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Genomic Organization of Infectious Salmon Anemia Virus

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GENOMIC ORGANIZATION OF INFECTIOUS SALMON ANEMIA VIRUS

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Infectious salmon anemia virus (ISAV) is an emerging pathogen of farmed Atlantic salmon (*Salmo salar*). The development of an effective ISA virus vaccine is a high priority for salmon producers in the U.S. and elsewhere. The process of developing a recombinant vaccine requires complete genetic characterization of the virus. Toward this end we have cloned, sequenced and determined the organization of the eight segments of single-stranded RNA from ISA virus isolate CCBB. The virus was grown in cell culture and purified by density gradient ultracentrifugation. Viral RNA was isolated from purified ISAV and used in the construction of two different cDNA libraries. After screening the libraries, individual ISA virus-specific cDNA clones were placed into eight groups and the DNA from a representative clone from each group was sequenced. Using Northern blot hybridization results, the eight representative clones were assigned to specific RNA segments of the ISA virus genome and a genetic map of ISA virus strain CCBB was constructed. In addition, N-terminal amino acid sequence analyses of purified ISA virus proteins correlated

protein product(s) to specific RNA segments and provided evidence for protein synthesis initiation sites. Finally, Western blot analysis identified viral proteins that were immunoreactive with ISA virus-specific serum from mice and Atlantic salmon.

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INTRODUCTION

Geographic Distribution

Infectious salmon anemia virus (ISAV) causes a lethal disease in salt-water farmed Atlantic salmon (*Salmo salar*). The disease was first described in Norway in 1984 but it is believed the disease was present as early as the 1970's (Thorud and Djupvik, 1988) and in Canada in 1994 (Lovely *et al.*, 1997). There have been recent outbreaks reported in Scotland (Mullins *et al* 1998), New Brunswick, Canada (Bouchard *et al.*, 1999) and in the United States off the coast of Maine (Clancy, 2001). ISA virus has recently been isolated in Chile from Coho salmon (*Oncorhynchus kisutch*) showing clinical signs of disease (Kibenge *et al.*, 2001). The distribution of the virus is probably linked to fish transport and local migration between individual fish farms (Nylund *et al.*, 2001).

Host Range and Susceptibility

The most significant host of infectious salmon anemia virus is the farmed Atlantic salmon due to potentially high mortalities, reports of up to100%, and resulting economic losses. ISA virus has been shown to agglutinate erythrocytes from different fish species with differing affinities. ISA virus agglutinates erythrocytes from Atlantic salmon, Rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), brown trout (*Salmo* trutta) and crucian carp (*Carassius carassius)*(Dannevig *et al.*, 1997). Transmission experiments with ISA virus have shown viral replication both in brown trout and in rainbow trout without causing disease (Dannevig *et al.*, 1997). ISA virus has also been isolated from diseased Coho

salmon *Oncorhynchus kisutch* in Chile (Kibenge *et al.*, 2001). It is apparent that ISA virus has the ability to replicate in fish species other than Atlantic salmon.

Pathology of ISA Virus Infected Atlantic Salmon

The pathological analysis of infectious salmon anemia virus has been studied in Atlantic salmon (Evensen *et al.*, 1991). Infectious salmon anemia in Atlantic salmon is characterized by progressive anemia with combined development of ascites, congestion of the foregut in early stages, enlargement and congestion of the liver and spleen. Changes in the liver are the most prominent with degeneration of the hepatocytes and presence of hemorrhagic necrosis in later stages of the disease. Congestion of the spleen is accompanied by erythrophagocytosis (Evensen *et al.*, 1991). The primary tissue tropism is endothelial cells of the vascular system of some salmonid species (Hovland *et al.*, 1994). There has been evidence that the Canadian isolate appears to be more destructive to the kidney than the liver. This may be due to genetic differences between the strains (Evensen *et al.*, 1991). Analysis of experimentally infected Atlantic salmon demonstrated that ISA virus could be found in most organs; liver, kidney, spleen, and heart from 1 day to 40 days post infection using RT-PCR. The results also showed that for the first 8 days post infection the head kidney and mid-kidney were predominately affected (Rimstad *et al.*, 1999).

Replication of ISA Virus

ISA virus was first propagated *in vitro* in Salmon head kidney cells (SHK-1). The viral infected SHK-1 cell began to appear vacuolated 12-14 days after infection. The appearance of more dead cells followed by the loss of surface adhesion (Dannevig *et al.*, 1995). ISA virus has been propagated in the Atlantic Salmon (AS)

cell line. Trypsin treatment was not necessary for viral replication in AS cells. The viral propagation was non-cytopathic to the cell line but heamadsorpotion of salmon erythrocytes to ISA virus infected AS cells was evident. Viral particles were observed with electron microscopy in ISA virus infected AS cells and not in uninfected AS cells (Sommer and Mennen, 1997). ISA virus was also successfully cultured using Chinook salmon embryo cells (CHSE-214) (Bouchard *et al.*, 1999). The optimal temperature for replication of ISA virus in SHK-1 and CHSE cells was 15°C with optimal yields of virus at $1x10^6$ TCID50/ ml (Falk *et al.*, 1997). The mechanism of entry of ISA virus into SHK-1 cells takes place in the following steps (i) the binding of ISA virus to neuraminidase-sensitive determinants on the cell surface, (ii) internalization of ISA virus and transport to endosomes and lysosomes, (iii) low-pH dependent fusion with endosomal membrane (Eliassen *et al.*, 2000). The addition of Actinomycin D inhibited ISA virus replication indicating that ISA virus is similar to other orthomyxoviruses in requiring a functional host cell RNA polymerase II for transcription of viral genes (Krossoy *et al.*, 1999).

Classification, Biochemical and Physiochemical Analysis of ISA Virus

ISA virus particles can be found in tissues of diseased Atlantic salmon and are pleomorphic, enveloped spheres ranging from 90-130 nm in diameter (Dannevig *et al.*, 1995; Nylund *et al.*, 1995). They are evenly covered with 13-15 nm spikes and contain filamentous nucloecapsid like structures that are released upon partial virion disruption (Sommer & Mennen, 1996). The buoyant density of ISA virus was determined to be 1.18 g/cc in both sucrose and CsCl gradients (Krossoy *et al.*, 1999). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed

ISA virus to consist of four major polypeptides with estimated molecular weights of 71, 53, 43, and 25 kDa (Dannevig *et al.*, 1997). The growth of another Norwegian ISA virus isolate in SHK-1 cells result in the corresponding 4 major viral polypeptides with molecular sizes of 71, 53, 43 and 24 kDa (Falk *et al.*, 1997). ISA virus is sensitive to chloroform which disrupts the envelope and results in loss of infectivity. Similar to other enveloped viruses, ISA virus is also sensitive to heat and low pH. ISA virus virions contain a receptor-destroying enzyme that appears to be an acetylesterase and in this regard is more similar to influenza C, as influenza A and B have neuraminidase activity. ISA virus acetylesterase receptor destroying activity does not affect receptor binding of influenza A or C, suggesting that the receptors are different for the viruses (Eliassen *et al.*, 2000).

The replication of ISA in SHK-1 cells was not inhibited by the addition of the DNA synthesis inhibitors 5-iodo-2-deoxyuridine (IdU) and 5-bromo-2-deoxyuridine (BrU) indicating that ISA virus has an RNA genome (Mjaaland *et al.*, 1997). Also tracing ^{32}P labeled virus using anti-ISA antibodies demonstrated that ^{32}P was found in the RNA fraction of nucleic acid extractions. Electrophoresis of ^{14}C labeled ISA virus virus revealed a genome consisting of 8 RNA segments. Treatment of RNA with Rnase A resulted in the disappearance of a positive hybridization reaction indicating a single stranded RNA genome (Mjaaland *et al.*, 1997). The data above as well as morphological electron microscopy studies indicate that ISA virus belongs to the *Orthomyxoviridae* family. Further phylogenetic evidence obtained through cDNA sequencing of the ISA virus polymerse gene *pb1* supports this grouping. The polymerase contains conserved motifs found in all *orthomyxoviridae* RNA-dependent

RNA polymerases (Krossoy *et al.*, 1999). The genes encoding NS, PB1, NP, and HA proteins are described below.

Phylogenetic Analysis of ISA Virus

The genomic relationship of the North American CCBB isolate of ISA virus genome segments 2 (436 bp) and 8 (151 bp) revealed significant difference between North American and Norwegian isolates (Blake *et al.*, 1999). Segments 2 and 8 of the North American ISA virus isolate exhibited 82.9% identity and 88% identity with segment 2 and 8 respectively with Norwegian islolate Sotra 92/93 (Blake *et al.*, 1999). Analysis of Norwegian ISA virus isolates revealed that European isolates maintained a 98 to 100% identity for gene segments 2 and 8 while the Canadian isolate showed 84 and 87 to 88% identity to European isolates for segments 2 and 8 respectively (Inglis *et al.*, 2000). This data is in agreement with other studies examining the relationship between European and North American ISA virus segments 2 and 8 (Inglis *et al.*, 2000).

From this data it was estimated that the divergence between the European isolates and the North American isolates may have been sometime around 1900. This date also coincides with the time when fish transportation between Europe and North America started (Nylund *et al.*, 2001).

Orthomyxovirus Replication and Genetics

The *Orthomyxoviridae* are enveloped viruses with a segmented single stranded RNA genome. The family is composed of Influenza A, B, C, Dhori, and Thogoto viruses. The genome of orthomyxoviruses influenza A, influenza B and ISA virus is each composed of eight RNA segments while influenza C, Thogoto, and

Dhori have seven segments (Fields, B.N., 1996). Each gene segment is packaged into a ribonucleoprotein (RNP) complex that contain in addition to the RNA segments the nucleoprotein (NP) and a polymerase complex (PB1, PB2, and PA). The NP serves as the core around which each segment of RNA is coiled (Mikulasova *et al.*, 2000). Each genome segment endcodes one protein except for 7 and 8 which encode two proteins via alternative splicing. The genome segments are numbered based on relative electrophoretic mobility in acrylamide gels from slowest to fastest (Brown, 2000).

Influenza A virus attaches to receptors of host cells through HA binding and is engulfed through receptor-mediated endocytosis. The low pH of host cell endosomes facilitates several reactions. It mediates cleavage of the HA protein which allows the viral and host cell membranes to fuse. Furthermore acidification of the interior of the virion through the M2 protein ion channel causes the M1 protein to dissociate from the RNP and the RNP is released into the cytoplasm. The RNP then enters the nucleus through nuclear pores (Mikulasova *et al.*, 2000). A similar method of entry has been described for ISA virus (Eliasson *et al.*, 2000).

Once in the nucleus replication of the virus begins. First, the viral genome (vRNA) acts as a template for the synthesis of mRNAs that are translated into viral proteins. Transcription of viral mRNAs takes place using a viral cap snatching mechanism where caps from newly synthesized host cell mRNAs are cleaved by the viral polymerase complex and used to initiate transcription of viral mRNA from vRNA. Viral mRNA transcription is terminated at a sequence of 5-7 uridines 16-17 nucleotides from the 5' end of the vRNA. Therefore viral mRNAs lack a portion of

sequence that corresponds to roughly 20 nucleotides on the 5' end of the vRNA. Second, the vRNA itself is copied into cRNA which is a full-length copy of the vRNA and it is not capped or polyadenylated. The cRNA is transcribed without a capped primer creating full-length copies of the vRNA thus successfully replicating the genome. RNP's are exported from the nucleus and assemble with M, HA, and NA proteins in the plasma membrane where the fully assembled virus buds from the host cell membrane. Through the use of mutants, variants, and in vitro mutagenesis specific biochemical functions of each gene segment have been defined (Brown, 2000). Each gene segment and product will be briefly discussed.

All the vRNA of influenza A virus contains conserved nucleotides at both the 5' and 3' ends (Mikulasova *et al.*, 2000). Each conserved region is 12-13 nucleotides in length and is untranslated. The conserved nucleotides at the 5' and 3' end are partially complementary and are thought to form panhandle structures. It has been demonstrated that the conserved ends are important viral polymerase promoter sites (Mikulasova *et al.*, 2000). The mRNA polyadenlyation signal for orthomyxoviruses is a stretch of 5-7 uridines 15-22 nucleotides downstream from the 5' end of the vRNA (Li and Palese, 1994). Similar conserved sequences have been described for ISA virus segments 2, 3, 6, and 8 (Krossoy *et al.*, 1999, 2001; Sandvik *et al.*, 2000; Rimstad *et al.*, 2001; Snow and Cunningham, 2001; Sandvik *et al.*, 2000).

Gene segments 1, 2 and 3 of influenza A correspond to the polymerase complex proteins PB2, PB1, and PA respectively. Most notably the PB1 protein contains four RNA-dependent RNA polymerase motifs that are conserved among RNA-dependent RNA polymersases. The RNA-dependent RNA polymerases

function to catalyze the addition of nucleotide triphosphates to elongating RNA chains (Brown, 2000). The putative PB1 RNA-dependent RNA polymerase of ISA virus has been described and contains the four conserved motifs found in all viral RNA-dependent RNA polymersases (Krossoy *et al.*, 1999). PB2 binds to host cell mRNA caps which are then used to prime viral mRNA transcription. PA has not been assigned any specific role in the replication cycle (Mikulasova *et al.*, 2000).

RNA segment 4 in influenza A virus corresponds to the HA protein. The HA protein is an integral membrane protein and a major virion glycoprotein that binds sialic acid on the host cell and determines host cell specificity. The HA is also a major antigenic protein. After the HA binds sialic acid on the host cell the virus is endocytosed. Inside the host endosome the low pH induces a conformational change in HA resulting in two subunits HA1 and HA2 that are connected by disulfide linkages (Webster *et al.*, 1992). Cleavage of the HA is required for infectivity since the amino terminus of HA2 fuses the host cell endosomal membrane facilitating viral entry into host cells. Influenza growth is inhibited by raising the pH of host cell endosomes using weak bases such as ammonium chloride, chloroquine, or amantadine as the high pH of the endosome inhibits cleavage of HA and selects for mutants with a HA capable of being cleaved at a higher pH (Brown, 2000). ISA virus has been shown to possess haemagglutinin (HA) activity and the viral gene responsible for this activity has been characterized (Krossoy *et al.*, 2001). The HA ISA virus gene was isolated from a cDNA library by immunoscreening positive clones. The HA gene contained an ISA virus specific 5' conserved region and a 1167 bp open reading frame encoding a polypeptide with a predicted molecular mass of

42.4 kDa that corresponds to an equivalent protein found in SDS-PAGE analysis of infected cells. Expression of the recombinant HA protein in insect cells resulted in the hemagglutination of salmon erythrocytes while addition of ISA virus-specific monoclonal antibody or a polyclonal rabbit serum inhibited hemagglutination of erythrocytes. Sequence analysis of the HA gene from five Norwegian isolates revealed a highly polymorphic region that may be useful in epidemiological studies (Krossoy *et al.*, 2001). Further characterization of a Scottish and Norwegian isolate from an independent lab confirms this data (Rimstad *et al.*, 2001). The HA proteins of orthomyxoviruses are known to contain carbohydrate side chains that affect the functionality of the protein (Keil *et al.*, 1984).

The carbohydrate side chains of the influenza A virus hemagglutinins are attached by N-glycosidic linkages. Complex type I, and mannose-rich type II as well as hybrid side chains are present. The three oligosaccharide types found on the mature HA differ from each other in completeness of processing. Evidence has shown that HA carbohydrates protect the peptide from proteolytic degradation and are involved in host cell receptor binding. The carbohydrate pattern of the virus is dependent on the host cell as well as the correct glycosylation sites Asn-X-Thr(Ser) and it has been shown that strain specific variation of glycosylation is determined by primary peptide structure. The amino acids tryptophan, proline and aspartic acid have been observed to interfere with glycosylation when present at the variable region of the glycosylation site (Keil *et al.*, 1984). Oligosaccharides found at variable sites in the same molecule have been shown to modulate antigenic properties of the protein (Klenk, 1990; Munk *et al.*, 1992; Skehel *et al.*, 1984), receptor binding (Robertson *et*

al., 1987), and proteolytic activation (Kawaoka *et al.*, Kawaoka & Webster, 1989; Ohuchi *et al.*, 1989; Ohuchi *et al.*, 1991). The significance of the carbohydrates at these putative sites on the HA of ISA virus is unknown but it is has been suggested that the differences may contribute to differences in the pathogenicity of different ISA virus strains.

Gene segment 5 of influenza A corresponds to the nucleoprotein (NP). The NP protein functions in virus assembly and RNA synthesis. NP contains two nuclear localization signals. The RNA binding site is found at the amino terminal 181 amino acid end of the protein (Brown, 2000). The NP is the major structural protein that interacts with the RNA segments to form the RNP. The NP protein is rich in arginine residues and has a net positive charge of +14 at pH 6.5. The NP is phorsphorylated but the extent is not known. NP is targeted to the nucleus and there are several homologous regions with the NP of influenza A and B viruses. A gene encoding the putative nucleoprotein (NP) of ISA virus has been identified (Snow *et al.*, 2000). The full-length open reading frame (ORF) of 1851 nuceotides identified a predicted protein of 616 amino acids. There was no significant sequence homology to other orthomyxovirus nucleoproteins in BLAST or FASTA databases. The gene contained the conserved sequence 5' GCAAAGA 3' preceding the ORF which has been identified in all other ISA virus genes (Snow *et al.*, 2000).

Segment 6 of influenza A corresponds to the neuraminidase (NA) protein. The NA protein is an integral membrane protein and is a major antigenic determinant that undergoes antigenic variation. This protein functions as a homotetramer to cleave sialic acid residues to release budding virions from the host cell membrane. This

enzyme may also be important in releasing virions from sialic acid containing inhibitors present in mucus membranes. Antibodies to NA prevent the release of the virus (Brown, 2000). The NA protein contains one hydrophobic domain that spans the lipid bilayer near the N-terminus of the protein. There is no evidence of post-translational cleavage of the NA polypeptide, the signal peptide is not removed and the initiating methionine is retained. A sequence of 6 amino acids is conserved in influenza A at the N terminus. This sequence is followed by a hydrophobic stretch that is probably the transmembrane domain of the NA stalk. This sequence does not appear to be conserved except for its hydrophobicity. Identity between N1 and N2 subtypes is estimated to be 39% in influenza A virus (Laver *et al.*, 1984). Influenza B gene segment 6 in addition to the ORF that encodes NA also contains a second ORF encoding the NB protein. Influenza B virus NA is synthesized from the second AUG initiation codon from the 5' end of the mRNA while the NB protein is synthesized from the first AUG initiation codon from the 5' end of the mRNA. NB is an integral membrane glycoprotein that is expressed at the plasma membrane of influenza B infected cells.

Segment 7 in influenza A encodes two proteins that are expressed via alternative splicing. The unspliced mRNA encodes the 252 amino acid matrix protein (M1). This protein is the most highly conserved in influenza A indicating evolutionary pressure to maintain function. It lies inside the lipid membrane and is the most abundant polypeptide in the virion. The M2 protein is 97 amino acids long and is a spliced transcript of segment 7. M2 is a minor component of the virion in terms of abundance but functions as an ion channel that transports protons into

virions during uncoating. M1 and M2 share the first 9 amino acids. After removal of the intron M2 is read in the +1 reading frame. The story is somewhat different for influenza B virus gene segment 7. The gene contains two overlapping reading frames suggesting that this gene is not spliced as with influenza A. Influenza C RNA segment 6 M1 protein is encoded by a single ORF which mRNA splicing introduces a stop codon after 242 residues. The matrix protein underlies the lipid envelope and supplies rigidity to the membrane. It is also believed that the matrix protein interacts with the cytoplasmic tails of HA, NA and M2 proteins. Interactions between the M1 protein and RNA have been demonstrated. The M1 protein also contains a zinc binding motif (cys-cys-his-his-type) and the domain is conserved in influenza A and B virus M1 proteins (Fields, B.N., 1996). Gene segment 8 of influenza A, B and C viruses encode two proteins. Influenza A NS1 protein is a 230 amino acid protein from unspliced segment 8 mRNA while NS2 is a 121 amino acid protein that contains the first 9 amino acids of NS1. NS2 differs as a 473 nt intron is excised. After the splice NS2 is read in the +1 reading frame. NS1 protein can be found in influenza virus infected cells but has not been detected in virions hence the designation non-structural. NS1 is also a phosphoprotein that contains two nuclear localization signals. NS1 has been shown to inhibit the interferon pathway. The role of NS2 is unclear, it was believed to be a nonstructural protein but now it is thought to be present in virions and form an association with the M1 protein.

Defective Interfering Particles

The repeated high-multiplicity passage of influenza virus results in the production of defective-interfering (DI) particles (Huang, 1988). Animal studies have

shown that a lethal dose of virus in mice inoculated at the same time with a preparation of DI particles leads to increased survival. Early investigations using embryonated eggs showed that the undiluted passage of influenza virus resulted in a high hemagglutinin to infectivity ratio where DI particles appear to interfere with standard viral replication (Huang, 1988). Most of influenza virus DI particles contain both the 5' and 3' end of the gene segment. The gene segment of origin is usually one of the polymerase genes from which the DI particle is derived from a single or multiple internal deletions. It is speculated that the host cell as well as viral strain may determine the amount and effect of DI particle abundance as it may provide a replicative advantage for the virus. With the repeated passage of virus larger DI RNAs disappear and smaller DI RNAs are formed. DI RNAs are capable of forming polyadenylated mRNA transcripts (Chambers *et al.*, 1984).

Molecular Evolution of Orthomyxoviruses

Orthomyxoviruses are segmented RNA viruses of negative polarity. Due to the segmented nature of the genome orthomyxoviruses have the ability to evolve rapidly. The influenza viruses are divided into types A, B and C according to the serological relatedness of their internal proteins. All influenza A viruses cross react serologically with nucleocapsid and M proteins. The influenza virus types are further divided into subtypes and strains according to serological differences of the surface glycoproteins hemagglutinin and neuraminidase. There are several mechanisms contributing to the evolution of these viruses. New strains of influenza virus can be produced by reassortment or the inclusion of different gene segments from different parental virus strains during co-infection of host cell with multiple

influenza strains. This event has been termed antigenic shift as novel arrangements of virus can create new antigenic epitopes which can lead to new pandemic strains. Viruses also evolve through point mutation and selection knows as antigenic drift as antigenic changes are not usually as dramatic. There are 14 HA and 9 NA subtypes which are found in all possible combinations in nature (Scholtissek. 1996). Mutation rates of influenza viruses as with other RNA viruses are high. The lack of proofreading mechanisms in the RNA polymerases results in replication errors on the order of 1 in 10^4 bases compared to 1 in 10^9 bases for high fidelity DNA polymerases (Webster *et al.*, 1992). The mutation rate for the European isolate of ISA virus segment 2 was estimated at 0.96×10^{-3} nucleotides site^{-1} yr^{-1} (Nylund *et al.*, 2000). The selection pressure on the virus ranges from the immune system of the host to tissue culture systems employed to propagate virus. Each virus gene may evolve differently due to selective pressures and evolutionary constraints. Genes coding for the surface glycoproteins HA and NA proteins are subject to strong selection pressure by neutralizing antibodies of the host immune system. Therefore these genes may evolve more rapidly and be replaced by reassortment more frequently. Genes encoding internal proteins such as the NP may not be subject to strong host immune selection, but do evolve host specificity. Polymerase genes appear to be more conserved as specific viral constraints may be imposed on the function of the protein. Furthermore conservation of functional polymerase genes would not affect the fitness of reassortment viruses (Webster *et al.*, 1992).

The quasispecies concept of evolution suggests that the high mutation rate of RNA viruses results in populations of multiple genetic variants. In terms of the

strong selection pressure from host immune systems a rare antigenic variant within the population may be successful in replication and be positively selected. This explanation appears to account for the influenza A HA gene evolution as a strain with a particular HA variant will spread through a population until immunity develops. This is probably true for ISA virus as evidence for a hypervariable region in the HA gene indicates differences between strains. The neutralist theory of evolution states that most of the evolutionary changes that take place are neither beneficial nor deleterious but rather neutral in terms of fitness of the organism. This also appears to be true for the influenza genome as many neutral changes are fixed in the genome as seen by the many variants within gene segments in nature (Smith and Palese, 1988).

Infectious salmon anemia (ISA) is caused by an unclassified virus most closely related to members of the Orthomyxoviridae (Krossoy et al., 1999). The primary tissue tropism is endothelial cells of the vascular system of some salmonid fish species (Hovland et al., 1994). Atlantic salmon (Salmo salar) are particularly susceptible to the virus with infection resulting in overt signs of disease and mortalities within farm populations ranging from none to 100% after several months. ISA viral particles are pleomorphic enveloped spheres 90-130 nm in diameter (Dannevig et al., 1995; Nylund et al., 1995). They are covered evenly with 13-15 nm surface spikes and contain filamentous nucleocapsid-like structures that are released upon partial virion disruption (Sommer & Mennen, 1996).

The virus hemagglutinates a variety of fish cells but not erythrocytes from mammals and birds (Falk et al., 1997). The virion contains an

acetylesterase receptor-destroying activity that does not affect influenza A or C hemagglutination suggesting that the receptors are different for the viruses. Recent evidence shows that ISA virus is similar to orthomyxoviruses in that it binds to sialic acid residues on host cell surfaces and undergoes fusion with the cell in acidic endosomes (Eliassen et al., 2000). The hemagglutinating and the acetylesterase activities seem to be carried out by two different proteins in the ISA virus (Rimstad et al., 2001).

Purpose and Scope of Study

The ISA virus genome is composed of eight segments of single-stranded, negative-polarity RNA. The genes encoding the putative PB1, NP, PA, HA and NS proteins have been described. However, definitive correlation of each gene to their corresponding genomic segment and to their encoded protein product has not been made. Analysis of the nucleotide sequences encoding the putative NS and PB1 shows that a minimum of two distinct genomic strains of ISA virus exists: the North American and European strain (Inglis et al., 2000). However, these gene sequences cannot be used to differentiate between the various European strains. Instead, the highly polymorphic region in the putative HA is used to identify and separate closely related species (Krossoy et al., 2001). Further resolution of the ISA virus genome and its' organization is an important step toward understanding the relationship between the individual virus isolates. Towards this goal, we describe here the genome structure of ISA virus isolate CCBB. The antigenic variation of ISAV has not been

clearly defined and it is not known whether cross-neutralizing fish immune response can be elicited. To address this, we have identified ISA virus proteins which are immunoreactive in Atlantic salmon. Together, these results are discussed for their impact on rational vaccine design.

Chapter 2

METHODS

Virus and Cell Lines

ISA virus strain CCBB was isolates in 1998 by Micro Technologies, Inc. (Richmond, ME) from infected Atlantic salmon in Back Bay, New Brunswick, Canada. Chinook Salmon Embryo (CHSE-214) cells obtained from Micro Technologies, Inc. (Richmond, Maine) were used for the propagation of ISA virus isolate CCBB. Cells were grown at 15 °C in minimum essential medium (MEM) containing Hank's salts and supplemented with L-glutamine (10 mM) and 5% fetal bovine serum (FBS; Gibco BRL; MEM-H5).

Virus and RNA Purification

Virus was prepared from ISA virus-infected CHSE-214 cell monolayers. Following complete cell lysis, the cell culture supernatant was filtered through a sterile 0.45 micron filter to remove extraneous cell debris. After dialysis against polyethylene glycol (PEG 8000; Sigma) to reduce the volume, the cell culture supernatant was centrifuged for two hours at 104,000-x *g* using a Beckman SW28 rotor and a Beckman L8-70M ultracentrifuge. The pelleted virus was resuspended in 1 ml TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA pH 7.5), layered on a 25, 35 and 45% discontinuous sucrose gradient and centrifuged for 3 hours at 132,000 x *g*

using Beckman SW28 rotor. Virus at the interface of the 35 and 45% sucrose layers was collected, resuspended in TNE and centrifuged for two hours at 104,000 x *g*. Viral RNA was isolated from the pelleted virus using Trizol (Gibco) as described by the manufacturer and then used to construct cDNA libraries.

cDNA Library Construction

Two different strategies were employed to clone the ISA virus genome. Approach 1: A common feature in the genome of orthomyxoviruses is the conserved sequence at the 3' end of viral RNA (Krossoy *et al.*, 1999). This characteristic was incorporated into the design of an ISA virus-specific primer: 5'AAGCAGTGGTAACAACGCAGAGT**AGCAAAGA** -3'. The region in bold is complementary to the 3' termini of ISA virus RNA (vRNA). The sequence in plain text is random and will non-specifically bind to the vRNA. First strand cDNA was synthesized from ISA virus RNA by reverse transcription with the ISA virus oligonucleotide. RNA (100 ng) isolated from purified ISA virus or CHSE-214 cells (control) was mixed with 1 of µl ISA virus primer (20 pmol/µl) in a total of 10 µl, incubated at 80 °C for 5 min and then combined with the following in a total of 20 µl: 4 µl of 5x first strand buffer (Gibco), 2 µl of 10 mM dNTP mix (Boehringer Mannheim), 1 µl 0.1 M DTT (Gibco) and 1 µl of Superscript II reverse transcriptase (15 U/µl Gibco). The mixture was incubated at 25 °C for 10 min and then at 42 °C for 1 hr.

The first strand ISA virus cDNA products synthesized by reverse transcription were PCR amplified using the ISA virus primer and random hexamers. To the first strand reaction, the following components were added in a total of 100 µl: 1.5 µl of

10 mM dNTP mix (Boehringer Mannheim, 1.25 µl of ISA virus primer (20 pmol/µl), 1 µl of random hexamers (25 pmol/µl; Gibco), 10 µl of 10x PCR buffer with Mg^{2+} (Boehringer Mannheim), 1 µl of Taq (5 U/µl; Boehringer Mannheim). After 35 cycles of 94 °C for 30 sec, 59 °C for 45 sec and 72 °C for 1 min, the PCR products were extended for 10 min at 72 °C.

The amplified cDNA products were separated by agarose gel electrophoresis, gel purified and then cloned into the pGEM-T vector as described by the manufacturer (Promega). *E. coli* DH5α (Gibco) was transformed with the ligation reactions and the ampicillin resistant colonies containing pGEM-T with cloned ISA virus cDNA were selected by blue/white screening. The white colonies were transferred to 96 well plates containing 200 of µl LB/ampicillin (250 µg/ml)/15% glycerol per well, grown overnight at 37 °C and stored at –20 °C.

Approach 2: First strand cDNA synthesis by reverse transcription results in double stranded products composed of one RNA strand and one cDNA strand. One method of synthesizing the second cDNA strand involves selectively replacing the RNA strand with DNA. This strategy was used in construction of the second ISA virus library.

First strand cDNA was synthesized from ISA virus RNA by reverse transcription with random hexamer primers. RNA (100 ng) isolated from purified ISA virus or CHSE-214 cells (control) was mixed with 1 µl of random hexamers (50 ng/µl; Gibco) in a total of 10 µl, incubated at 65 °C for 5 min, placed on ice for 2 min and then combined with the following in a total of 20 µl: 4 µl of 5x first strand buffer (Gibco), 2 µl of 10 mM dNTP mix (Boehringer Mannheim), 1 µl of 0.1 M DTT

(Gibco) and 1 µl Superscript II reverse transcriptase (15 U/µl; Gibco). The mixture was incubated at 25 °C for 10 min and then at 50 °C for 50 min.

Second strand synthesis was performed using the TimeSaver cDNA synthesis kit (Pharmacia). The first strand reaction was added to the second strand reaction mix, incubated at 12 °C for 30 min and then at 22 °C for 1 hr. After spin column purification, the blunt ended, double stranded cDNA were cloned into dephosphorylated, *Sma*I digested pUC18 (Pharmacia) as outlined by the manufacturer. *E. coli* DH5α (Gibco) was transformed with the ligation reactions and the ampicillin resistant colonies containing pUC18 with cloned ISA virus cDNA were selected by blue/white screening. The white colonies were transferred to 96 well plates containing 200 µl of LB/ampicillin (250 µg/ml)/15% glycerol per well, grown overnight at 37 °C and stored at –20 °C. Clones from the library were provided by Dr. Clouthier.

Selection and Identification of ISA Virus Clones from the cDNA Libraries

The contents of one 96 well plate were transferred to one Hybond N^+ membrane (Amersham) placed on top of an LB agar plate containing ampicillin (250 µg/ml). Clones were grown on the filters at 37 °C overnight and the filters were processed on soaking pads saturated with the following solutions: 0.5 N NaOH (7 min); 1 M Tris-HCl pH 7.4 (2 min); 1 M Tris-HCl pH 7.4 (2 min); 0.5 M Tris-HCl pH 7.4, 1.5 M NaCl (4 min). The filters were transferred to a bath of 2x SSC (1x SSC is 0.15 M NaCl, 0.015 M Na_3 citrate), 1% sodium dodecyl sulfate (SDS) to remove the cellular debris and then soaked in 2x SSC to remove residual SDS. After a brief wash in chloroform, the filters were air dried and then baked at 80 °C for 2 hrs.

Prehybridization of the filters for 2 hr in 6x SSC, 0.5% SDS, 5x Denhardt's and 0.1mg/ml of *E. coli* tRNA (Sigma) was followed by hybridization with a probe labelled with [α^{32}P] dCTP by nick translation. Nick translation was performed as outlined by the manufacturer (Amersham). Probes consisted of gel purified DNA that had been restriction enzyme digested from the plasmids of randomly selected library clones. Library clones were grouped based on the probe to which they hybridized. Clones were selected by Dr. Clouthier.

Preparation of DNA for Nucleotide Sequence Analysis

Plasmid DNA isolated from representative clones of each group was sequenced at the University of Maine Core Sequencing Facility. The plasmid templates were prepared using Qiaprep columns (Qiagen). The sequences were analyzed by BLAST searches through the National Center for Biotechnology Information server. Only those sequences that matched other orthomyxovirus sequences or that did not match non-viral sequences were analyzed further.

Northern Blot Hybridization

Northern blot analysis was used to correlate each representative sequence with a specific ISA virus genomic segment. Total RNA was isolated from CHSE-214 cell monolayers or CHSE-214 cell monolayers infected with ISA virus using Trizol (Gibco) as outlined by the manufacturer. The RNA was separated on a 2% agarose gel containing formaldehyde and transferred onto Hybond N^+ membrane (Amersham) in 10x SSC by capillary action as described in Fourney *et al.* (1992). The probes used for Northern blot analysis were gel purified, restriction enzyme fragments digested from the plasmids of appropriate cDNA library clones. The probes were labelled

with [α^{32}P]dCTP (NEN) by nick translation (Amersham) and hybridized to the blots at 42°C for 18 hr in ULTRAhyb™ (Ambion). The membranes were washed for 2 x 5 min in 2X SSC-0.1% SDS at 42 °C and then for 2 x 15 min in 0.1X SSC- 0.1% SDS at 42 °C. The results were recorded on Kodak X-OMAT AR film.

Construction of Full-Length clones of Each ISA Virus Genome Segment

Full-length sequence for each of the ISA virus-specific clones was generated by rapid amplification of cDNA ends (RACE) PCR using the RLM-RACE kit (Ambion). The 5' and 3' primers used are listed in Table 1. The PCR products were cloned into either pCR®2.1-TOPO® or pGEM-T as directed by the manufacturers (Invitrogen or Promega, respectively) and then sequenced.

Table 1. 5' and 3' RACE primers used to construct ISA virus CCBB gene segments

Primer Name	Length	Tm	Sequence	Gene segment amplified
2C51	20	57	tcaacacaaaaccactggag	4
2C52	19	56.7	cttgattcgtctccagtgg	4
2B102	22	57.1	tggcttctttcctgtcggactc	7
2B101	22	55.2	caggaacctttgagtccgacag	7
4D82	20	55.8	acgcaggtgaatgatgccag	4
4D81	23	57	tggagggcaatgaggttagacag	4
5E71	18	57	ttgtggtggtggtgtttg	5
5E72	22	57.9	ccgacatttcaccttctaagac	5
5E6L	20	60	ggtagcatgattgggaccac	1
5E6R	20	59.9	cagttttgctttgccctctc	1
1-12L	20	59	acttctccaaaaggcattcg	3
1-12R	20	59	actgccccagacactcttgt	3
5E6Aint	20	60.4	catctgtgtaccttggatgg	1
5E7fullf	22	55.1	agttaaagatggcttttctaac	5
5E7fullR	28	54.7	ctatttatacaattaataatgcataatc	5
5E65prime	21	58.7	cgttgttaccactgcttaatc	1
5prime5E6CNSS	20	62.2	cactgtctcccaccaaactc	1
4D8consF	20	61.9	caagatggataacctccgtg	4
4D8consR	22	57.5	tacatatcgtcaaacacacaac	4
ISA2Cf	24	61.9	gaagactacttgccttaccagatg	4
ISA2Cr	22	68	tactccccacaacaccccaatc	4
Haforward	19	62	agcaaagatggcacgattc	6
Hareverse	24	61	tgcacttttctgtaaacgtacaac	6

Generation of Anti-ISA Virus Immune Sera

Anti-ISA virus antibodies were generated in Atlantic salmon injected with tissue culture supernatant from ISA virus-infected CHSE cell monolayers (Opitz *et al.*, 2000). Mouse polyclonal and monoclonal antibodies (mAbs) to ISA virus were generated by Rob Beecroft (Immuno-Precise Antibodies Ltd.) in BALB/c mice immunized by intraperitoneal injections with purified ISA virus. Fusion of mouse spleen cells with X63-Ag8.6.5.4 BALB/c parental myeloma cells and cloning of hybridomas were performed as previously described (Richardson *et al.*, 1986). Hybridoma tissue culture supernatants were screened against both ISA virus and CHSE cells dried onto the wells of standard ELISA plates. Atlantic salmon sera was provided by Dr. Clouthier.

SDS-PAGE and Western Blot Analysis

Whole cell lysates of naïve and ISA virus-infected CHSE cells as well as purified ISA virus were screened for the presence of immunoreactive antigens with sera from vaccinated and challenged Atlantic salmon. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (1970). Proteins were solubilized with SDS-PAGE sample buffer and separated by SDS-PAGE on 5% stacking gel and 12% resolving gel. Immunoreactive protein bands were visualized by Western blot analysis. Briefly, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (Bio-Rad Laboratories). The membranes were blocked with 3% skim milk buffer and then incubated with sera from vaccinated and challenged Atlantic salmon followed by incubation with mouse

anti-salmonid immunoglobulin 5F12 mAb (Immuno-Precise Antibodies Ltd.). Alternatively, the proteins on the membranes were screened with anti-ISA virus mouse polyclonal and monoclonal antibodies. Immunoreactive proteins were detected with goat anti-mouse immunoglobulin G-alkaline phosphatase conjugates (Southern Biotechnology Associates, Inc) and visualized with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium (Sigma). SDS-PAGE and Western Blot analysis was provided by Dr. Clouthier.

Computer Analyses

AssemblyLIGN 1.0.9b (Oxford Molecular Group) was used to order the overlapping sequenced DNA fragments for the construction of full-length clones. The programs contained in MacVector™ 6.5.3 (Oxford Molecular Group) were used to identify open reading frames and regions of local similarity. The nucleotide and predicted amino acid sequence for each open reading frame were analyzed by BLAST searches through the National Center for Biotechnology Information server (Altschul *et al.*, 1990; Pearson & Lipman, 1988) or the Influenza database (Los Alamos National Laboratory). The most likely cleavage sites for signal peptidase in HA and 5:E-7 were determined using SignalP V1.1 (Nielsen *et al.*, 1997). Transmembrane helices were predicted using TMHMM-1.0 http://www.cbs.dtu.dk/services/TMHMM-1.0/.

N-terminal Amino Acid Sequence Analysis

The proteins of purified ISA virus were separated by SDS-PAGE, blotted onto PVDF membrane (BioRad) and stained with 0.1% Coomassie blue R-250 in 40% methanol/1% acetic acid. The stained protein bands were cut out of the membrane and subjected to N-terminal amino acid sequence analysis using an Applied

Biosystems model 470A gas-phase sequencer or a model 473 liquid-phase sequencer with on-line phenylthiohydantoin analysis. N-terminal amino acid analysis was provided by Dr. Clouthier.

Chapter 3

RESULTS

Genetic Organization of ISA Virus

The genetic organization, size and nucleotide sequence of the CCBB ISA virus isolate was determined. To accomplish this, two cDNA libraries were made using ISA viral genomic RNA from purified virus and were screened using randomly chosen cDNA sequences from each library. Eight distinct cDNA hybridization groups were identified. The DNA from a representative clone from each group was sequenced and then used to perform Northern blot hybridization on total RNA from naïve or ISA virus-infected CHSE cells (Fig. 1). RNA segments visible by Northern blot hybridization were correlated to the proper nucleotide sequence by consecutively probing one RNA blot with all eight probes. The specificity of each cDNA clone and the migration of the corresponding RNA segment was confirmed by the same method using RNA isolated from purified ISA virus. Eight RNA segments were identified with segments 1 and 2 comigrating at 2.4 kb. The ISA virus RNA segment corresponding to each cDNA clone is summarized in Table 2. The genome segments are numbered with respect to mobility in agarose gels from slowest to fastest and comprise a genome of 14.3 kb.

The cDNA sequence of each ISA viral RNA segment was predicted to contain one open reading frame with the exception of segments 7 and 8 each of which

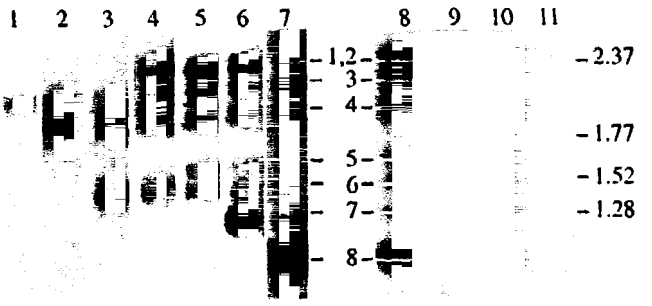

Figure 1. Northern blot analysis. Northern blot analysis of total cellular RNA from ISA virus infected CHSE-214 cells, lanes 1-8 are indicate addition of a ISA virus probe corresponding to each genomic segment of ISA virus. Lanes 9 and 10 are duplicates of lane 8 and the numbers internal to the pictures represent each genome segment, segments 1 and 2 co-migrate. The RNA mass references can be seen to the far right..

Table 2. Summary of groups formed from screening ISA virus cDNA libraries. Provided by Dr. Clouthier.

[1] Library 3 has a total of 768 clones

[2] Library 5 has a total of 1364 clones

Probe	Number of positive clones	
	cDNA Library 3[1]	cDNA Library 5[2]
5:E-6	33	DNP
PB1	41	0
NP (1-1#2; 5-1#1)	6; 43	212; 1144
2:C-5; 4:D-8	5; 38	DNP
5:E-7	14	DNP
2:B-10	50	DNP
NS	10	6
CHSE	100	DNP

encoded two proteins. The length of each gene and the corresponding encoded polypeptide(s) and the predicted molecular weights of the translated proteins are summarized in Table 3.

Comparison of the cDNA nucleotide and predicted amino acid sequences of the open reading frames to those listed in the GenBank and Influenza databases showed that RNA segments 1, 4, 5 and 7 of ISA virus isolate CCBB were unique. Using similar analyses, RNA segments 2, 3, 6 and 8 were found to encode PB1, NP, HA and NS, respectively.

ISA Virus Proteins

Purified ISA virus proteins were separated by SDS-PAGE and visualized by Coomassie blue staining (Fig. 2). SDS-PAGE analysis of purified ISA virus CCBB revealed seven putative ISA virus proteins with estimated molecular masses between 25-72 kDa (Fig. 2). The four prominent proteins were 72, 47, 42, and 25 kDa. N-terminal amino acid sequence analysis was successful with three of the seven proteins (Table 4).

N-terminal amino acid sequence analysis was used to correlate viral proteins with the predicted translation of open reading frames encoded by RNA segments of ISA virus isolate CCBB.

The identity of immunoreactive polypeptides encoded by the RNA segments of ISA virus isolate CCBB were determined by Western blot analysis performed on naïve and ISA virus-infected CHSE-214 cells (Fig. 3). Sera, collected from Atlantic salmon injected with tissue culture supernatant containing live ISA virus, reacted with the 72 and 42 kDa proteins of ISA virus (Fig. 3A). Purified ISA virus was used to

Table 3. Gene segments and predicted proteins of ISA virus CCBB. Provided by Dr. Clouthier.

[1] Based on the average length determined from Northern blot analysis with 2-5 replicates per probe.

[2] Inferred analogues from Influenza A, B and C genome designations.

Seg-ment	Clone	Length of segment[1] (kb)	Length of ORF (bp)	Encoded protein[2]	Nascent poly-peptide length (aa)	Molecular weight predicted (kDa)
1	5:E-6	2.4	NA	P1	NA	NA
2	PB1	2.4	2245	PB1	708	80.5
3	1-1#2; 5-5#1	2.2	1851	NP	617	68.0
4	2:C-5/4:D-8	1.9	1737	P2	579	65.3
5	5:E-7	1.6	1353	P3	451	49.8
6	HA	1.5	1185	HA	395	43.1
7	2:B-10	1.3	771	P4	257	28.6
			441	P5	147	16.3
8	NS	1.0	705	P6	235	26.5
			552	P7	184	20.3

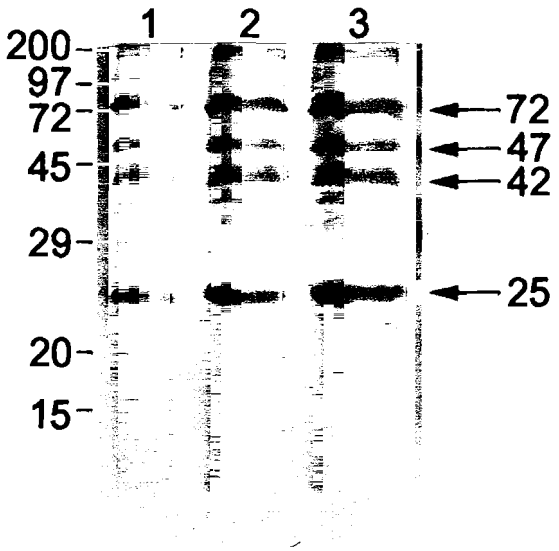

Figure 2. SDS-PAGE of purified ISA virus. Purified virus lanes 1, 2 and 3. Arrows indicate the predominate proteins found in purified virions and the molecular mass in kDa. Provided by Dr. Clouthier.

Table 4. N-terminal amino acid sequence analysis of ISA virus proteins. Table indicates protein molecular weight (MW), the determined amino acid sequence (sequence analysis), and identity of protein based on translated gene sequence data. Provided by Dr. Clouthier.

Protein Size (kDa)	Sequence analysis	Similarity analysis
40	RLXLRNHPDTTWIGDSRSDQSRXNQ (N-terminal sequence)	HA
42	Same N-terminal sequence as 40 kDa protein	HA
47	EPXIXENPTXLAI (N-terminal sequence)	Segment 5

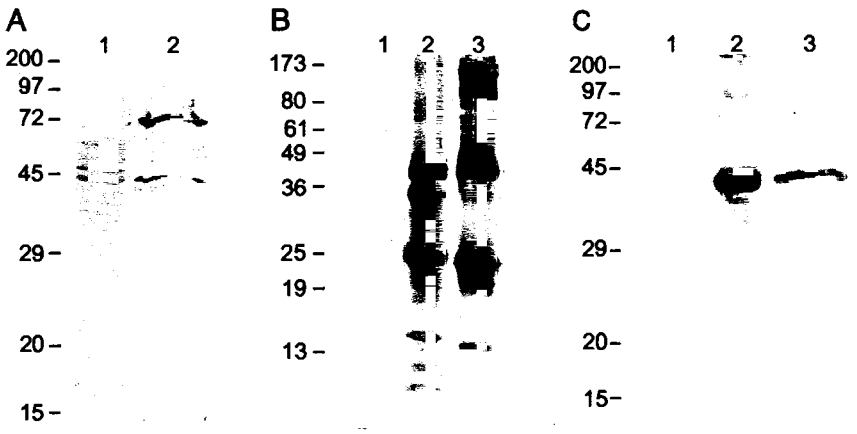

Figure 3. Western Blot of ISA virus proteins. Sera from fish challenged with ISA virus react with a 72 kDa and 42 kDa protein in CHSE-214 cells that were infected with ISA virus (3A lane 2) and is unreactive in unifected CHSE-214 cells (3A lane 1). Mouse polyclonal antibodies recognize 3 proteins in purified virus (3B lane 3) and six proteins in cellular extract (3B lane 2) and is unreactive in uninfected cells (3B lane 1). Mouse monoclonal antibodies only recognize a 42 kDa protein in purified ISA virus (3C lanes 2 and 3) lane 1 is a negative control. Provided by Dr. Clouthier

generate ISA virus-specific mouse polyclonal and monoclonal antibodies. Of the six immunoreactive proteins present in the cellular preparation of ISA virus and recognized by the mouse polyclonal sera, three were present in the purified ISA virus sample (42, 25 and 15 kDa; Fig. 3B). Only the 42 kDa protein was recognized by the monoclonal antibody (Fig. 3C). For each serum tested, no reaction was observed with the naïve CHSE sample indicating that the immunoreactive proteins were derived from ISA virus.

Complementary DNA (cDNA) Libraries

The results of cDNA libraries are listed below in table 3. The library using RNA from purified ISA virus followed by reverse transcription with random primers resulted in cloning all ISA virus CCBB gene fragments.

Northern Blots

Clones from both libraries that were sequenced and did not match known genes in genbank and that were suspected to be of viral origin were used for Northern blot analysis. Northern blotting resulted in grouping ISA virus positive clones into gene segment groups and allowed relative sizes of the gene segments to be determined. Each gene segment of ISA virus CCBB was used to probe ISA virus infected and uninfected CHSE-214 cells. Figure 1 shows seven distinct bands from eight different gene segment probes on the same Northern blot. Probes for segment one and two co-migrated at 2.4 kb and appear as a single bands. None of the bands present in ISA virus infected cells are present in uninfected cells (Figure 1, lane 4).

ISA Virus CCBB Genome

Using Northern blot analysis and 5' and 3' RACE the genetic organization of ISA virus CCBB was determined. Table 4 briefly describes characteristics of each gene segment for ISA virus CCBB.

Segment 1

The partial gene coded by segment 1 of ISA virus isolate of CCBB is listed in the appendix. A translated protein has not been offered since the full-length transcript is not fully described here.

Segment 2

Segment 2 of ISA virus isolate CCBB contains a 2245 bp ORF encoding a 708 amino acid polypeptide with an estimated molecular mass of 80.5 kDa (Table 3). The predicted amino acid sequence of PB1 encoded by RNA segment 2 was 82.2% and 84.5% similar to the predicted translation of the PB1 proteins from Norwegian and Scottish (AJ002475, AF262392, respectively) isolates of ISA virus (Table 5). The complete mRNA and predicted translation is in the appendix.

Segment 3

Segment 3 of ISA virus isolate CCBB contains an 1851 bp ORF encoding a 617 amino acid polypeptide with an estimated molecular mass of 68.0 kDa (Table 3). The assignment of NP to the open reading frame on RNA segment 3 was based on nucleotide sequence similarity to the influenza A RNA binding region (data not shown) and to the NP sequence described by Snow & Cunningham (2001). The predicted protein sequence for CCBB ISA virus NP was highly conserved, sharing

Table 5. Percent comparisons of HA, NP and NS from ISA virus strain CCBB to those from other ISA virus isolates. Provided by Dr. Clouthier.

ISA virus strain and accession number	HA		NP		NS	
	DNA	Protein	DNA	Protein	DNA	Protein
Norway/Bremnes AF302799	79.3	84.8				
Norway/H-Sotra/91	80.0	85.3				
Scottish/390/98 AJ276859	79.3	84.3				
Maine	99.6	99.2				
Scottish/390/98 AJ276858			84.7	96.6		
Norway/Glesvaer/90 AF262382					88.2	75.6
Scottish/390/98 AJ242016					88.4	76.1
Canada/Bay of Fundy/97AF262389					98.9	97.9

96.6% identity to that reported for the Scottish NP (AJ276858) (Table 5). The complete mRNA and predicted translation can be seen in the appendix.

Segment 4

Gene segment 4 of ISA virus CCBB isolate contains a 1737 bp ORF encoding a 579 amino acid with a predicted molecular mass of 65.3 kDa (Table 3). Similarity searches using BLAST and the Influenza Data Base revealed no significant homology. The complete mRNA and predicted translation is in the appendix.

Segment 5

Gene segment 5 from ISA virus CCBB isolate encodes a 1353 bp ORF encoding a 451 amino acid polypeptide with a predicted molecular mass of 49.8 kDa (Table 3). Analysis of segment five indicates a high probability for a transmembrane helix. Table six indicates the orientation of the protein in the membrane.

Table 6. Prediction of transmembrane helices in ISA virus genome segment 5. Based on TMHMM-1.0 http://www.cbs.dtu.dk/services/TMHMM-1.0/. The complete mRNA and predicted translation can is in the appendix.

Location relative to membrane	Amino acids
Outside	1-419
Transmembrane helix	420-442
Inside	443-449

Segment 6

The ISA virus CCBB isolate segment 6 contained an 1185 bp ORF encoding a polypeptide with a predicted molecular mass of 43.1 kDa (Table 3). The complete mRNA and predicted translation can be viewed in the appendix. The predicted translation of the open reading frame encoded by RNA segment 6 shared 84.8, 84.3 and 99.2% identity to the predicted HA protein sequences for ISA virus isolates from Norway (AF302799), Scotland (AJ276859) and Maine, respectively (Table 5). Comparison of amino acid sequences derived from N-terminal sequencing to proteins listed in the GenBank data bases showed that the amino acid sequences from the 40 and 42 kDa proteins were similar to amino acids 17-41 in the predicted translation of the Norwegian and Scottish ISA hemagglutinin genes (AF302799, AJ276859, respectively) and were identical to the corresponding region of ISA virus isolate CCBB. The region of overlap did not include the first 16 amino acids of the predicted translation indicating that these amino acids are not present in the virion HA. Furthermore, the translated sequence of HA from ISA virus isolate CCBB had a predicted signal peptidase cleavage site between Ser-16 and Arg-17. Together, the information shows that after cleavage of the signal sequence, the mature HA begins at Arg-17. N-terminal amino acid sequence analysis revealed that the 40kDa protein was probably a C-terminally truncated form of the 42kDa HA. Similar analysis indicated that the thirteen N-terminal amino acids from the 47kDa protein were unique and identical to amino acids in the predicted translation of ISA virus RNA segment 5. The translated sequence of the 47 kDa protein had a predicted signal

sequence of 17 amino acids and the N-terminal amino acid sequence results confirmed that the cleavage site was located between Cys-17 and Glu-18. Comparisons of HA proteins from Maine, Norway, and Scotland indicated a hyper-variable region starting at amino acid 337 (Table 7). It is believed that this hyper-variable region will be useful for epidemiological studies of ISA virus isolates. The potential gylcosylation sites were predicted using Net O Glyc 2.0, which determines possible glycosylation sites, based on primary amino acid sequence. There appears to be one site at amino acids 333-335 that is conserved among all ISA virus HA proteins (Table 8).

Segment 7

The ISA virus CCBB isolate mRNA segment 7 gene contains two open reading frames. The first predicted ORF is 771 bp encoding a 257 amino acid protein with an estimated molecular mass 28.6 kDa. The second predicted ORF is 441 bp encoding a 441 amino acid protein with an estimated molecular weight of 16.3 kDa (Table 3). The complete mRNA and predicted translations are located in the appendix.

Segment 8

ISA virus CCBB gene segment 8 has two potential ORFs. The first ORF is 705 bp encoding a 235 amino acid polypeptide with an estimated molecular mass of 26.5 kDa. The second putative ORF is 552 bp encoding a 184 amino acid polypeptide with an estimated molecular weight of 20.3 kDa (Table 3). Our results confirmed that segment 8 encoded NS as previously reported by Mjaaland *et al.* (1997). The predicted translation of NS1 was 75.6, 76.1 and 97.9% identical to the

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protein NP and the surface proteins HA and NA. However, the surface proteins are the only antigens capable of inducing neutralizing antibody and therefore a protective immune response (Suarez and Schultz-Cherry, 2000). The HA-specific antibodies in fish sera indicate that ISA virus HA may play a similar role in protecting Atlantic salmon against ISA. Although antibody responses are also made to the influenza virus internal protein NP, the antibodies are not neutralizing and thus not protective (Suarez and Schultz-Cherry, 2000). Instead, the highly conserved sequence for NP from influenza A is the major target of cross-reactive cytotoxic T-lymphocytes generated against all influenza virus subtypes in mice and man (Yewdell and Hackett, 1989). The high sequence conservation results in a NP type –specific antigen that is used to differentiate between influenza A, B, and C (Lamb and Krug, 1996). The nucleocapsid protein from the Scottish strain of ISA virus is 96.6% identical to the CCBB isolate indicating that the NP protein is highly conserved. These findings suggest that the NP encoded by RNA segment 3 may be an important antigen for ISA vaccine design and typing of ISA viruses.

Further characterization of the HA for ISA virus isolate CCBB reveals three potential N-linked glycosylation sites (Table 8) (Kornfeld & Kornfeld, 1985): one unique to the North American isolates of ISA virus (155-157) and one conserved among the European and North American isolates of ISA virus (333-335). Of interest also is the putative N-glycosylation site in the hypervariable region of HA from Norwegian ISA virus isolate H-Sotra/91. Although the requirement for N-glycosylation is intrinsic to a given protein, carbohydrates at conserved glycosylation sites in the HA from influenza A act synergistically by promoting and stabilizing a

conformation compatible with transport, trimerization and folding of the protein (Roberts *et al.*, 1993). Oligosaccharides found at variable sites in the same molecule have been shown to modulate antigenic properties (Klenk, 1990; Munk *et al.*, 1992; Skehel *et al.*, 1984), receptor binding (Robertson *et al.*, 1987) and proteolytic activation (Kawaoka *et al.*, 1984; Kawaoka & Webster, 1989; Ohuchi *et al.*, 1989; Ohuchi *et al.*, 1991). The significance of the carbohydrates on these putative binding sites in the HA of ISA virus is unknown. However, if the influenza virus is used as a model, the carbohydrates on HA may contribute to differences in the pathogenicity and the clinical signs of disease that are observed between the North American and European isolates of ISA virus. Furthermore a predicted transmembrane domain has been identified (Table 9) for the putative HA demonstrating its role at an integral membrane protein.

Segment 5 has a single open reading frame of 1353 nucleotides coding for 451 amino acids (M_r = 49,800). N-terminal amino acid sequence analysis confirms that the 47 kDa protein visible in Coomassie blue-stained SDS polyacrylamide gels of purified ISA virus is encoded by segment 5. Furthermore, the analysis identifies the protein synthesis initiation site and confirms the presence of a 17 amino acid signal sequence that is probably cleaved from the protein after its insertion in the rough endoplasmic reticulum membrane. The function of the protein encoded on segment 5 remains unknown but P3 is likely the 53kDa protein reported for other ISA virus strains (Falk *et al.*, 1997; Kibenge *et al.*, 2000). In this study, antibodies in serum from Atlantic salmon infected with ISA virus do not recognize P3. However, the hydrophobic signal sequence of 17 amino acids, potential glycosylation sites and

prediction of a transmembrane domain suggest that it is a surface glycoprotein. P3 may be the enzyme responsible for the acetylesterase activity detected in ISA virions (Falk *et al.*, 1997). If this is the case, then like influenza A and B viruses, ISA virus has two virion surface glycoproteins: one with the acetylesterase activity and the other with hemagglutinin activities.

RNA segment 7 of ISA virus isolate CCBB is 1022 nucleotides in length; the largest open reading frame extends from the ATG codon at nucleotides 47-49 to a termination codon at nucleotides 815-817 and encodes a 257 amino acid protein (M_r = 28,600). This segment also contains a second open reading frame that codes for 147 amino acids (M_r = 16,300). RNA segment 7 of influenza A also encodes two proteins, the membrane protein M_1, which is the most abundant polypeptide in the virion and an integral membrane protein M_2 that has ion channel activity (Allen *et al.*, 1980; Winter & Fields, 1980; Pinto *et al.*, 1992). The M_2 protein from influenza A, B and C is encoded by a spliced segment 7 transcript differing from M_1 mRNA due to the excision of an intron between nucleotides 71 and 740 (Lamb *et al.*, 1981). If ISA virus uses a similar coding strategy, M_2 from ISA virus isolate CCBB may also be expressed via alternative splicing. Comparison of the nucleotide and translated protein sequences of segment 7 from ISA virus isolate CCBB and influenza A, B and C did not reveal any significant homology. However, the similarity of genome structure and organization between the orthomyxoviruses suggests that segment 7 of the ISA virus genome encodes the matrix proteins. A 25 kDa and a 15 kDa protein were detected in cellular and purified ISA virus by Western blot analysis using anti-

ISA virus polyclonal mouse sera. The identity of these proteins is unknown but may correspond to M_1 and M_2, respectively.

The eighth RNA segment of ISA virus isolate CCBB contains two open reading frames: the first is comprised of 703 nucleotides and codes for a 235 amino acid protein (M_r = 26,500); the second is comprised of 552 nucleotides and codes for a 184 amino acid protein (M_r = 20,300). The protein sequence for the largest open reading frame is 76% and 98% homologous to the predicted translation of putative NS_1 from two European and one Canadian isolate of ISA virus, respectively. Furthermore, Mjaaland *et al.* (1997) found that the corresponding sequence from a Norwegian isolate of ISA virus hybridized to the smallest segment of its genome indicating that segment 8 of ISA virus isolate CCBB encodes the putative NS_1 and NEP (NS_2) proteins. NS_1 from influenza A virus is an auxiliary factor that plays a crucial role in inhibiting interferon-mediated responses in the host (Garcia-Sastre *et al.*, 1998). Like the HA, the differences detected in the putative NS_1 protein sequences of ISA virus may contribute to the observed differences in the host response to the European and North American isolates of the virus.

The North American and European isolates of ISA virus are genetically different and probably represent distinct strains of ISA virus. The nucleotide variation of ISA virus isolates indicates that genomic drift occurs and that either genomic shift does not occur or has not been observed due to either sampling errors, host-range barrier or geographic confinement. Since the North American and European isolates of ISA virus share Atlantic salmon as their common host, geographical proximity seems to be the primary barrier to reassortment. The

possibility of and the consequences of genetic reassortment between isolates of ISA virus are unknown. Thus, studies to determine if reassortment occurs in ISA viruses and the consequences with respect to pathogenicity of the virus are required. Genetic studies indicate that virulence in influenza viruses is multigenically determined (Rott, 1979; Ward, 1997). The virulence of reassortant viruses resulting from crosses between different influenza parental strains depends not only on which gene has been replaced but also the parental origin of that gene confirming that multiple alleles control virulence. Reassortment studies with the various strains of ISA virus may also provide insight into the biochemical structure/function of a given gene with respect to the phenotype of the reassortant virus. These studies will provide the basis for rational design of a vaccine that targets the various antigenic subtypes and evolution patterns of the ISA virus.

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APPENDIX

Given below are the mRNA gene segments for ISA virus CCBB. All genes are written using the conventional 5' to 3' system. The predicted translations of each gene segment are given below the individual genes.

Segment 1.

ISA virus CCBB partial mRNA.

GGTAGCATGATTGGGACCACCCATGTTTGTTCACGAAGTTAGCTTTTAGTG
TGAATGAGCTTGTGCGTGCAAACCCTAGCTTGTCAGGTACTGAATGGAAT
GGAACCACTCTGGAAGTGACTTCTTGACATTTTGAAGATACAAGTACTGG
TGAAGGAACCTCTACTCTGATTCCCTTTGTGCATCCATGTTGTGTACAGCA
ACTTGTGATGTCCAACATTCCTGTTTTGATTCTGTCAGTTGTAAGTCCTTTT
TCGACACCACAATCCGTAGTGGGTTCGAGAGCCATCATTCTCCCTTTCTTT
CCTTTGCACTCCTCAGGTGGGCATCCTCTTGTCACGTATCCAACAGTACCT
CCACAGATGAAAACCTTTGGTCCTTTTCTTCCACATTTGGAAATGTCACTG
TCTCCACCAAACTCAGACACCTTGATTTTTCCTAGCACAATGTCTTGTTTG
GTTAGGTTGACAAATGTTGTGTCTAACACAGNTTTTACTGCACAGTTCTCC
ATCTGTGTACCTTGGATGGCTTCGAATAGTGTC

Segment 2.

ISA virus CCBB mRNA. Shown in bold are the ATG translation start sites and TGA stop sites.

CCATGGCCGCGGGATTGAACGCTCTTTAATAACC**ATG**GAAACTCTAGTAG
GAGGGCTGCTGACTGGAGAAGATTCTCTGATCAGTATGTCAAACGATGTA
TCTTGTCTTTATGTTTACGATGGACCAATGAGAGTTTTCTCTCAGAACGCA

TTAATGCCAACTCTGCAAAGTGTAAAAAGAAGTGACCAATTTTCCAAAGG

GAAAACAAAGAGATTTATCATTGACCTGTTCGGAATGAAGAGAATGTGGG

ACATCGGAAACAAACAGTTGGAAGACGAGAACTTAGACGAAACTGTAGG

CGTGGCTGACTTGGGGCTGGTGAAATATCTAATCAACAACAAGTACGATG

AAGCAGAAAAGACAAGTTTAAGGAAGTCAATGGAAGAAGCATTCGAAAA

ATCCATGAACGAAGAATTTGTGGTTTTAAACAAAGGAAAGTCTGCAAACG

ACATCATTTCAGACACAAATGCGATGTGCAAATTCTGTGTAAAGAACTGG

ATAGTGGCAACAGGTTTCAGGGGAAGAACGATGTCAGATTTAATTGAACA

CCATTTCAGATGCATGCAAGGGAAACAGGAGGTGAAAGGATACATTTGG

AAACACAAGTACAACGAAAGGCTTAAAAGAAAACAGCTAAGCAAAGAAG

AAGTGAAATTCGACAGAGAAGAATATACTTCAAGAAGCTTCAGACTACTC

TCTTTCTTGAAGAACAGCGAGAGGACCAAACTCGAGCCGAGAGCAGTGTT

CACAGCAGGAGTTCCATGGAGGGCATTCATCTTCGTCCTAGAACAGACAA

TGCTGGTGGTAAACAAACTGGACCCGAATTCAGTGATATGGATGGGAAGT

GATGCAAAGATAAACACCACAAACTCCAGGATAAAGGAAATAGGGATGA

AAAATCAAGGACAAACACTAGTGACACTCACAGGAGATAACTCCAAATA

CAACGAGAGCATGTGCCCAGAGGTGATGATGGTGTTCCTAAGAGAACTAG

GAATAAAAGGACCAATGTTGGAAGTACTGGACTATGCGCTGTGGCAATTT

TCACAGAAGAGTGTAAAACCTGTCGCACCTATAAAGAAGAGAACCGGCA

AGTCTACCGTGGTGATAAAAGCAGATTCCGTTAAGGAGTGTAGAGATGCC

TTCAACGAAAAGGAACTGGAGCTGATTCAAGGAGTTGAATGGATGGACG

ACGGATTTGTGAGAGTGAGGAGAGGAATGTTGATGGGAATGGCAAACAA

CGCTTTTACCACAGCTTCTACAATTGCCTCCTCTTTTAGTTTCACACCAGA

AGCTGTGTACACATTACAGAGCTCAGACGACTTCGTTACAGGTAGCTGTG

GAAGAGACGTGCAACACGCAAGACAAAGGCTAGAGATGGCTCTTAAAGT

GAGCAAAGCCGCAGGTCTGAACGTATCACAGAAGAAGTCATTCTACGTTG

AAGGGACAACTTTCGAGTTCAACTCTATGTTCGTAAGAGACGGTAAAGTG

ATGGCAAACGGAGGAAACTTTGAGAACATGACAGTTCCTGGAGGATTAG

GACCATCTACAGATCTCTTTGTCGTGGGGAAACAAGCAAGAAACTCCATG

TTGAGAGGCAACCTATCCTTCAGCCAGGCGATGGAGATGTGCAAAATAGG

AATCACAAATGTTGAGAAAGTTTACTATGGAAACAGAAAATACCAGGAG

CTGAAAAATGAGATAAGAGAGAAATGTGGAGAAGAAACGATGTCCATAC

CAGAGAGCATGGGAGGAGACAGGAAACCAAGACCGTGGGAATTACCTCA

GAGCTTTGATGGAATTGCCTTAAAAGAAGCTGTGAACAGAGGACATTGGA

AAGCTGCCAAGTACATCAAATCTTGCTGCAGCATAGAGTTCGATGAAGAA

GGAGACCAATCTTGGGACACTTCGAAAACAGCACTTGTGGTCATAAGGAA

AAATGAAACGGACATGAGAAGAAGAACTGTTAAAACGAGGAACCCAAAA

GATAAAATCTTCAATGATGCAATGAACAAGGCCAAAAGGATGTACGAAA

CAGTCGTGGACAGAAACCCATTACTAGGTCTGAAGGGGAAGGGAGGTAG

ACTGACAGTAAAAGACTTGAAAGCAAGGAAGCTTATTGATGAAGTAGAA

GTTGTTAAGAAGAAAAAGCATGTTTGAAATCACTAGTGCGGCCGCCTGCA

GG

Segment 2.

Translation of PB1 ISA virus CCBB mRNA. Shown in bold are the conserved motifs found in all RNA-dependent RNA polymerases.

METLVGGLLTGEDSLISMSNDVSCLYVYDGPMRVFSQNALMPTLQSVKRSD

QFSKGKTKRFIIDLFGMKRMWDIGNKQLEDENLDETVGVADLGLVKYLINN

KYDEAEKTSLRKSMEEAFEKSMNEEFVVLNKGKSANDIISDTNAMCKFCVK

NWIVATGFRGRTMSDLIEHHFRCMQGKQEVKGYIWKHKYNERLKRKQLSKE

EVKFDREEYTSRSFRLLSFLKNSERTKLEPRAVFTAGVPWRAFIFVLEQTMLV

VNKLDPNSVIWMGSDAKINTTNSRIKEIGMKNQGQTLVTLTGDNSKYNESMC

PEVMMVFLRELGIKGPMLEVLDYALWQFSQKSVKPVAPIKKRTGKSTVVIKA

DSVKECRDAFNEKELELIQGVEWMDDGFVRVRRGMLMGMANNAFTTASTI

ASSFSFTPEAVYTLQ**SSDDF**VTGSCGRDVQHARQRLEMALKVSKAAGLNVS

QKKSFYVEGTTF**EFNS**MFVRDGKVMANGGNFENMTVPGGLGPSTDLFVVG

KQARNSMLRGNLSFSQAMEMCKIGITNVEKVYYGNRKYQELKNEIREKCGE

ETMSIPESMGGDRKPRPWELPQSFDGIALKEAVNRGHWKAAKYIKSCCSIEF

DEEGDQSWDTSKTALVVIRKNETDMRRRTVKTRNPKDKIFNDAMNKAKRM

YETVVDRNPLLGLKGKGGRLTVKDLKARKLIDEVEVVKKKKHV

Segment 3.

ISA virus CCBB mRNA. Shown in bold are the ATG start site and TGA stop site.

AGCAAAGATTGCTCAAATCCCAAAAATAATACAGAAAACGTATAAGAGA

TGGCCGATAAAGGTATGACTTATTCTTTTGATGTCAGAGACAACACCTTG

GTTGTAAGAAGATCTACCGCTACTAAAAGTGGCATTAAGATCTCCTACAG

AGAGGATCGAGGAACATCACTTCTCCAAAAGGCATTCGCCGGGACAGAA

GATGAATTCTGGGTGGAGTTAGATCAAGATGTCTACGTTGACAAAAAGAT

TAGAGAATTCCTGGTAGAAGAGAAAATGAAGGACATGAGCACAAGAGTG

TCTGGGGCAGTGGCAGCAGCAATTGAAAGATCAGTTGAATTTGACAATTT

CTCAAAAGAAGCAGCAGCTAACATTGAAATGGCTGGTGTAGATGATGAA

GAAGCTGGAGGAAGTGGTCTGGTAGACAACAGAAGGAAGAACAAAGGG

GTCTCAAACATGGCCTACAATCTGTCTCTATTCATAGGGATGGTGTTTCCT

GCTCTCACTACTTTCTTCAGTGCTATCCTATCAGAAGGTGAAATGAGCATC

TGGCAAAATGGACAAGCAATCATGAGAATTCTGGCACTGGCAGATGAAG

ACGGAAAGAGACAAACAAGAACAGGAGGACAGAGGGTGGACATGGCTG

ATGTAACCAAGCTGAACGTAGTCACGGCTAACGGAAAAGTCAAGCAAGT

TGAAGTAAACTTGAACGATCTCAAAGCAGCATTCAGGCAGAGTAGACCTA

AAAGATCGGACTACAGAAAAGGGCAAGGTTCCAAGGCTACAGAATCAAG

CATCTCCAACCAATGTATGGCACTGATTATGAAATCTGTGCTGTCAGCAG

ACCAACTTTTTGCTCCGGGAGTGAAGATGATGAGGACGAACGGTTTCAAT

GCGTCGTACACAACACTGGCAGAAGGGGCAAACATTCCGAGCAAGTACC

TAAGACACATGAGGAACTGCGGAGGAGTAGCTCTGGACCTGATGGGAAT

GAAGAGGATCAAAAACTCACCTGAAGGAGCCAAGTCTAAGATCTTTTCCA

TCATCCAGAAGAAAGTAAGAGGAAGATGTCGCACAGAGGAGCAACGCCT

CCTGACTAGCGCACTGAAAATCAGCGACGGTGAAAACAAGTTCCAGAGA

ATCATGGACACTCTATGTACAAGCTTCCTGATTGACCCTCCAAGAACTAC

CAAATGCTTCATTCCACCTATTTCCAGTCTCATGATGTACATCCAAGAAGG

CAACTCTGTACTGGCAATGGATTTCATGAAAAACGGAGAGGACGCCTGCA

AGATCTGCAGAGAAGCCAAACTGAAAGTGGGGGTAAACAGTACGTTCAC

AATGTCAGTAGCTAGAACATGCGTTGCAGTGTCAATGGTTGCAACAGCTT

TTTGTTCTGCAGATATCATCGAGAATGCAGTGCCTGGTTCCGAAAGGTAC

AGATCCAACATCAAGGCTAACACAACCAAACCAAAAAAGGACTCCACTT

ACACAATTCAAGGACTTAGATTGTCTAACGTGAGGTATGAAGCAAGACCT

GAAACATCACAAAGCAACACAGACAGAAGTTGGCAAGTGAACGTGACTG

ACAGCTTCGGAGGACTTGCTGTGTTCAACCAAGGGGCAATTAGAGAAATG

CTAGGAGACGGAACATCAGAGACAACTAGTGTGAACGTCAGAGCCCTGG

TGAAGAGAATTCTGAAATCAGCTTCAGAGAGGAGTGCAAGAGCTGTAAA

GACATTTATGGTGGGAGAACAAGGGAAATCAGCTATTGTTATCTCTGGTG

TGGGACTGTTCTCTATTGACTTTGAAGGGGTAGAGGAAGCGGAAAGAATA

ACTGACATGACACCTGAAATTGAGTTTGACGAGGACGACGAGGAAGAGG

AAGACATTGACATTTAGAGTGACAATTATGTAACTTCCTAATTACCCTATA

TTGTTTGAATATATAATGAAACTATTGTGTGTTAAAGGTTGTGGGTTTGAT

TATTAAATTTAAA**TTGA**AACGGTATTGACGATATTTACAAAAAAAAAAAA

AAAAAAAAAAAA

Segment 3.

Translation of NP ISA virus CCBB mRNA.

MADKGMTYSFDVRDNTLVVRRSTATKSGIKISYREDRGTSLLQKAFAGTEDE

FWVELDQDVYVDKKIREFLVEEKMKDMSTRVSGAVAAAIERSVEFDNFSKE

AAANIEMAGVDDEEAGGSGLVDNRRKNKGVSNMAYNLSLFIGMVFPALTTF

FSAILSEGEMSIWQNGQAIMRILALADEDGKRQTRTGGQRVDMADVTKLNV

VTANGKVKQVEVNLNDLKAAFRQSRPKRSDYRKGQGSKATESSISNQCMAL

IMKSVLSADQLFAPGVKMMRTNGFNASYTTLAEGANIPSKYLRHMRNCGGV

ALDLMGMKRIKNSPEGAKSKIFSIIQKKVRGRCRTEEQRLLTSALKISDGENK

FQRIMDTLCTSFLIDPPRTTKCFIPPISSLMMYIQEGNSVLAMDFMKNGEDAC

KICREAKLKVGVNSTFTMSVARTCVAVSMVATAFCSADIIENAVPGSERYRS

NIKANTTKPKKDSTYTIQGLRLSNVRYEARPETSQSNTDRSWQVNVTDSFGG

LAVFNQGAIREMLGDGTSETTSVNVRALVKRILKSASERSARAVKTFMVGEQ

GKSAIVISGVGLFSIDFEGVEEAERITDMTPEIEFDEDDEEEEDIDI

Segment 4.

ISA virus CCBB mRNA. Shown in bold are the ATG start site and TGA stop site.

CAAG**ATG**GATAACCTCCGTGAATGCATAAACCGCAAAAGAAGACTACTT

GCCTTACCAGATGTTCCTGAAACTTCGGATGCCTTTCTAAGTGATTTGAGA

CATCTATACATGTGTGTTGCTTTCTGTGATCAACACAAAACCACTGGAGAC

GAATCAAGATTCACCAACCTGGAATTACTTGACCAAGATGAAGCACTAGG

TGCCCAAAGAGCTTTTGAAGCCAAACATGGAATAAAAGGAGGTTCTTTAG

GAGACGTTCTTGACCATGAACTGAAAAAGGTCATTGAATTTACTTTTACTT

CTGGAAGTTTGTATATTGCCGAACAAAGAAAAAGAAAGACTCAAGCAGA

CTCAATAATTGTGTGCGTTTCAGAAGGACTTAACGACTTCAGCGTATCAC

ACGGAGTGCTAGACATGGGACTTGTGGAAACAGGGGTGAATGCAGTAAG

AGATTTCTGCACACAAAACGGAATACCAATGAAGATAAATCAGGTAGGA

TCCACGAGAACACCAACACCGATCAGCACATGCAAAATCTCTGAACAAAT

AACACGACARATAAACAGTACAATTACTGAAAGGAAAATGGAAACAGTA

CTGGCAGCAATCGCAATTAAACCAGAACTCAAAYYAACTCAGAAAGGAT

GCAGMMCTTGTAAAGAACTAGAAGATGAAAATATTCTGTGGATGGACCC

TCAATTCTGTGAAATTGATGAAAGTTTTCCTTACAGAGGAGGGCCATACG

GGAACTTCCTGCAAGAATTGCTGCTTACAACCAACGACGTAGAGACCAAC

GGGAAAGACAGAGAAGAAGTAGTAAAGAASATACTGGATAACAAGGCGT

TCACCGTTGAAAGTGGTGAATGCATAATAACACTTCCAGACAAAATGACT

TGTTTCGGAGAACARGAGAAGAAGAGACCAGCAACAATAGACGAAGTGA

GAACCGCAGGAGAAAGGTTTGAACAGAGTGTTAAACCGAAAACCCAAAG

ATATGGAAGGTTATCAGACAAATGGATGGAGCTTGAAAAGTTTATCTTTA

CTGCAAGCAAAACAGAAGTGGATACTTTCCTTTCTGTAGGGACCGAAAGA

CTTGAGTCGGTTGGAGTGTGTGTCGGAGCTTTACACAGAGCGACCACAAC

CAGGATAATTAGACCTATGATTCAAGGAGGGAAATGTTGGGGGATGATGT

TCAAAACAAAGTCCAAAATGGGAGACACGAGGAAGGAAGGATACTGTCA

CGCAATCATTTTCGGAAAAGGGGAAGATAAATCAGGACAAAACAAGATG

ACAATGATGGGGAAAACAGTACATTGGCATCTAAGAGTAGTTAAGTCTAA

AGGAGACTGGATGGCGCAACAACTCTGTGCAAACAAAAGCAGAATATGG

CAACATGACCCTGAGCTAGTAACAGAAGGAGTGACAGTTCTAATGACGCC

TTTTTCTCAGAAAATTGCAACCATTAGTAGATGGAGGGCAATGAGGTTAG

ACAGCATGTTTCATGTTTCTAGTGCCTGGCATCATTCACCTGCGTGTGAAG

CTGCATCGGCAATGCTGAGAAAGTTTGTGGAGATAGTACATGCCATCAAC

CAGAAAAGAGATTGGGGTGTTGTGGGGAGTATGGAGGACATGGTGAAGG

AAGTGGAGGAAATAGGGGAGCACTTGCAGACGGCATGTGAYTTTAGAGT

TTACAACATKTGCAAAGCCTTGATTCAGAAAATTGCAGTCAGTACCCAA**T**

GAGTGGTTATTTACTTGTAAATTGTTGTGTGTTTGACGATATGTATTT

Segment 4.

Translation of P2 the putative PA ISA virus CCBB mRNA.

MDNLRECINRKRRLLALPDVPETSDAFLSDLRHLYMCVAFCDQHKTTGDESR
FTNLELLDQDEALGAQRAFEAKHGIKGGSLGDVLDHELKKVIEFTFTSGSLYI
AEQRKRKTQADSIIVCVSEGLNDFSVSHGVLDMGLVETGVNAVRDFCTQNGI
PMKINQVGSTRTPTPISTCKISEQITRQINSTITERKMETVLAAIAIKPELKXTQK
GCXXCKELEDENILWMDPQFCEIDESFPYRGGPYGNFLQELLLTTNDVETNG
KDREEVVKXILDNKAFTVESGECIITLPDKMTCFGEQEKKRPATIDEVRTAGE
RFEQSVKPKTQRYGRLSDKWMELEKFIFTASKTEVDTFLSVGTERLESVGVC
VGALHRATTTRIIRPMIQGGKCWGMMFKTKSKMGDTRKEGYCHAIIFGKGE
DKSGQNKMTMMGKTVHWHLRVVKSKGDWMAQQLCANKSRIWQHDPELV
TEGVTVLMTPFSQKIATISRWRAMRLDSMFHVSSAWHHSPACEAASAMLRK
FVEIVHAINQKRDWGVVGSMEDMVKEVEEIGEHLQTACDFRVYNXCKALIQ
KIAVSTQ

Segment 5.

ISA virus CCBB mRNA. Shown in bold are the ATG start site and TGA stop site.

AGTTAAAG**ATG**GCTTTTCTAACAATTTTAGTCTTGTTCCTTTTTAAAGAGG
TTCTTTGTGAACCTTGTATTTGTGAGAACCCAACATGTCTAGGAATAACAA
TCCCACAGGCAGGTTTCGTAAGAAGCGCTCCAGGAGGTGTACTTCTAACT
GAGACAATCACGGAAAGACCACAACTAACAGAGTGGACAACCTCCAGAC
CGAAGCTTGAAGAAACTCTCTGGTTAGATGGGGAAACAAAGAACGGAAA
AGTATCTCAGACACTATTCGAAGCCATCCAAGGTACACAGATGGAGAACT

GTGCAGTGAAAGCTGTGTTAGACACAACATTTGTCAACCTAACCAAACAA

GACATTGTGCTAGGAAAAATCAAGGTGTCTGAGTTTGGTGGAGACAGTGA

CATTTCCAAATGTGGAAAGAAAAGGGACTACAGGGTTTTTCATTCTGTGG

AAGGGTACTTGTTGGGAATACGTGACAAGAGGAATGCCCCACCTGAGGA

AGTGCAAAGGAAAAGAAAGGGGAGAATGATGGGCTCTCGAACCCCACTA

CGGATTTGTGGTGTCGAAAAAGGACTTACAACTGACAGAATCAAAACAG

GAATGTTGGACATCACAAGTTGCTGTACACAACATGGATGCACAAAGGGA

ATCAGAGTAGAGGTTCCTTCACCAGTACTTGTATCTTCAAAATGTCAAGR

AGTCACTTTCAGAGTGGTTCCATTCCATTCAGTACCTGACAAGCTAGGGTT

TGCACGCACAAGCTCATTCACACTAAAAGCTAACTTCGTGAACAAACATG

GGTGGTCCAAGTATAATTTCAACCTAAGAGGATTTCCTGGAGAAGAGTTC

ATTAAGTGTTGTGGATTTACGTTGGGAGTCGGAGGAGCGTGGTTTCAAGC

CTACTTAAATGGAATGGTTCAAGGTGACGGTGCCGCATCTGCAGACGACG

TGAAAGAGAAACTCAACGGAATAATCGACCAGATAAACAAAGCGAACAC

ACTTCTTGAAGGAGAAATTGAAGCAGTGAGGAGGATTGCCTATATGAACC

AAGCATCAAGTCTTMAGAACCAAGTGGAAATCGGACTAATAGGTGAATA

TTTGAACATTAGCAGTTGGTTGGAGACTACTACATTAACTAAAACAGAAG

AAGGCTTGATGAAGAATGGCTGGTGTCAGTCTAACACGCACTGCTGGTGT

CCACCTAAACCTACAATTGTTCCCACCATTGGATATGTTGACAGTATAAA

AGAAGTAACGGGTACAAGTTGGTGGATGGTTATGATACATTACATTATTG

TGGGGTTAATAGTTATTGTGGTGGTGGTGTTTGGTTTAAAACTATGGGGAT

GTCTTAGAAGG**TGA**AATGTCGGTCTAAAAATTCTTTTTCTGTACATTACTA

AAGGGTAGCTTAACCAAGGTGTTTATGTATATAGACTATTATTGGATAAG

TTAGAAATTTGTATCTGATTATGCATTATTAATTGTATAAATAG

Segment 5.

Translation of P3 the putative acetylesterase ISA virus CCBB mRNA.

MAFLTILVLFLFKEVLCEPCICENPTCLGITIPQAGFVRSAPGGVLLTETITERP

QLTEWTTSRPKLEETLWLDGETKNGKVSQTLFEAIQGTQMENCAVKAVLDT

TFVNLTKQDIVLGKIKVSEFGGDSDISKCGKKRDYRVFHSVEGYLLGIRDKRN

APPEEVQRKRKGRMMGSRTPLRICGVEKGLTTDRIKTGMLDITSCCTQHGCT

KGIRVEVPSPVLVSSKCQXVTFRVVPFHSVPDKLGFARTSSFTLKANFVNKHG

WSKYNFNLRGFPGEEFIKCCGFTLGVGGAWFQAYLNGMVQGDGAASADDV

KEKLNGIIDQINKANTLLEGEIEAVRRIAYMNQASSLXNQVEIGLIGEYLNISS

WLETTTLTKTEEGLMKNGWCQSNTHCWCPPKPTIVPTIGYVDSIKEVTGTSW

WMVMIHYIIVGLIVIVVVVFGLKLWGCLRR

Segment 6.

ISA virus CCBB mRNA. Shown in bold are the ATG start and TGA stop sites.

AGCAAAG**ATG**GCACGATTCATAATTTTATTCCTACTGTTGGCGCCTGTTTA

CAGTCGTCTATGTCTTAGAAACCATCCTGACACCACCTGGATAGGTGACT

CCCGAAGCGATCAATCAAGGGTGAACCAACAGTCTCTTGATCTGGTTACA

AACTTCAAGGGAATTCTACAAGCCAAGAACGGGAATGGTCTCATGAAGC

AGATGAGCGGAAGGTTCCCAAGTGATTGGTACCAACCTACTACAAAGTAT

AGGATTCTATACATTGGTACAAACGACTGCACTGAGGGCCCTAACGACGT

GATCATACCGACGTCAATGACACTAGACAATGTGGCAAGGGACCTGTACC

TGGGAGCATGTCGAGGAGATGTAAGAGTGACACCAACCTTCGTGGGAGC

AGCTGAGCTTGGACTGATTGGGAGAACAGATGCCTTAACAGAATTTTCTG

TAAAGGTGCTGACTTTCAACAACCCTACTATTGTAGTAGTTGGACTAAAT

GGAATGTCAGGAATCTACAAGGTCTGCATTGCTGCCTCTTCTGGAAACGT

AGGCGGAGTCAACTTGGTGAACGGATGCGGATACTTCAGCGCTCCTCTGA

GATTCGACAACTTCAAAGGACAGATCTACGTGTCAGACACCTTTGAAGTC

AGAGGAACAAAGAACAAATGTGTCATACTTAGATCTTCTAGCAATGCTCC

TTTGTGTACACATATCAAAAGAAACATTGAGTTGGATGAGTACGTTGACA

CACCAAACACTGGGGGCGTATATCCTTCTGATGGGTTTGATTCTCTTCACG

GCTCTGCTTCGATTAGAACTTTTTTAACAGAGGCACTGACATGTCCAGGTG

TAGATTGGGACAGAATTGATGCAGCTTCATGCGAGTATGACAGTTGTCCT

AAACTTGTGAAAGAATTTGACCAAACAGGGCTCGGAAACACAGATACTC

AAATAATGAGAGAGCTAGAAGCACAAAAGGAGATGATTGGTAAACTTGG

CAGAAACATTACAGACGTAAACAACAGAGTAGATGCTATTCCACCACAG

CTTAGCAACATCTTCATCTCTATGGGAGTGGCAGGTTTTGGGATAGCACTG

TTTCTAGCAGGGTGGAAGGCTTGTGTTTGGATAGCAGCTTTCATGTATAAG

TCTAGAGGTAGAAACCCACCTGCAAATCTGTCTGTTGCT**TGA**TACTAAGA

CAAACAAAGTTTTCAAATAATCAAATGTTTTCTAATGTAATGTAAAATTCA

AATCGTATGTGATATTATTATTTTGAAGACGTTCTTGATGTTGTACGTTTA

CAGAAAAGTGCATTTTTTACT

Segment 6.

Translation of the putative HA ISA virus CCBB mRNA.

MARFIILFLLLAPVYSRLCLRNHPDTTWIGDSRSDQSRVNQQSLDLVTNFKGI
LQAKNGNGLMKQMSGRFPSDWYQPTTKYRILYIGTNDCTEGPNDVIIPTSMT
LDNVARDLYLGACRGDVRVTPTFVGAAELGLIGRTDALTEFSVKVLTFNNPT
IVVVGLNGMSGIYKVCIAASSGNVGGVNLVNGCGYFSAPLRFDNFKGQIYVS
DTFEVRGTKNKCVILRSSSNAPLCTHIKRNIELDEYVDTPNTGGVYPSDGFDS
LHGSASIRTFLTEALTCPGVDWDRIDAASCEYDSCPKLVKEFDQTGLGNTDT
QIMRELEAQKEMIGKLGRNITDVNNRVDAIPPQLSNIFISMGVAGFGIALFLAG
WKACVWIAAFMYKSRGRNPPANLSVA

Segment 7.

ISA virus CCBB mRNA. Shown in bold are the ATG start site and TGA stop site.

CTGGCCTTGAAACATGTGATTTAAATACAAAGAAAA**ATG**TTCAGAACATGT
CTGGATTTAACTTCGAGGTAATGGTGCCGGAACAAGGAGGAAAAGTGGTC
TTCAGCCTTACTGAAACGGGGTCATGTGTCTCGTTTTACGGAGATGATGA
ACCAGGTGAAGGGTCCTGCGAACTTGCCTCTGAAAACATGGATTTTCCAA
GTTGTCCTCTGGGGAATGGAGATGACTTCTGTCTGTCGCTGGCGCTAAGC
ACAATGAGATGGTCTGGGATGACCAAGAGAAACAACTTCATGGACAGAT
TCATTGGAAGTTTTGTTCATTGTACACCAGTGATGATCTGGTCGTATGGAA
ATTTGTCCAAGAAAAGCCATCACAAAATGGTTTGCCACACTTGCCCAGAC
GAGTACAAGTTCAGTGACAAGGACGAGATGCAGGGATACTATGAGGAAT

GTCTAGAGGCTTCTACTGACATTTTCCTTGATGAACTTGCTACTGTTGTTA

CAGGTGGCTTCTTTCCTGTCGGACTCAAAGGTTCCTGGGGAGGATGGTAC

CTCAAGTACGTCAGGTATGCTGGACCTCTTGCGGGATCAAGTGGATTCAT

TGTCAATCAACGATTCTACGACAGAGCCCAAAACAAGACTGGATCCAGG

GTTGTATCCATGGTTGAAATGGACGGAGACGGCTTATCGTTCATCTACGA

GAAGCCTAGCGTCTACCATAGTGATGGGTGCACTGGTTCAGCAGCGAGGT

TCTGGAAACGGGATCACAATGAGAGAGCTGGAGT**TGA**GCTTAGGGCTGG

ACTTCACTTCAGAATGTGATTGGTTGAAAACTTGTTATGTAAACAAGAATT

TTGTGTTTTTGTCAGAAAAAGAAATTGCTGTAAACATGGAAGTTGAAAAA

TTCATTTGTAATGAGAACTAAAGATGTCTTTGTGTTCAAATTTTAACTAAT

GACAATATATGAAATATGTCGTACATGGTGTTGATGATAATTTTTAAAAC

GAAAAGGAGAATTTTTACTTTT

Segment 7.

Translation of P4 the putative M1 ISA virus CCBB mRNA.

MSGFNFEVMVPEQGGKVVFSLTETGSCVSFYGDDEPGEGSCELASENMDFPS

CPLGNGDDFCLSLALSTMRWSGMTKRNNFMDRFIGSFVHCTPVMIWSYGNL

SKKSHHKMVCHTCPDEYKFSDKDEMQGYYEECLEASTDIFLDELATVVTGG

FFPVGLKGSWGGWYLKYVRYAGPLAGSSGFIVNQRFYDRAQNKTGSRVVS

MVEMDGDGLSFIYEKPSVYHSDGCTGSAARFWKRDHNERAGVELRAGLHFR

M

Segment 7.

Translation of P5 the putative M2 ISA virus CCBB mRNA.

MNLLLLLQVASFLSDSKVPGEDGTSSTSGMLDLLRDQVDSLSINDSTTEPKTR

LDPGLYPWLKWTETAYRSSTRSLASTIVMGALVQQRGSGNGITMRELELSLG

LDFTSECDWLKTCYVNKNFVFLSEKEIAVNMEVEKFICNEN

Segment 8.

ISA virusCCBB mRNA shown in bold are the ATG start sites and TAA stop sites.

TGCAAAGATTGGCTATCTACC**ATGCATG**AGAGAAGCAAACCCAAAACCA

CGGGAGCTGATCAGACATGCCTTGAAGAAGAAAAAGAGACCAGAGGTGG

TTTACGCAATGGGAGTTCTTCTGACACTGGGGGGAGAGAGCGGACTGACC

GTGGAGTTTCCTGTTCCAGAAGGAAAAACTGTGAAGGTCAAAACCTTGAA

CCAATTGGTGAACGGGATGATCAGTCGAGCGACGATGACCCTCTACTGTG

TGATGAAAGATCCACCATCGGGAGGCATGGCAACGCTGATGAGAGACCA

CATCAGGAACTGGCTGAAGGAGGAATCAGGATGCCAGGACGCGGATGGT

GGAGAGGAAAAATGGGCAATGGTGTATGGTATGATTTCACCCGACATGGC

AGAGGAGAAGACGATGCTGAAGGAGCTGAAAACAATGCTACACAGCAGG

ATGCAGATGTATGCTCTGGGTGCAAGTTCGAAAGCCCTAGAGAATTTAGA

AAAGGCCATCGTCGCTGCAGTTCATCGACTTCCGGCATCCTGCTCGACAG

AGAAGATGGTGCTTCTGGGGTACCTGAAG**TAA**GCTTCAAAGAAAGAATG

GAAGCGGAGAAGAAGAAACTGAAAGAGCTGGACGACAAGATCTACAAGC

TAAGGAGAAGATTGAGGAAGATGGAGTACAAGAAAATGGGGATCAACCG

AGAAATCGACAAATTGGAAGACTCTGTACAA**TAA**AATCACTAGT

Segment 8.

Translation of P6 the putative NS1 ISA virus CCBB mRNA.

MHERSKPKTTGADQTCLEEEKETRGGLRNGSSSDTGGRERTDRGVSCSRRKN
CEGQNLEPIGERDDQSSDDDPLLCDERSTIGRHGNADERPHQELAEGGIRMPG
RGWWRGKMGNGVWYDFTRHGRGEDDAEGAENNATQQDADVCSGCKFESP
REFRKGHRRCSSSTSGILLDREDGASGVPEVSFKERMEAEKKKLKELDDKIYK
LRRRLRKMEYKKMGINREIDKLEDSVQ

Segment 8.

Translation of P7 the putative NEP ISA virus CCBB mRNA.

MREANPKPRELIRHALKKKKRPEVVYAMGVLLTLGGESGLTVEFPVPEGKTV
KVKTLNQLVNGMISRATMTLYCVMKDPPSGGMATLMRDHIRNWLKEESGC
QDADGGEEKWAMVYGMISPDMAEEKTMLKELKTMLHSRMQMYALGASSK
ALENLEKAIVAAVHRLPASCSTEKMVLLGYLK

BIOGRAPHY OF THE AUTHOR

Trent L. Rector was born in Belfast, Maine on November 26, 1974. He was raised in Damariscotta, Maine and graduated from Lincoln Academy High School in 1993. He attended the University of Maine and graduated in 1998 with a Bachelors' degree in Microbiology. Trent is a candidate for the Master of Science degree in Microbiology from The University of Maine in December, 2001.