Cloning and identification of the infectious salmon anaemia virus haemagglutinin

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Infectious salmon anaemia virus (ISAV) is an orthomyxo-like virus that causes serious disease in Atlantic salmon (Salmo salar). Like the orthomyxoviruses, ISAV has been shown to possess haemagglutinin (HA) activity. This study presents the cloning, expression and identification of the ISAV HA gene, which was isolated from a cDNA library by immunoscreening. The HA gene contained an ISAV-specific conserved nucleotide motif in the 5' region and a 1167 bp open reading frame encoding a protein with a predicted molecular mass of 42–44 kDa. The HA gene was expressed in a baculovirus system. A monoclonal antibody (MAb) shown previously to be directed against the ISAV HA reacted with insect cells infected with recombinant baculovirus. Salmon erythrocytes also adsorbed to these cells and adsorption was inhibited by the addition of either the ISAV-specific MAb or a polyclonal rabbit serum prepared against purified virus, confirming the virus specificity of the reaction. Immunoblot analyses indicated that ISAV HA, in contrast to influenza virus HA, is not posttranslationally cleaved. Sequence comparisons of the HA gene from five Norwegian, one Scottish and one Canadian isolate revealed a highly polymorphic region that may be useful in epidemiological studies.

Introduction

Infectious salmon anaemia (ISA) is a viral disease that causes severe problems in the Atlantic salmon farming industry in Norway, Canada and Scotland (Mullins et al., 1998; Rodger et al., 1998; Thorud & Djupvik, 1988). The aetiological agent of ISA has been shown to share several characteristics with members of the Orthomyxoviridae family (Falk et al., 1997; Koren & Nylund, 1997; Krossøy et al., 1999; Mjaaland et al., 1997). The ISA virus (ISAV) has a negative-stranded RNA genome consisting of eight single-stranded segments that range from 1 to 2.3 kb (Mjaaland et al., 1997). It has also been demonstrated that the sequences of the 5’ mRNAs start with a heterogeneous sequence followed by eight or nine conserved residues. These conserved residues show a distinctive nucleotide similarity to corresponding orthomyxovirus sequences (Krossøy et al., 1999; Sandvik et al., 2000). Furthermore, the predicted secondary structures of the terminal sequences of two ISAV genomic segments have been shown to correspond to the panhandle structures described for influenza viruses (Sandvik et al., 2000). To date, the sequences of only two ISAV genomic segments have been published. Segment 8 encodes two proteins of unknown function and shares no significant identity with any other known sequence (Mjaaland et al., 1997). Segment 2 has been reported to encode the putative polymerase protein (PB) and phylogenetic analysis carried out using this sequence places ISAV within the Orthomyxoviridae family (Krossøy et al., 1999).

Influenza A and B viruses contain two surface glycoproteins, the haemagglutinin (HA) with receptor binding and membrane fusion activities and the neuraminidase (NA) with...
receptor-destroying activity. In contrast, influenza C viruses contain only a single surface glycoprotein, the HA–esterase–fusion protein (HEF), which has all three activities. HA and HEF are synthesized as precursor proteins that generate the C terminus of HA1/HEF1 and the N terminus of HA2/HEF2 after cleavage, respectively. This cleavage event primes the membrane fusion potential of HA/HEF, which is required for the virus to be infectious. Fusion of the viral and cellular membranes takes place as the fusion activity of HA/HEF is triggered by the low-pH environment of the endosome (reviewed by Skehel & Wiley, 2000; Wiley & Skehel, 1987). ISAV has been shown to possess both haemagglutinating and receptor-destroying activity; the latter has been suggested to be an acetylcholinesterase (Falk et al., 1997). Trypsin treatment was used to show that the membrane fusion activity is activated by proteolytic activity (Falk et al., 1997). Recently, Eliassen et al. (2000) demonstrated that ISAV replicates in a manner similar to the influenza viruses, with binding of virus particles to NA-sensitive determinants on cell surface glycoproteins or glycolipids. Furthermore, it was shown that virus particles are internalized into endosomes and lysosomes where a low pH-dependent fusion with the cell membrane occurs.

Among influenza viruses, the most polymorphic gene has been shown to be that of the HA (reviewed by Webster et al., 1992). Accordingly, this sequence is useful to identify and separate closely related isolates. Sequence comparisons of ISAV gene segments 2 and 8 have shown distinct differences between isolates originating from the eastern region of North America and Europe (Blake et al., 1999; Cunningham & Snow, 2000; Krossøy et al., 2001), but it has not been possible to differentiate between the European isolates using these segments.

In the present study, we report the cloning, expression and identification of the gene encoding the ISAV HA. We present data indicating that ISAV HA, in contrast to influenza virus HA, is not posttranslationally cleaved. A highly polymorphic region that may be useful as an epidemiological marker is also described.

Methods

Virus isolation and construction of a cDNA library. Kidney samples from Atlantic salmon (Salmo salar L.) were taken during an ISA outbreak in Bremnes, Norway in 1998. Virus was isolated and propagated in Atlantic salmon kidney (ASK) cells as described previously (Devold et al., 2000). RNA was isolated from ISAV-infected ASK cells using Trizol reagent (Life Technologies). RNA was isolated from ISAV-infected cells on days 2, 3 and 4 post-infection (p.i.). RNA was pooled and mRNA was isolated using the Dynabeads mRNA Purification kit (Dynal). A sample of 2 µg mRNA was used for cDNA synthesis with the cDNA Synthesis kit (Stratagene). A unidirectional bacteriophage lambda cDNA library was then constructed using the Uni-ZAP XR vector and Gigapack III Gold packaging extract (Stratagene).

Screening of the bacteriophage lambda cDNA library. Library screening was performed using an anti-ISAV HA monoclonal antibody (MAb), 3H6F8 (Falk et al., 1998), and a polyclonal anti-ISAV rabbit sera (see below) with the picoBlue immunoscreening kit (Stratagene). PCR products from clones suspected to be derived from ISAV were produced using vector-specific primers and the PCR products were then sequenced. One set of internal gene-specific PCR primers was constructed for each sequence and this primer pair was then used on cDNA from ISAV-infected and uninfected cells to determine whether the sequence was viral or cellular. For one of the clones, tentatively designated 9Z, the PCR primer pair 9ZF1 (5’ cgcgcttacagctgtaccttgct tt 3’) and 9ZR1 (5’ gggttgatattcatctcgctgta 3’) was used. The pBlueScript plasmid was then excised from the ISAV-positive clones using the ExAssist helper phage and the SOLR strain of E. coli (Stratagene). Complete sequencing was performed on the isolated plasmids. To obtain full-length cDNA sequences, 5’ RACE was performed with the 5’RACE system, version 2.0 (Life Technologies). 5’ RACE products were cloned into the pCR 2–1-TOPO vector using the TOPO TA Cloning kit (Invitrogen) and sequenced as described below.

DNA sequencing and assembly. Plasmids and PCR products were sequenced using the BigDye Terminator Sequencing kit and an ABI 377 DNA analyser (PE Biosystems). Sequences were assembled using the Sequercher software (Gene Codes Corporation). GenBank searches were performed using BLAST software, version 2.0.

Preparation of antisera. ISAV was purified on a continuous sucrose gradient using the Glesvaer 90 isolate, as described previously (Falk et al., 1997). Polyclonal antisera was prepared by immunizing rabbits three times at 6 week intervals using approximately 50 µg of purified ISAV for each immunization. The first and second immunizations were administered subcutaneously in Freund’s complete and Freund’s incomplete adjuvant (Difco), respectively. The third immunization was administered intravenously in saline. Animals were bled 10 days after the third immunization. Eurogentec prepared the peptide antiserum against the 9Z protein by using the peptides MGDSRSDQSRVNPQSC and CPKMVKDFDQTLSGLNT coupled to keyhole limpet haemocyanin. The conjugated peptides were pooled and injected into two rabbits, according to Eurogentec’s procedures.

Northern blot. Northern blotting was performed with the Northern Max kit (Ambion). Briefly, approximately 15 µg of total RNA from either ISAV-infected ASK (3 days p.i.) or uninfected cells was separated by formaldehyde–agarose gel electrophoresis and blotted onto a positively charged nylon membrane (Boehringer Mannheim). The DIG-labelled RNA molecular mass marker 1 (Boehringer Mannheim) was also run in parallel. PCR using the primer pair 9ZF1/9ZR1 produced a 552 bp DNA probe that was DIG-labelled and used according to the DIG High Prime Labelling and Detection Starter kit 2 (Boehringer Mannheim).

Baculovirus expression of 9Z cDNA. 9Z cDNA was amplified using primers 5’ tgggctgcaagatgcaagcttc 3’ and 5’ aaggactcgtgctcttcttcataac 3’ and cloned into the pFastBac1 vector (Life Technologies). The construct was transformed into TOP 10 cells (Invitrogen) and the isolated plasmids were used to transform DEH10Bac competent cells (Life Technologies). Recombinant baculoviruses were constructed according to the recommendations of Life Technologies.

Immunofluorescence in Sf9 cells. Sf9 cells infected with 9Z-recombinant or non-recombinant baculovirus were grown in microtitre plates at 28 °C. At 5 days p.i., cells were fixed in formol–calcium and washed. Cells were then incubated with anti-ISAV HA MAb 3H6F8 for 1 h at 37 °C. After washing with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) was added and cells were incubated at room temperature for 1 h. After washing, cell nuclei were stained with propidium iodide, mounted using
Identification of the ISAV HA

Table 1. Origins, abbreviations and accession numbers of ISAV isolates used for sequence comparisons

<table>
<thead>
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<th>Year of isolation</th>
<th>Strain reference no.*</th>
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* Strain reference number at the National Veterinary Institute, Oslo, Norway.
† Submitted by E. Rimstad, S. Mjaaland and S. Sandvik (unpublished data).

Results

Cloning and sequence determination of ISAV cDNA

Immunoscreening of the bacteriophage lambda cDNA library with the HA reactive MAb 3H6F8 was negative, but screening with a polyclonal serum prepared against purified, whole virus identified a possible ISAV clone with an open reading frame. The clone was then propagated in ASK cells and RNA was extracted with Trizol. Reverse transcription was performed using M-MLV reverse transcriptase (Promega) with random hexamers as primers. cDNA sequences corresponding to the HA gene were amplified by PCR using the sense primer 5'-gtagagttcgtgtgatggaggc-3' and the anti-sense primer 5'-gggctggtggtggctggtgtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtg
Fig. 1. Deduced amino acid sequence alignment of the ISAV HA proteins. Only amino acids that are different from the Bremnes 98 isolate are shown. Amino acid identities are indicated by dots. Sequences that are not determined are indicated by nonbreaking spaces (—) and amino acid deletions are indicated by dashes. Potential glycosylation sites are boxed and a potential transmembrane region is underlined. Strain reference numbers are defined in Table 1.

reading frame (ORF) of 1167 bases. The clone was tentatively designated 9Z and database searches revealed that its sequence was similar (98%) to the ISAV gene segment 7, encoding an unidentified protein (submitted previously to GenBank by E. Rimstad, S. Mjaaland and T. Sandvik, accession no. AF220607). No significant identity to other sequences was found and this prevented us from predicting a function or any three-dimensional structure of the protein. Crystal structure analyses has shown previously that the HA and HEF proteins from influenza A and C viruses, respectively, have a high degree of three-dimensional structural similarities, even if the primary structures of these proteins only show a low degree of amino acid identity (12%) (Rosenthal et al., 1998). Therefore, detailed structural and biochemical analyses are needed to identify any structural similarities between the 9Z protein and other orthomyxovirus proteins. Computer analysis using the TMHMM program identified a putative membrane-spanning region (aa 350–372) and a high degree of α-helix structure in the C-terminal end of the protein (Fig. 1). However, other algorithms identified additional membrane-spanning/hydrophobic regions with a lower scores. Therefore, other experimental studies are necessary before certain conclusions can be drawn. The 9Z ORF encodes a protein with a predicted molecular mass of 42-4 kDa and the primer pair 9ZF1/9ZR1 amplified a product of the expected size from ISAV-infected ASK cells, but not from uninfected cells. The specificity of this reaction was confirmed by hybridization of this PCR product to RNA from ISAV-infected ASK cells, but not to RNA from uninfected cells. The cDNA also contained the conserved nucleotide sequence 5′ agcaaaga 3′ at the 5′end, as reported earlier for ISAV genomic segments (Krossøy et al., 1999; Sandvik et al., 2000).

Expression of 9Z and identification of gene products

Western blot analysis of the recombinant 9Z protein expressed in baculovirus-infected Sf9 cells revealed two major and one minor protein with molecular masses of approximately 39, 78 and 34 kDa, respectively. Similar results were obtained by using either the polyclonal antibody against whole virus or
the peptide antiserum (Fig. 2A, B). The lysate of Sf9 cells infected with non-recombinant baculovirus was negative with both antisera (Fig. 2C, D). Furthermore, immunoprecipitation of the recombinant protein using the anti-ISAV HA MAb revealed the same bands (39, 78 and 34 kDa) as observed with the recombinant protein lysate (Fig. 2E), linking the MAb with the recombinant protein observed with Western blot. No staining was observed in the controls, i.e. when non-recombinant baculovirus was precipitated or when an irrelevant MAb was used to precipitate the recombinant protein.

A Western blot of purified virus, detected using the polyclonal antibody against whole virus, revealed four major proteins with estimated molecular masses of 24, 43, 53 and 71 kDa (Fig. 2G), which is in agreement with previous reports (Falk et al., 1997; Kibenge et al. 2000). When the peptide antiserum was used to stain the blot containing purified virus, one major band that co-localized completely with the 43 kDa viral protein was detected (Fig. 2F), confirming the link between the 43 kDa protein and the 9Z recombinant protein. Also, this relatively wide protein band (39–46 kDa) partly co-localized with the 39 kDa band seen in the blots containing the recombinant protein.

As demonstrated by immunofluorescence, the anti-ISAV HA MAb bound to insect cells expressing the recombinant 9Z protein, whereas it did not bind to cells infected with non-recombinant baculovirus (Fig. 3A, B). The polyclonal antiserum and the peptide serum, however, reacted specifically with Sf9 cells infected with the 9Z-recombinant baculovirus.

A distinct adsorption of salmon erythrocytes to Sf9 cells infected with 9Z-recombinant baculovirus was observed (Fig. 3C), but no adsorption to cells infected with non-recombinant baculovirus was observed (Fig. 3D). This haemadsorption was inhibited by the addition of the anti-ISAV HA MAb (Fig. 3E), but not by the addition of an irrelevant MAb (Fig. 3F). Similarly, the polyclonal anti-ISAV whole virus antiserum (but not the pre-serum) also inhibited adsorption (Fig. 3G), confirming that the reaction was virus-specific. Complete inhibition with the MAb was obtained with dilutions up to 1:20, whereas the polyclonal antiserum inhibited haemadsorption when diluted up to 1:100. The peptide serum showed no inhibiting effect. It has been shown previously that ISAV will not haemagglutinate erythrocytes from brown trout (Falk et al., 1997) and this was confirmed in the present study, as erythrocytes from this species did not adsorb to Sf9 cells expressing the recombinant 9Z protein (Fig. 3H).

Sequence comparisons of ISAV isolates

Sequence alignment of the predicted 9Z proteins from different ISAV isolates is presented in Fig. 1. Pairwise comparisons of the sequences from the seven isolates show identities varying between 84 and 98% at the amino acid level, with the Canadian (Bay of Fundy 97) isolates clearly separated from the European isolates. All sequences contained two potential N-glycosylation sites, except the Canadian isolate, which contained an additional site at position 151–153 (Fig. 1). However, this sequence (NPT) is considered to be weak glycosylation sequon that is probably not in use (Feldmann et al., 1988). One of the N-glycosylation sites is located at the cytoplasmic side of the predicted transmembrane region, which, if this prediction is correct, leaves us with only one potential N-glycosylation site that is shared by all isolates. A highly polymorphic region (HPR), corresponding to a predicted difference in molecular mass of 1–4 kDa between the isolates Hitra 99 and Bremnes 98/Loch Nevis 98, was identified in close proximity to the predicted membrane-spanning region.

Discussion

This work describes the cloning and identification of a cDNA fragment corresponding to an ISAV gene segment. The viral origin of this cDNA sequence was confirmed by using PCR and hybridization reactions. Analysis of the nucleotide sequence in the 5′ region of this cDNA demonstrated conserved sequences similar to those for ISAV segments described previously (Krossøy et al., 1999; Sandvik et al., 2001). The gene sequence was found to encode a protein with a predicted molecular mass of 42–4 kDa, which is in agreement with a viral protein of 43–46 kDa detected previously in purified ISAV preparations (Falk et al., 1997; Kibenge et al., 2000). MAb 3Hsf8 has been shown to be HA-specific based on its ability to inhibit haemagglutination, neutralize virus infection in cell culture and to bind to the surface of ISAV particles in immune electron microscopy reactions (Falk et al., 1998). MAb 3Hsf8 probably binds to a conformational epitope, as no reaction with purified ISAV has been demon-
Fig. 3. Immunofluorescence and haemadsorption assays on Sf9 cells. HA-recombinant (A) and non-recombinant (B) baculovirus-infected Sf9 cells were incubated with an HA-specific MAb and detected using FITC-labelled goat anti-mouse IgG (green). Cell nuclei were stained with propidium iodide. For the haemadsorption assay, a 0–25% solution of erythrocytes from Atlantic salmon (C–G) or brown trout (H) was added to Sf9 cells infected with HA-recombinant (C, E–H) or non-recombinant (D) baculovirus. Haemadsorption was inhibited with the anti-ISAV HA MAb (E), an irrelevant MAb (F) and a polyclonal anti-ISAV serum (G). Unattached erythrocytes were removed by washing and remaining cells were counterstained in DADE Diff Quick before examination in an inverted microscope.
strated by Western blotting (Falk et al., 1998), possibly explaining why screening of the lambda library with MAb 3H6F8 was negative. However, when immunofluorescence with this MAb was performed on Sf9 cells expressing recombinant 9Z protein, distinct labelling of the cell membrane was observed. Furthermore, it was shown that Atlantic salmon erythrocytes adsorbed to these cells. The specificity of haemadsorption was demonstrated by inhibition of binding using both the MAb 3H6F8 and the polyclonal antiserum raised against purified whole virus preparations. Finally, the recombinant baculovirus-infected insect cells did not adsorb red blood cells from brown trout, which is in agreement with previous observations (Falk et al., 1997). Accordingly, it is probable that the described sequence encodes the ISAV HA.

As shown by Western blotting analyses, peptide antiseras were used on baculovirus-expressed recombinant protein, a narrow band with a slightly smaller molecular mass was detected, although it showed partial co-localization with the native protein. The ISAV HA sequences probably contain only one N-glycosylation site outside the predicted transmembrane sequence. We have not investigated if this site is glycosylated, but it is known that recombinant glycoproteins expressed in insect cells may exhibit a slightly lower molecular mass when compared with native proteins following SDS–PAGE (Wagner et al., 1996). The differences observed might therefore be related to different glycosylation patterns in insect cells as compared with salmon cells, which may result in truncated oligosaccharides. Western blot analyses with recombinant protein revealed two major and one minor protein bands. The molecular mass of the larger of the two major proteins was approximately twice the mass of the smaller and we believe that this protein probably represents a tightly bound dimer that could not be separated by the SDS–PAGE dissociation buffer. The minor protein probably represents a breakdown product or a non-complete recombinant protein. The relationship of these polypeptides to the recombinant HA protein was demonstrated further by the binding of all three proteins to the anti-ISAV HA MAb, as shown by immunoprecipitation.

The predicted molecular mass of HA is similar to that observed by SDS–PAGE and Western blotting using purified virus preparation as antigen. In contrast to the influenza viruses, it appears that no proteolytic cleavage of the ISAV HA occurs. Or, that only a small part undetectable by gel-electrophoresis is removed. It is known from studying influenza viruses that uncleaved HA precursors show specific haemagglutinating activity and that this activity remains unaffected by cleavage into HA1/HEF1 and HA2/HEF2 (Herrler et al., 1979; Lazarowitz et al., 1973). However, proteolytic cleavage of the precursors is required to trigger the pH-dependent activation of the fusion activity of these proteins (Huang et al., 1981; Kitame et al., 1982; Maeda & Ohnishi, 1980; Maeda et al., 1981; Ohuchi et al., 1982; White et al., 1981). It has been demonstrated previously that addition of trypsin to the culture medium during ISAV replication has a beneficial effect on the production of infectious virus particles (Falk et al., 1997). A low pH-dependent endosomal fusion activity in the ISAV replication cycle has also been demonstrated (Eliassen et al., 2000), suggesting that the binding and uptake of ISAV is, in principle, the same as that for influenza viruses. However, our results indicate that the ISAV HA is different compared with the influenza virus HA (with regard to proteolytic cleavage) and that the presence of two disulfide-linked chains similar to influenza virus HA1–HA2 is unlikely. It is possible that fusion activity is linked with another, as yet unidentified, surface protein. It has been shown that three polypeptides of 24, 43 and 53 kDa are associated with the detergent-soluble fraction of purified viruses (K. Falk, unpublished data) and it is tempting to speculate that this profile represents the matrix protein, the HA and a possible fusion protein, respectively.

Most of the sequence variation in the HA gene was found between the isolate from Canada and the European isolates and the difference is slightly more than that found when segments 2 and 8 are used for comparison (Blake et al., 1999; Cunningham & Snow, 2000; Krossøy et al., 2001). The higher sequence variation in the HA gene is to be expected, as natural selection from host immune systems should drive the evolution of this surface protein, while the internal or non-structural proteins most probably encoded by segments 2 and 8 may have virus-specific functional constraints on evolution (Webster et al., 1992). The HPR provides most of the variation between the European isolates, while the difference between the Canadian isolate and the European isolates is evenly spread throughout the sequence. The significance of this polymorphic region is not yet understood, but it may be suitable as an epidemiological marker in the study of ISAV isolate distribution: the changes between the isolates seem to be non-random changes. However, sequences from more ISAV isolates are required to verify this observation. At present, there is only limited information available about the ISAV genes and their products. The present study of the HA sequence, with the identification of a HPR that is potentially useful in epidemiological studies, should add some valuable information to the situation.

In conclusion, this study reports the identification of the ISAV HA. We tie the haemagglutinating activity to the 43 kDa ISAV protein and support our conclusion by the following observations: (i) a MAb shown previously to react with the ISAV HA reacted with insect cells expressing the 9Z gene; (ii) the 9Z-expressing insect cells also adsorbed erythrocytes from Atlantic salmon, but not from brown trout, which is in agreement with the properties of ISAV; (iii) haemadsorption was inhibited by both the anti-ISAV HA MAb and the polyclonal ISAV whole virus antiserum; (iv) the polyclonal antiserum, which was shown to react with all the major viral proteins, also recognized the recombinant 9Z protein; (v) an
antiserum prepared against two peptides derived from the 9Z/HA sequence recognized one major band in purified virus preparations that co-localized with the 43 kDa virus protein; (vi) a protein band that partly co-localized with the 43 kDa protein was detected in lysates of insect cells expressing the recombinant protein; and (vii) this recombinant protein was also immunoprecipitated with the anti-ISAV HA MAb. The ISAV HA seems to differ from corresponding proteins in influenza viruses in that it does not appear to be post-translationally cleaved and, therefore, does not carry fusion activity, which may be found on a separate surface protein. However, further experiments are needed to clarify these points. Compared with members of the Orthomyxoviridae family, the differences in the ISAV HA support the opinion that ISAV represents a new group of orthomyxoviruses (Falk et al., 1997; Krossøy et al., 1999).

The authors wish to thank John Claessens (Intervet International) for comments on the manuscript and Lindsey Moore (Centre for Research in Virology, University of Bergen), Connie Folkestad Husøy (Intervet Norbio), Judith Tiggesler and Suzanne van Riet (Intervet International) for expert technical assistance. Lars Hamre (Department of Fisheries and Marine Biology, University of Bergen) is thanked for supplying brown trout. The HIV-1 reverse transcriptase MAb was kindly supplied by Dr Anne Marie Szilvay (Department of Molecular Biology, University of Bergen). This study was funded by Intervet International and by grant no. 128044/122 from the Norwegian Research Council.

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Received 1 November 2000; Accepted 26 February 2001