ATT ATG   1890
       G   N   I   V   V   E   K   I   F   A   G   P   I   M

1891  GGT CCC TCT GCT CAA CTA GGA CTG TCC CTA CTT GTG AAC GAC   1932
       G   P   S   A   Q   L   G   L   S   L   L   V   N   D

1933  ATC GAG GAC GGA GTT CCA AGG ATG GTA TTC ACC GGC GAA ATC   1974
       I   E   D   G   V   P   R   M   V   F   T   G   E   I

1975  GCC GAT GAC GAG GAG ACA ATC ATA CCA ATC TGC GGT GTC GAC   2016
       A   D   D   E   E   T   I   I   P   I   C   G   V   D
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2017  ATC AAA GCC ATC GCA GCC CAC GAA CAA GGG CTG CCA CTC ATC   2058
       I   K   A   I   A   A   H   E   Q   G   L   P   L   I

2059  GGC AAC CAA CCA GGA GTG GAC GAG GAG GTG CGA AAC ACA TCC   2100
       G   N   Q   P   G   V   D   E   E   V   R   N   T   S

2101  CTG GCC GCA CAC CTG ATC CAG ACC GGG ACC CTG CCC GTA CAA   2142
       L   A   A   H   L   I   Q   T   G   T   L   P   V   Q

2143  CGC GCA AAG GGC TCC AAC AAG AGG ATC AAG TAC CTG GGA GAG   2184
       R   A   K   G   S   N   K   R   I   K   Y   L   G   E

2185  CTG ATG GCA TCA AAT GCA TCC GGG ATG GAC GAG GAA CTG CAA   2226
       L   M   A   S   N   A   S   G   M   D   E   E   L   Q

2227  CGC CTC CTG AAC GCC ACA ATG GCT CGG GCC AAA GAA GTC CAG   2268
       R   L   L   N   A   T   M   A   R   A   K   E   V   Q

2269  GAC GCC GAG ATC TAC AAA CTT CTT AAG CTC ATG GCA TGG ACC   2310
       D   A   E   I   Y   K   L   L   K   L   M   A   W   T

2311  AGA AAG AAC GAC CTC ACC GAC CAC ATG TAC GAG TGG TCA AAA   2352
       R   K   N   D   L   T   D   H   M   Y   E   W   S   K

2353  GAG GAC CCC GAT GCA CTA AAG TTC GGA AAG CTC ATC AGC ACG   2394
       E   D   P   D   A   L   K   F   G   K   L   I   S   T

2395  CCA CCA AAG CAC CCT GAG AAG CCC AAA GGA CCA GAC CAA CAC   2436
       P   P   K   H   P   E   K   P   K   G   P   D   Q   H

2437  CAC GCC CAA GAG GCG AGA GCC ACC CGC ATA TCA CTG GAC GCC   2478
       H   A   Q   E   A   R   A   T   R   I   S   L   D   A

2479  GTG AGA GCC GGG GCG GAC TTC GCC ACA CCG GAG TGG GTC GCG   2520
       V   R   A   G   A   D   F   A   T   P   E   W   V   A

2521  CTG AAC AAC TAC CGC GGA CCA TCT CCC GGG CAG TTC AAG TAC   2562
       L   N   N   Y   R   G   P   S   P   G   Q   F   K   Y

2563  TAC CTG ATC ACT GGA CGA GAA CCA GAA CCA GGC GAC CAG TAC   2604
       Y   L   I   T   G   R   E   P   E   P   G   D   Q   Y

2605  GAG GAC TAC ATA AAA CAA CCC ATC GTG AAA CCG ACC GAC ATG   2646
       E   D   Y   I   K   Q   P   I   V   K   P   T   D   M

2647  AAC AAA ATC AGA CGT CTA GCC AAC AGT GTG TAC GGC CTC CCA   2688
       N   K   I   R   R   L   A   N   S   V   Y   G   L   P

2689  CAC CAG GAA CCA GCA CCA GAG GAG TTC TAT GAT GCA GTT GCA   2730
       H   Q   E   P   A   P   E   E   F   Y   D   A   V   A

2731  GCT GTA TTC GCA CAG AAC GGA GGC AGA GGT CCA GAC CAG GAC   2772
       A   V   F   A   Q   N   G   G   R   G   P   D   Q   D

2773  CAA ATG CAA GAC CTC AGG GAG CTA GCA AGA CAG ATG AAA CGA   2814
       Q   M   Q   D   L   R   E   L   A   R   Q   M   K   R

2815  CGA CCC CGG AAC GCC GAT GCA CCA CGG AGA ACC AGA GCG CCA   2856
       R   P   R   N   A   D   A   P   R   R   T   R   A   P

2857  GGA AAA CCG GCA CCG CCG GAC GTC TCA AGG TTC ACC CCC AGC   2898
       G   K   P   A   P   P   D   V   S   R   F   T   P   S

2899  GGA GAC AAC GCT GAG GTG     2916
       G   D   N   A   E   V
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Fig. IV.3. Amino acid alignment between three IPNV isolates. Deduced amino acid sequences for the large open reading frame of the A segments for IPNV-Sp, -Jasper (Ja), and -N1 are shown using a single amino acid code. The concensus (Con) sequence for all three isolates is shown.

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 SP  MNTNKATATYLKSIMLPETGPASIPDDITERHILKPETSSYNLEVPESGSGILVCFPGAPGSR
 JA  MSTSKATATYLRSIMLPENGPASIPDDITERHILKQETSSYNLEVSESGSGLLVCFPGAPGSR
 N1  MNTNKATATYLKSIMLPETGPASIPDDITERHILKQETSSYNLEVSESGSGILVCFPGAPGSR
CON  M T KATATYL SIMLPE GPASIPDDITERHILK ETSSYNLEV ESGSG LVCFPGAPGSR

 SP  IGAHYRWNANQTGLEFDQCWRASQDLKKAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATF
 JA  VGAHYRWNLNQTALEFDQWLETSQDLKKAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATF
 N1  IGAHYRWNANQTGLEFDQWLETSQDLKKAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATF
CON   GAHYRWN NQT LEFDQ    SQDLKKAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATF

 SP  EGSLSEVESLTYNSLMSLTTNPQDKANNQLVTKGVTVLNLPTGFDKPYVRLEDETPQGLQSMN
 JA  EGSLSEVESLTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGFDKPYVRLEDETPQGPQSMN
 N1  EGSLSEVESLTYNSLMSLTTNPQDKVNNQLVTKGVTVLNLPTGFDKPYVRLEDETPQGLQSMN
CON  EGSLSEVESLTYNSLMSLTTNPQDK NNQLVTKG TVLNLPTGFDKPYVRLEDETPQG QSMN

 SP  GARMRCTAAIAPRRYEIDLPSQSLPPVPATGTLTTLYEGNADIVNSTTVTGDINFSLAEQPAN
 JA  GARMRCTAAIAPRRYEIDLPSERLPTVAATGTPTTIYEGNADIVNSTAVTGDITFQLEAEPVN
 N1  GAKMRCTAAIAPRRYEIDLPSQRLPPVPATGTLTTLYEGNADIVNSTTVTGDINFSLAEQPAN
CON  GA MRCTAAIAPRRYEIDLPS  LP V ATGT TT YEGNADIVNST VTGDI F L   P N

 SP  ETRFDFQLDLMGLDNDVPVVTVVSSVLATNDNYRGVSAKMTQSIPTENITKPITRVKLSYKIN
 JA  ETRFDFILQFLGLDNDVPVVTVTSSTLVTADNYRGASAKFTQSIPTEMITKPITRVKLAYQLN
 N1  ETKFDFQLDFMGLDNDVPVVTVVSSVLATNDNYRGVSAKMTQSIPTENITKPITRVKLSYKIN
CON  ET FDF L   GLDNDVPVVTV SS L T DNYRG SAK TQSIPTE ITKPITRVKL Y  N

 SP  QQTAIGNVATLGTMGPASVSFSSGNGNVPGVLRPITLVAYEKMTPLSILTVAGVSNYELIPNP
 JA  QQTAIANAATLGAKGPASVSFSSGNGNVPGVLRPITLVAYEKMTPQSILTVAGVSNYELIPNP
 N1  QQTAIGNVATLGTMGPTTVSFSSGNGNVPGVLRPITLVAYEKMTPLSILTVAGVSNYESYPNP
CON  QQTAI N ATLG  GP  VSFSSGNGNVPGVLRPITLVAYEKMTP SILTVAGVSNYE  PNP

 SP  ELLKNMVTRYGKYDPEGLNYAKMILSHREELDIRTVWRTEEYKERTRVFNEITEKTSDLPTSK
 JA  DLLKNMVTKYGKYDPEGLNYAKMILSHREELDIRTVWRTEEYKERTRAFKEITDFTSDLPTSK
 N1  ELLKNMVTRYGKYDPEGLNYAKMILSHREELDIRTVWRTEEYKERTRVFNEITDFSSDLPTSK
CON   LLKNMVT YGKYDPEGLNYAKMILSHREELDIRTVWRTEEYKERTR F EIT   SDLPTSK

 SP  AWGWRDIVRGIRKIAAPVLSTLFPMAAPLIGTADQFIGDLTKTNAAGGRYHSMAAGGRYKDVL
 JA  AWGWRDLVRGIRKVAAPVLSTLFPMAAPLIGAADQFIGDLTKTNSAGGRYLSHAAGGRYHDVM
 N1  AWGWRDIVRGIRKVAAPVLSTLFPMAAPLIGMADQFIGDLTKTNAAGGRYHSMAAGGRHKDVL
CON  AWGWRD VRGIRK AAPVLSTLFPMAAPLIG ADQFIGDLTKTN AGGRY S AAGGR  DV

 SP  ESWASGGPDGKFSRALKNRLESANYEEVELPPPSKGVIVPVVHTVKSAPGEAFGSLAIIIRGE
 JA  DSWASGSEAGSYSKHLKTRLESNNYEEVELPKPTKGVIFPVVHTVESAPGEAFGSLVVVIPGA
 N1  ESWASGGPDGKFSRALKNRLESANYEEVELPPPSKGVIVPVVHTVKSAPGEAFGSLAIIIPGE
CON   SWASG   G  S  LK RLES NYEEVELP P KGVI PVVHTV SAPGEAFGSL   I G

 SP  YPSFLDANQQVLSHFANDTGSVWGIGEDIPFEGDNMCYTALPLKEIKRNGNIVVEKIFAGPIM
 JA  YPELLDPNQQVLSYFKNDTGCVWGIGEDIPFEGDDMCYTALPLKEIKRNGNIVVEKIFAGPAM
 N1  YPELLDANQQVLSHFANDTGSVWGIGEDIPFEGDNMCYTALPLKEIKRNGNIVVEKIFAGPIM
CON  YP  LD NQQVLS F NDTG VWGIGEDIPFEGD MCYTALPLKEIKRNGNIVVEKIFAGP M

 SP  GPSAQLGLSLLVNDIEDGVPRMVFTGEIADDEETIIPICGVDIKAIAAHEQGLPLIGNQPGVD
 JA  GPSSQLALSLLVNDIDEGIPRMVFTGEIADDEETVIPICGVDIKAIAAHEHGLPLIGCQPGVD
 N1  GPSAQLGLSLLVNDIEDGVPRMVFTGEIADDEETIIPICGVDIKAIAAHEPGLPLIGNQPGVD
CON  GPS QL LSLLVNDI  G PRMVFTGEIADDEET IPICGVDIKAIAAHE GLPLIG QPGVD

 SP  EEVRNTSLAAHLIQTGTLPVQRAKGSNKRIKYLGELMASNASGMDEELQRLLNATMARAKEVQ
 JA  EMVANTSLASHLIQSGALPVQKAQGACRRIKYLGQLMRTTASGMDAELQGLLQATMARAKEVK
 N1  EEVRNTSLAAHLIQTGTLPVQRAKGSNKRIKYLGELMASNASGMDEELQRLLNATMARAKEVQ
CON  E V NTSLA HLIQ G LPVQ A G   RIKYLG LM   ASGMD ELQ LL ATMARAKEV

 SP  DAEIYKLLKLMAWTRKNDLTDHMYEWSKEDPDALKFGKLISTPPKHPEKPKGPDQHHAQEARA
 JA  DAEVFKLLKLMSWTRKNDLTDHMYEWSKEDPDAIKFGRLVSTPPKHQEKPKGPDQHTAQEAKA
 N1  DAEIYKLLKLMAWTRKNDLTDHMYEWSKEDPDALKFGKLISTPPKHPEKPKGPDQHHAQEARA
CON  DAE  KLLKLM WTRKNDLTDHMYEWSKEDPDA KFG L STPPKH EKPKGPDQH AQEA A

 SP  TRISLDAVRAGADFATPEWVALNNYRGPSPGQFKYYLITGREPEPGEQYEDYIKQPIVKPTDM
 JA  TRISLDAVKAGADFASPEWIAENNYRGPSPGQFKYYMITGRVPNPGEEYEDYVRKPITRPTDM
 N1  TRISLDAVRAGADFATPEWVALNNYRGPSPGQFKYYLITGREPEPGDEYEDYIKQPIVKPTDM
CON  TRISLDAV AGADFA PEW A NNYRGPSPGQFKYY ITGR P PG  YEDY   PI  PTDM

 SP  NKIRRLANSVYGLPHQEPAPEEFYDAVAAVFAQNGGRGPDQDQMQDLRELARQMKRRPRNADA
 JA  DKIRRLANSVYGLPHQEPAPDDFYQAVVEVFAENGGRGPDQDQMQDLRDLARQMKRRPRPAET
 N1  NKIRRLANSVYGLPHQEPAPEEFYDAVAAVFAQNGGRGPDQDQMQDLRELARQMKRRPRNADA
CON   KIRRLANSVYGLPHQEPAP  FY AV  VFA NGGRGPDQDQMQDLR LARQMKRRPR A

 SP  PRRTRAPGKPAPPDVSRFTPSGDNAEV*
 JA  RRQTKTPPRAATSSGSRFTPSGDDGEV*
 N1  PRRTRAPAEPAPPGRSRFTPSGDNAEV*
CON   R T  P   A    SRFTPSGD  EV*
```

Fig. IV.4. Western immunoblot of the _trpE_-VP2 fusion proteins with anti-Sp sera. Lane 1, IPNV-Buhl infected fish tissue culture cell lysate; lane 2, prestained low molecular weight markers from BioRad at 75 kDa, 50 kDa, 39 kDa, 27 kDa and 17 kDa; lanes 3 and 4, pB10 induced bacterial cell lysate; lane 5, pA43 induced bacterial cell lysate; lane 6, bacterial cell lysate containing the pATH vector with no insert; lane 7, bacterial cell lysate without a plasmid; lane 8, low molecular weight markers; lane 9, purified IPNV-Buhl. The arrow in lane 5 indicates the position of the _trpE_-VP2 fusion protein encoded by the recombinant plasmid, A43.

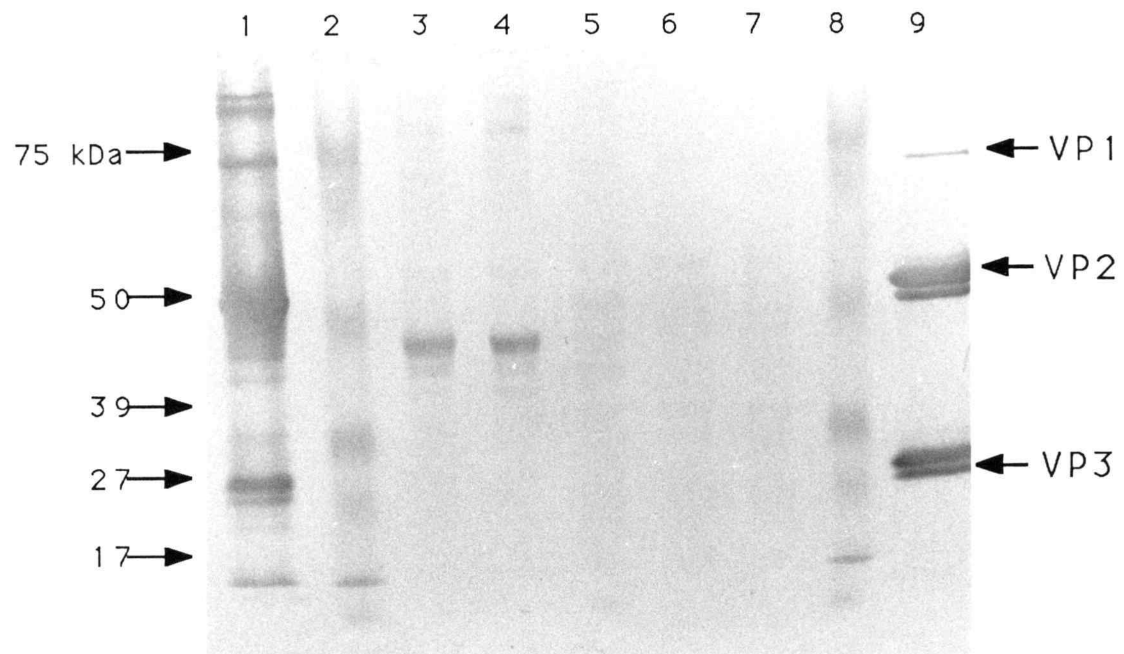
1
2
3
4
5
6
7
8
9
75 kDa
50
39
27
17
VP1
VP2
VP3

Fig. IV.5. Immunoblots of _trpE_-VP2 fusion proteins with antisera to the heterologous IPNV strains, IPNV-Buhl (WB serotype) and IPNV-EVE (Ab serotype). A.) Reactivity with antisera to IPNV-Buhl. Lanes 1 and 2 contain lysates from uninduced and induced cells containing the pATH1 expression vector with no viral insert; lanes 3 and 4, lysates from induced and uninduced cells containing pA43; lanes 5 and 6, lysates from uninduced and induced cells containing pB10; lane 7, prestained low molecular weight markers from BioRad at 75 kDa, 50 kDa, 39 kDa, 27 kDa and 17 kDa; lanes 8 and 9, lysates from uninduced and induced cells containing pB10, lane 10, purified IPNV. B.) Reactivity with antisera to IPNV-EVE. Lanes 1 and 2 contain lysates from uninduced and induced cells containing pB10; lanes 3 and 4, lysates form uninduced and induced cells containing pA43.

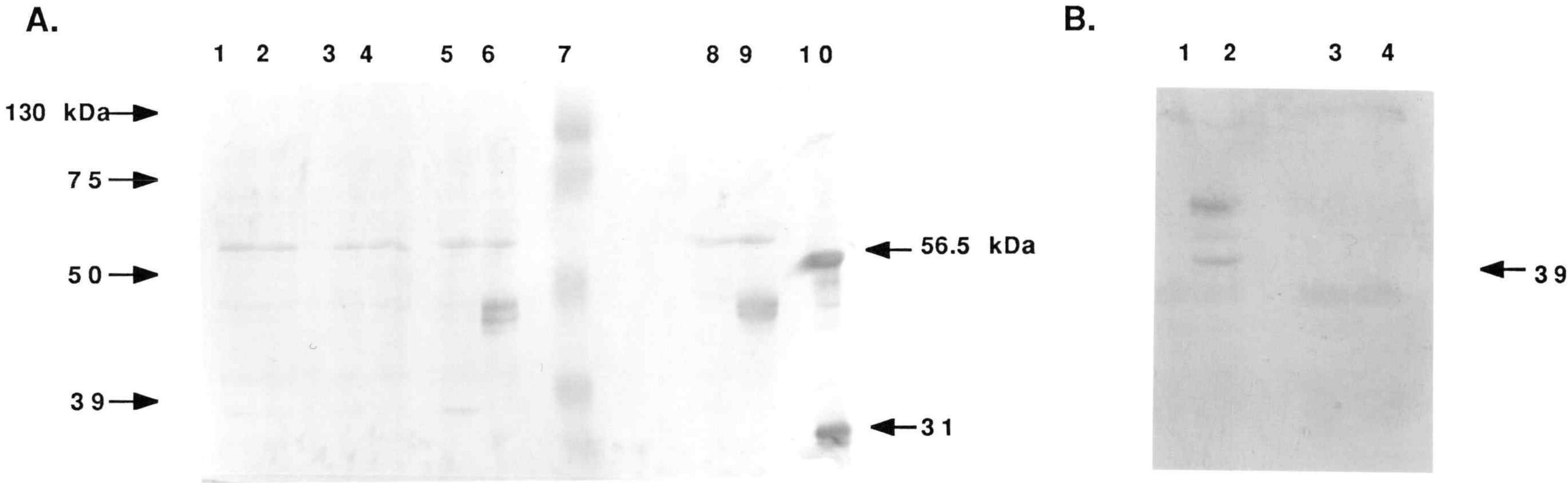
A.
1 2 3 4 5 6 7 8 9 10
130 kDa
75
50
39
56.5 kDa
31
B.
1 2 3 4
39

Fig. IV.6. Radioimmuneprecipitation of the trpE fusion protein B10. The trpE fusion proteins were expressed in E. coli and labeled with ^{35}S methionine. The samples in lanes 1-4 were precipitated with the Mab AS-1. The samples in lanes 5 and 6 were non-precipitated. Lane 1, ^{35}S methionine labeled fusion pTA1 (Manning and Leong, 1990) which contained the entire A segment; lane 2, ^{35}S methionine labeled fusion protein B10; lane 3, ^{35}S methionine labeled fusion protein A43; lane 4, ^{35}S methionine labeled pATH 3, which expressed the trpE protein; lane 5, ^{35}S methionine labeled fusion protein B10; lane 6, ^{35}S methionine labeled fusion protein A43.

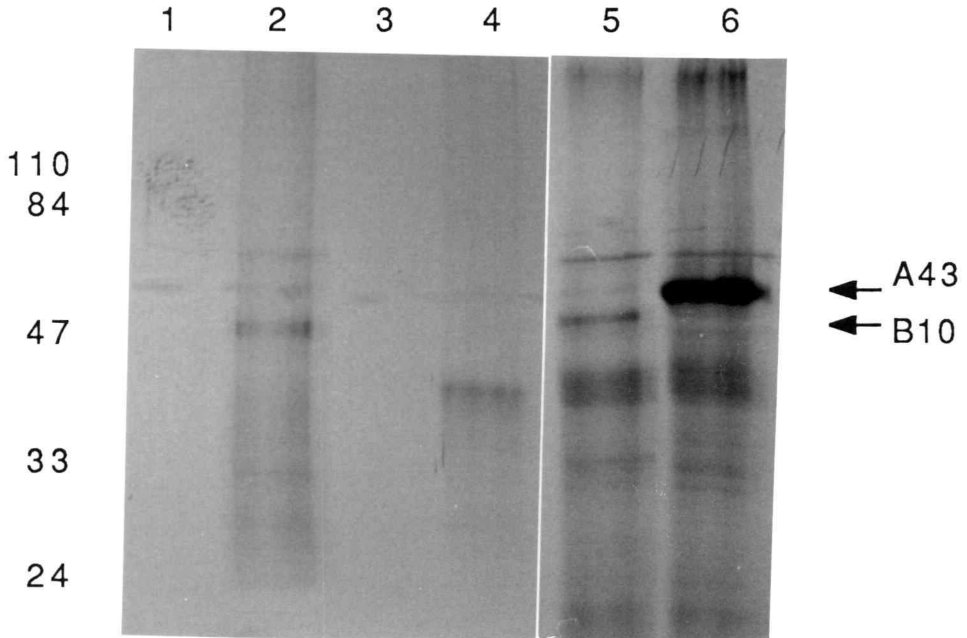
1
2
3
4
5
6
110
84
47
33
24
A43
B10

Table IV.1. Comparison of the IPNV A segment similarity[a] at the nucleotide and amino acid level.

Virus	Nucleotide	Amino acid
Sp vs. Ja	75.7	83.7
Sp vs. N1	96.5	96.8

[a]The % similarity (identical residues) between the Sp, Jasper (Ja) and N1 isolates of IPNV are reported for the nucleotide and amino acid sequences.

Table IV.2. Comparison of the IPNV protein similarity.

	% Similarity[a]		
	VP2	NS	VP3
Sp vs. Ja	88.8	79.2	78.0
Sp vs. N1	95.8	97.9	97.6

[a]The % similarity (identical amino acids) for VP2, NS and VP3 were calculated for the Sp, Jasper (Ja) and N1 isolates of IPNV.

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V. Sequence Analysis and Bacterial Expression of Infectious Pancreatic Necrosis Virus Genome Segment B and its Encoded VP1 Protein: a Putative RNA-Dependent RNA Polymerase

ABSTRACT

The sequence of the B genome segment of infectious pancreatic necrosis virus as determined for both the Sp and Jasper serotypes. The sequences are 2630 and 2784 basepairs long, respectively, and contain a single large open reading frame encoding the VP1 protein. The VP1 protein is the putative RNA-dependent RNA polymerase (RdRp) of IPNV. The proteins exhibit an 88% homology with each other, but only 41% with infectious bursal disease virus (IBDV) VP1 protein, another member of the birnavirus family. Despite the low overall homology between the birnavirus VP1 proteins, homologous regions were detected within the central portion of the proteins. Concensus sequences associated with GTP-binding proteins and RdRps were identified in the VP1 sequences. However, unlike the RdRps associated with single-stranded plus RNA viruses, the birnavirus RdRP lacks the Gly-Asp-Asp motif characteristic of this enzyme family. Additionally, we have expressed the IPNV-Sp VP1 protein as a trpE fusion protein in Escherichia coli. The trpE-VP1 fusion protein was isolated and used to produce polyclonal rabbit antisera. This antisera can recognize both the native VP1 and the trpE-VP1 fusion proteins.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is a member of the Birnaviridae. This virus family is characterized by a genome containing two segments of double-stranded RNA enclosed in a non-enveloped icosahedral shell (Matthews, 1982). The virus causes an acute, contagious disease of salmonid fishes. Other members of the birnavirus family include infectious bursal disease virus (IBDV) in chickens and Drosophila X virus (DVX).

The physical map of the larger A segment of the viral genome has been determined to be in the order from the 5' end of the sense strand, VP2, the major capsid protein; NS, the nonstructural protease; and VP3, the minor capsid protein (Huang et al. 1986; Nagy et al., 1987). The entire nucleotide sequence of the A segment for three different viral isolates has been determined and found to encode a polyprotein from a single open reading frame (Duncan et al., 1987; Haverstein et al., 1990; Mason et al. manuscript in preparation). This polyprotein is processed by the autocatalytic NS protease to yield the functional proteins VP2, NS and VP3 (Duncan et al., 1987; Manning et al., 1990).

The sequence analysis of the viral segment B is reported here. This segment encodes VP1, the putative viral RNA-dependent RNA polymerase (RdRp), which directs the synthesis of non-polyadenylated mRNAs corresponding in size to the A and B genome segments. VP1 is approximately 90 kDa as estimated by gel migration and it is also presumed to be the protein linked to the 5' end of the genome in the virion (VPg) (Persson and MacDonald,

1982). However, the lack of a reagent to VP1 has not allowed confirmation that the VPg is the VP1 protein We report here the expression of the B segment, coding VP1, as a fusion protein with the Escherichia coli trpE protein from a pATH vector construction. The purified fusion protein was used to generate VP1 specific polyclonal rabbit antisera.

Recently, the nucleotide sequence of the B genome segment of IBDV was examined for homology to other viral RdRps (Morgan et al., 1988). No homology between the predicted VP1 sequence and the sequences of putative ssRNA-dependent RNA polymerases (ssRdRp) was observed. Subsequently, the IBDV sequence was re-examined and consensus sequence homology was detected (Kamer and Argos, 1984; Argos, 1988). We have determined the nucleotide sequences for the B segments of IPNV-Jasper and IPNV-SpS and compared their predicted amino acid sequences of their encoded VP1 proteins with other RdRps. Sequence analysis revealed extensive homology between IPNV and IBDV VP1 proteins in central regions of the proteins and the presence of several conserved domains associated with RdRps (Poch et al., 1989) and GTP binding proteins. Unlike other RdRps, however, IPNV lacks the conserved Gly-Asp-Asp motif, the proposed catalytic site for the RdRp enzyme family.

MATERIALS AND METHODS

Cloning and Sequence Analysis

The virus was grown and cDNA synthesis was performed as previously described (Duncan et al., 1991). The sequence of the IPNV genome segment B for both virus strains was determined by dideoxy-chain termination procedure (Sanger et al., 1977). Sequence analysis was carried out by sequencing overlapping subclones in both directions. Plasmid sequencing (Wang et al., 1988) was performed using synthetic oligomeric primers to confirm regions of the Sp sequence. Primers complementary to Sp nucleotide positions 96-117, 681-697, 805-821, 1746-1763, 2079-2096 and 2371-2388 were used for sequence analysis using the strategy outlined in Fig. V.1.

Preparation of VP1 Antisera

A recombinant plasmid containing the trpE promoter and trpE gene fused to the IPNV-Sp VP1 gene was constructed. The VP1 gene was digested with the restriction endonuclease BamHI and ligated into the BamHI site of the pATH vector. This resulted in a 39 amino acid truncation from the amino terminus of VP1 in order to create a functional in-frame clone. The construction was verified by DNA sequence analysis by the dideoxy-chain termination method.

Recombinant colonies were screened for the production of functional fusions by induction of the bacterial fusion protein with indoleacrylic acid (10 µg/ml). Bacterial lysates were

separated by 7.5% SDS-PAGE and protein transfer blots were developed using antisera to the bacterial trpE protein and they were examined for the production of a 120 kDa band, the product of the trpE-VP1 fusion. Preparative SDS-PAGE was used to purify the trpE-VP1 fusion protein, which was then electroeluted and used to inject New Zealand White rabbits for polyclonal antisera production.

RESULTS

Cloning and Sequence Analysis

The virus was grown and cDNA synthesis was performed as previously described (Duncan et al., 1991). The sequence of the IPNV genome segment B for both virus strains was determined by dideoxy-chain termination procedure. The sequence was determined by sequencing overlapping subclones in both directions. Plasmid sequencing (Wang et al., 1988) was performed using synthetic oligomeric primers to confirm regions of the Sp sequence. The amino acid sequences of the Sp and Jasper isolates were aligned and compared to those of other viral polymerases. The consensus sequence and amino acid substitutions are shown in Fig. V.2. Amino acid sequence analysis revealed the presence of several conserved sequence motifs which may be involved in enzymatic activity for this class of proteins. The VP1 proteins contain the described concensus sequences. Motif A contains the invariant Asp residue strictly conserved in the birnaviruses. The gaps between motifs are characteristic of the polymerase family. The distance between motifs A and B is 50-90 residues, which is consistent with dsRNA viral polymerases. Motif B is also conserved in the birnaviruses. The spacing between motifs B and C and motifs C and D is 20 to 30 residues, which is consistent with previous reports. As previously reported (Kamer and Argos, 1984; Argos, 1988; Poch et al., 1989), the Gly-Asp-Asp sequence of motif C (Fig. V.3) is highly conserved for RdRps. While IBDV contains the conserved D-D, the IPNV sequences do not contain the catalytic

core sequences, suggesting that catalytic activity functions differently in the birnavirus class of RdRps. The IPNV VP1 proteins represent the only putative RdRps that deviate from the Gly-Asp-Asp motif.

Bacterial Expression of VP1

The VP1 gene was ligated into the pATH vector (provided by Dr. T. J. Koerner and Dr. A. Tzagoloff, Columbia University) as a *trpE* fusion. The *trpE*-VP1 fusion protein was used to immunize rabbits for the production of polyclonal antisera. The expression of VP1 as a fusion protein in *E. coli* did provide a means of obtaining enough antigen to produce anti-VP1 specific sera in rabbits. We have developed an antisera reagent specific for the VP1 protein of IPNV. The antisera produced against VP1 was able to recognize both the *trpE*-VP1 fusion protein and the virion VP1 in Western immunoblots for the Sp (Fig. V.4) and Jasper (data not shown) isolates of IPNV.

DISCUSSION

An understanding of the replication of RNA viruses can provide insight into mechanisms for the control their expression. Thus, we have sought information on the structure and function of RdRps with the eventual aim of using the viral polymerase as a target.

RdRps display amino acid sequence conservation over several domains of the protein (Fig. V.2 and Fig. V.3.). These similarities are present despite the variety in morphology, genetic organization and host specificity. Conserved amino acid sequence motifs were first described by Kamer and Argos (1984) for the picornavirus polymerase and putative RdRps from other plus-stranded RNA viruses from plants and animals. A conserved G-D-D motif flanked by hydrophobic residues has been proposed as the catalytic site for RdRps. Recently, four consensus sequences have been reported to be shared by RdRPs of plus-, minus- and double-stranded RNA viruses, as well as homology to cellular polymerases (Poch et al., 1989). A 15 amino acid sequence surrounding the conserved G-D-D has been defined for polymerases. The rules governing the consensus sequence indicate that the residues flanking the core are hydrophobic. Mutational analysis of the core region in both the HIV reverse transcriptase and bacteriophage Q-betas RdRp destroyed or significantly reduced polymerase function.

Although the Gly-Asp-Asp (G-D-D) sequence motif is highly conserved in RdRps, the IPNV VP1 protein does not contain this motif. This result raises the possibility that the G-D-D sequence

is not part of the catalytic core of RNA polymerases. The birnaviruses are also distinctly different in this region because they contain charged or polar hydrophilic residues rather than the hydrophobic flanking residues associated with the G-D-D motif. The birnavirus IBDV VP1 does contain the D-D residues of the G-D-D motif, but its flanking amino acid residues are hydrophilic. Thus, it appears that the birnaviruses may contain a novel catalytic site and represent a new family of RdRps or G-D-D does not constitute the catalytic core of RdRps.

The availability of a reagent specific for VP1 will allow confirmation of the identity of the VPg protein of IPNV. Immunogold labeling experiments are currently underway to examine if VP1 is the VPg protein.

Fig. V.1. Physical map and sequencing strategy of the IPNV B segments for both the Sp and Jasper isolates. A restriction map of the B segment is shown, subclones generated for sequencing are represented by bars below the restriction map. Arrows indicate the regions sequenced and the direction of the sequencing.

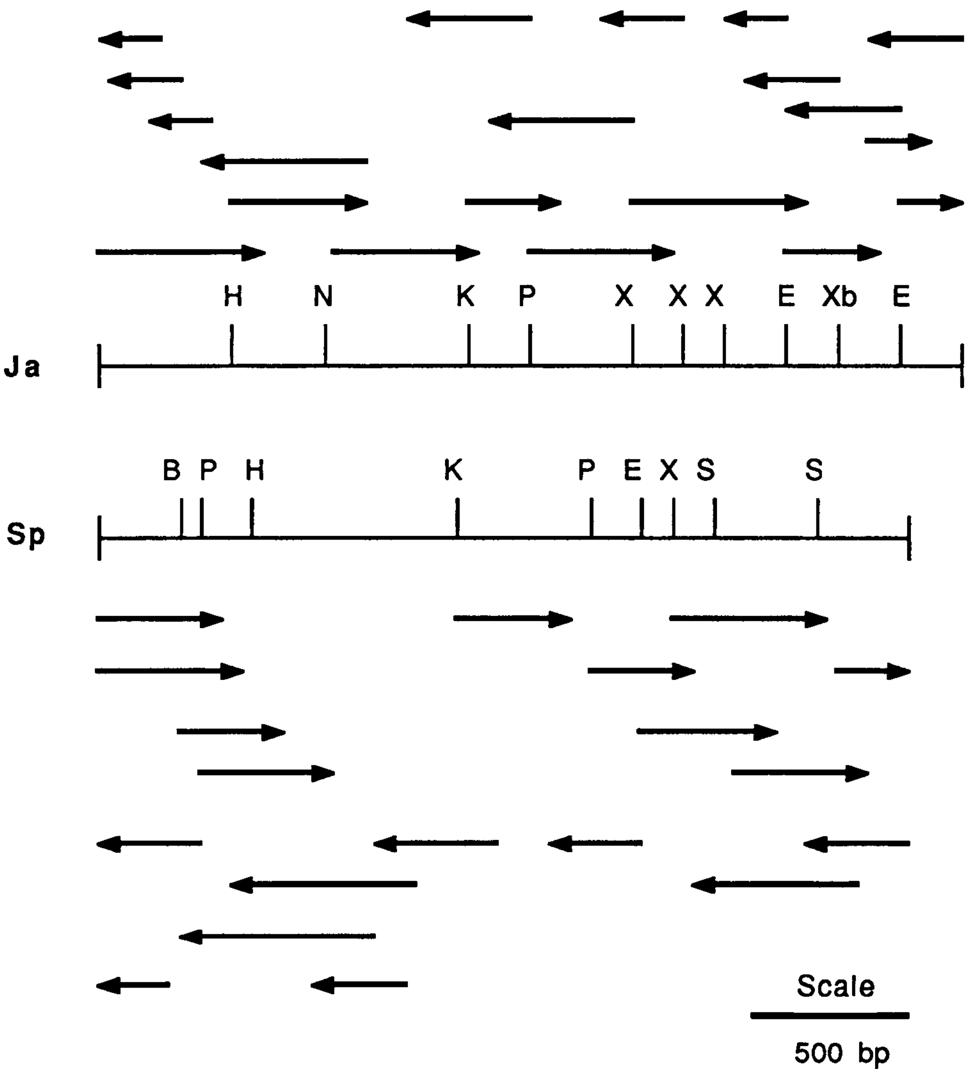

H
N
K
P
X
X
X
E
Xb
E
Ja
B
P
H
K
P
E
X
S
S
Sp
Scale
500 bp

Fig. V.2. IPNV segment B amino acid similarity. The concensus (Con) sequence for the B segment is shown between IPNV-Sp (Sp) and IPNV-Jasper (Ja) listed above and below the concensus sequence, respectively. The conserved polymerase concensus sequences are boxed and designated A, B, C and D.

```
Sp                   N      Q                          A   EH
Con   MSDIFNSPQNKASIL ALMKST GDVEDVILPKRFRPAKDPLDSPQAAA FLK  KYRILRPRA
Ja                   T      T                          Q   DN

Sp          I         AA  D     EM N                R    D
Con   IPTMVE ETDAALPRL  MV DGKLK  V VPEGTTAFYPKYYPFH PDHD VGTFGAPDITLL
Ja          L         RQ  E     DT S                K    E

Sp                                                                  LA
Con   KQLTFFLLENDFPTGPETLRQVREAIATLQYGSGSYSGQLNRLLAMKGVATGRNPNKTPK  GY
Ja                                                                  TV

Sp        M R M       P  N            Q    A          A
Con   TNEQ A L EQTLPIN PK EDPDLWAPSWLI YTGD STDKSYPHVT KSSAGLPYIGKTKGD
Ja        L K L       T  H            N    L          I

Sp                     K       G AA  VLS                   K   L
Con   TTAEALVLADSFIRDLG AATSADP A  KK   DFWYLSCGLLFPKGERYTQ DWD KTRNIW
Ja                     R       E GV  TIT                   V   K

Sp                  S   D                       I        EH  QEQ
Con   SAPYPTHLLLSMVS PVM ESKLNITNTQTPSLYGFSPFHGG  RIMTIIR  LD   DLVMIY
Ja                  T   N                       MD       DS  NDE

Sp           D                          R                        Y G
Con   ADNIYLQ NTWYSIDLEKGEANCTPQHMQAMMYY LTRGWTNEDGSPRYNAPTWATFAM V PS
Ja           N                          L                        N A

Sp         T          T                           R    S       A
Con   MVVDS CLLMNLQLKT GQGSGNAFTFLNNHLMSTIVVAEWKAG PNPM KEFMDLE KTGINF
Ja         S          Y                           K    T       E

Sp           D  SI M   D   L            RV              V    L
Con   KIERELK LR  I EAV TAP DGYLADGSDLPP  PGKAVELDLLGWSA YSRQ EMFVPVLE
Ja           E  ET V   E   Q            IR              I    M

Sp           V         S                     I       I
Con   NERLIAS AYPKGLENK LARKPGAEIAYQIVRYEAIRL GGWNNPL ETAAKHMSLDKRKRLE
Ja           A         A                     V       L

Sp                  T           S TA       L     ETE  V D  P     G
Con   VKGIDVTGFLDDWN MSEFGGDLEGI L  PLTNQTL DINTP   FD K RP TPRSP KTL
Ja                  N           T SE       V     LDS  P A  Q     K

Sp    A   A                               H H     A TS F GD      E
Con    EVT AITSGTYKDPKSAVWRLLDQRTKLRVSTLRD A ALKPA S  D W  ATEELA QQQ
Ja    D   T                               Q L     S SV N AE      Q

Sp                   A      V      S  TS             A
Con   LLMKANNLLKSSLTE REALET QSDKII GK  PEKNPGTAANPVV YGEFSEKIPLTPTQK
Ja                   T      I      A  SN             G

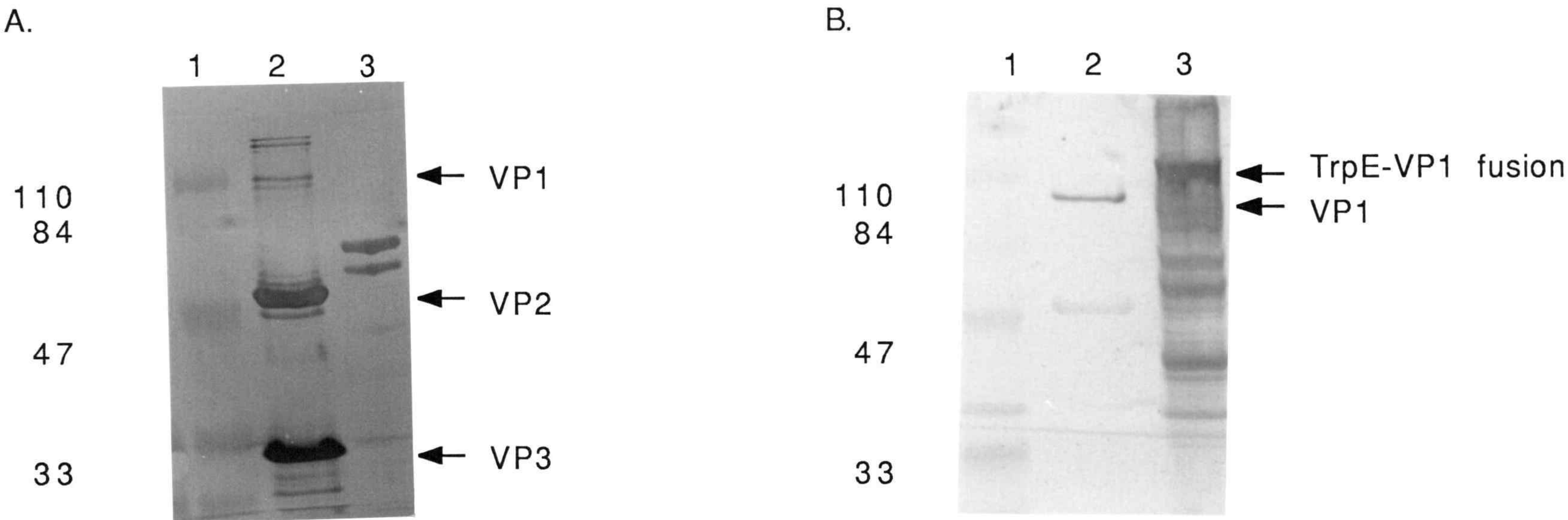
Sp                *
Con   KNAKRREKQRRN
Ja                Q
```

Fig. V.3. Amino acid similarity between the RNA-dependent RNA polymerases of several dsRNA viruses. Shown are four conserved sequence motifs, A, B, C and D (Poch et al., 1989) in which the polymerases of dsRNA viruses share with other viral polymerases. Con= concensus sequences, Sp= IPNV-Sp, Ja= IPNV-Jasper, IBDV= infectious bursal disease virus, BTV= blue tongue virus and Reo= reovirus type 1.

Motif:		A			B				
CON		D			SG		T		N
Sp	QDNTWYSI	D	LEKGEAN	LQLKTTGQG	SG	NAF	T	FLN	N	HLMSTIVV
Ja	QNNTWYSI	D	LEKGEAN	LQLKTYGQG	SG	NAF	T	FLN	N	HLMSTIVV
IBDV	WSNTWYSI	D	LEKGEAN	LQIKSYGQG	SG	NAA	T	FIN	N	HLLSTLVL
BTV	GYTLEQII	D	FGYGEGR	DLALIDTHL	SG	ENS	T	LIA	N	SMHNMAIG
Reo	PPNQSINI	D	ISACDAS	FTHMTTTFP	SG	STA	T	STE	H	TANNSTMM

Motif:		C				D		
CON		GDD			G		R/K	
Sp	IERL	EKD	LRSI	LL	G	WSAVYS	R	QL
Ja	IERE	LKN	RETI	LL	G	WSAIYS	R	QM
IBDV	IERS	IDD	IRGK	LL	G	WSATYS	K	DL
BTV	EQYV	GDD	TLFY	KC	G	HEASPS	K	TM
Reo	YVCQ	GDD	GLMI	KY	G	EEFGW*	K	YD

Fig. V.4. Western immunoblots of the _trpE_-VP1 fusion protein developed with anti-Sp and anti-_trpE_-VP1 fusion protein sera. Lane 1, molecular weight markers; lane 2, purified IPN Sp virus; lane 3, bacterial expressed _trpE_-VP1 fusion protein A.) Developed with antisera against IPNV-Sp. B.) Developed with antisera against the _trpE_-VP1 fusion protein.


A.
1
2
3
110
84
47
33
VP1
VP2
VP3
B.
1
2
3
110
84
47
33
TrpE-VP1 fusion
VP1

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VI. CONCLUSION

This report examines the protein expression of IPNV and the characterization of a virally-encoded proteinase. Proteinases catalyzing cleavage events can be virally-encoded or reside in the host prior to a viral infection. There is a difference in the location in the cell of polyproteins cleaved by virus-encoded and host-encoded proteinases. Polyproteins that reside in the cytoplasm are cleaved by virally encoded enzymes. (Rice and Strauss, 1981). Evidence that IPNV is proteolytically processed by a virally-encoded proteinase includes that IPNV, a dsRNA virus, replicates entirely in the cytoplasm of the cell. A single mRNA species is detected and processing occurs efficiently with in vitro translations and proteins expressed in E. coli (Manning and Leong, 1990).

The processing of the polyprotein of IPNV was examined and the viral proteinase NS has been partially characterized. The essential coding sequences of NS were determined using plasmid deletion mapping and NS was found to be encoded between amino acids 452 and 736. NS has previously been reported to have proteolytic activity and is responsible for the cleavages at the pVP2-NS and NS-VP3 junctions (Duncan et al., 1987). Similarly, reports by Jagadish et al. (1988) implicate the VP4 protein (IBDV nomenclature for NS) of IBDV in proteolytic processing. Site-directed mutagenesis of the large segment of IBDV demonstrated that when 10 codons were inserted in-frame near the N-terminus

of VP4 normal processing occurred. Processing could be interrupted by the in-frame insertion of four codons in the central portion of VP4 (Jagadish et al., 1988), resulting in the accumulation of polyprotein. These data, in addition to the site-specific mutagenesis and proteinase inhibitor studies, provide evidence that NS is responsible for the proteolytic processing of the A segment polyprotein.

We present evidence that NS is an autocatalytic proteinase. Previously, it was reported that antibodies specific for IPNV were unable to inhibit the proteolytic processing of the A segment. It was suggested that polyprotein processing may occur rapidly prior to antibody binding (Manning et al.,1990). Additionally, the presence of the 38 kDa protein in vivo and in vitro suggests that NS is unable to act in trans. The 38 kDa protein appears to be produced from internal ribosomal initiation downstream of the 5' end of NS from an in-frame ATG codon (Manning et al., 1990). The 38 kDa protein therefore carries a cleavage site for NS at the NS-VP3 junction. However, due to the existence of this protein the cleavage site of the 38 kDa does not appear to be utilized.

Further characterization of the proteinase is required to determine its cleavage and catalytic sites, however, the information obtained in this study indicates that the NS protein is a novel proteinase. The unique properties of this enzyme make it an ideal target for antiviral therapy. The function of this molecule to process the viral structural and non-structural proteins indicate that its structure and function should be highly conserved.

The NS molecule may be a suitable model for the comparison of evolutionary relationships between dsRNA and ssRNA viruses that utilize virally encoded proteinases for their protein expression.

The lack of the canonical GDD sequence in the VP1 polymerase protein and the finding that none of the putative RdRps from dsRNA viruses fulfil the rules defined by Argos (1988) for core polymerase sequences, suggests that the dsRNA viral polymerases are intrinsically different from their ssRNA viral counterparts. Alternatively, the conserved GDD sequence may represent a highly conserved structural motif, and not the catalytic site, which is more diverged in the case of the dsRNA viruses. Since the VP1 protein of the birnaviruses is unique, this protein could be targeted with antivirals in order to control the IPN disease.

Since the nucleotide of the A and B segments of IPNV-Sp have been determined, comparisons between other birnaviruses and dsRNA viruses can be made. We have mapped segments of VP2 with polyclonal and monoclonal antisera and found that the N-terminal portion of VP2, amino acid residues 99-206 of VP2, carried by B10, contained a nonlinear epitope that was common to three serotypes and six newly proposed serotypes. This region of 107 amino acids carried a common immunoprotective portion of VP2, while the region containing amino acid residues 207-314 maps to a serotype specific portion of VP2, as it was only recognized by homologous anti-IPNV-Sp sera. Examination of the amino acid sequence comparison between IPNV-Sp, -Jasper and -N1 revealed

that VP2 was highly conserved from amino acids 1-210 between all three isolates and the region spanning amino acids 211-333 displayed heterogeneity. These results are in agreement with a comparison of the deduced amino acid sequence of IPNV-N1 and IPNV-Jasper capsid proteins by Havarstein et. al. (1990) which revealed heterogeneity from amino acid 206-350 and it was proposed that the region contained serotype-specific epitope(s), which was confirmed with this study. This region constituted a variable region when compared with both IPNV-Jasper and IBDV. The variable region of VP2 corresponded to a segment of IBDV-VP2 recognized by a neutralizing monoclonal antibody. It was suggested that amino acids 183-337 of VP2 carried serotype specific epitopes for both IPNV and IBDV (Havarstein et al., 1990). The information obtained for the major capsid protein through sequence similarities and the reactivity with polyclonal and monoclonal antisera, will expedite the production of an effective vaccine against IPNV. Additionally, the evolutionary relationships between dsRNA and ssRNA viruses can be more closely examined. The identification of novel polymerase sequences will also lend to our understanding of the replication of dsRNA viruses.

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