We report here the first full-length sequence of the eight ssRNA genome segments of the infectious salmon anemia virus (ISAV, Glesvaer/2/90 isolate), a salmonid orthomyxovirus-like. Comparison of ISAV genome sequence with those of others orthomyxovirus reveals low identity values, and a remarkable feature is the extremely long 5’ end UTR of ISAV segments, which all contain an additional conserved motif of unknown function. In addition to the genome nucleotide sequence determination, specific mAbs have been produced through mice immunization with sucrose-purified ISAV. Four mAbs directed against the haemagglutinin-esterase glycoprotein, the nucleoprotein and free or actin-associated forms of the matrix protein have been characterized by (i) indirect fluorescent antibody test; (ii) virus neutralization; (iii) radioimmunoprecipitation and (iv) Western blot assays. These mAbs will potentially be useful for the development of new diagnostic tests, and the nucleotide sequences will help to establish a reverse genetics system for ISAV.

Infectious salmon anemia (ISA) was first observed in Norway from Atlantic salmon (Salmo salar L.) in 1984 (Thordal & Djupvik, 1988). ISA outbreaks have since caused considerable economic losses to the Norwegian salmon farming industry and have also been reported in Scotland (Rowley et al., 1999), in the eastern coast of Canada (Bouchard et al., 1999; Lovely et al., 1999) and has been associated with extensive outbreaks in Chile since June 2007 (Godoy et al., 2008). The mortality rate in contaminated salmon farms may vary from 15 to 100 % over several months. Although, ISA virus (ISAV) outbreaks were observed only in Atlantic salmon, the virus was detected from apparently healthy animals in other species (Raynard et al., 2001). The only exception was the isolation of ISAV from diseased Coho salmon (Oncorhynchus kisutch) in Chile in 1999 (Kibenge et al., 1999). Experimental infections showed that other species could be susceptible to ISAV infection, especially juvenile rainbow trout (Oncorhynchus mykiss) for which mortality was recorded after both virus injection and bath immersion, which may represent a threat to the trout industry (Biacchesi et al., 2007).

The aetiological agent of ISA was identified as an orthomyxovirus-like with a negative-stranded RNA genome consisting of eight segments (Dannevig et al., 1995; Falk et al., 1997; Mjaaland et al., 1997), and constitutes the only member of the genus Isavirus (Kawaoka et al., 2005). ISAV is an enveloped virus composed of four major structural proteins: the nucleoprotein (66–72 kDa), the matrix protein (22–25 kDa) and two envelope glycoproteins that are the haemagglutinin-esterase glycoprotein (42 kDa) and the fusion protein (47–50 kDa) (Clouthier et al., 2002; Falk et al., 2004). Sequence characterization of ISAV genome segments is almost fully achieved, except for several segment end UTRs (Sandvik et al., 2000). It is now evident that the gene coding assignment to each segment and the putative functions of the ISAV proteins are unique and significantly different from those of other orthomyxoviruses. The ISAV genome encodes at least 10 proteins. ISAV genome segments 1–6 encode one protein each and by analogy to other orthomyxoviruses and additional characterizations, the ISAV proteins are as follows: PB2, component of RNA polymerase (Snow et al., 2003); PB1, RNA-dependent RNA polymerase core motif (Krossoy et al., 1999); NP, nucleoprotein (Aspehaug et al., 2004; Ritchie et al., 2001; Snow & Cunningham, 2001); PA; component of RNA polymerase (Ritchie et al., 2001); F, fusion protein (Aspehaug et al., 2005; Clouthier et al., 2002), and HE, haemagglutinin-esterase glycoprotein containing both receptor-binding and receptor-destructing enzyme domains (Hellebo et al., 2004; Kristiansen et al., 2002; Krossoy et al., 2001; Rimstad et al., 2001). Similar to influenza viruses, the two smallest ISAV
genome segments encode at least two proteins each. Segment 7 produces a collinear transcript mRNA (S7 ORF1) encoding an antagonist of type I interferon (IFN) response, whereas S7 ORF2, protein, identified as a putative nuclear export protein, is encoded by a spliced mRNA (Bierin et al., 2002; Garcia-Rosado et al., 2008; Kibenge et al., 2007; McBeath et al., 2006; Ritchie et al., 2002). Segment 8 produces an mRNA transcript containing two overlapping ORF. The smaller ORF1 encodes the matrix protein (M) and the S8 ORF2 was recently shown to be involved in the modulation of type I IFN response together with S7 ORF1 (Bierin et al., 2002; Garcia-Rosado et al., 2008).

In the present study, we used the ISAV Glesvaer/2/90 isolate amplified by passages onto the salmonid-derived cell line TO (Wergeland & Jakobsen, 2001) maintained in L15 medium (Invitrogen) in the absence of FBS at 15°C cell line TO (Wergeland & Jakobsen, 2001) maintained in isolate amplified by passages onto the salmonid-derived In the present study, we used the ISAV Glesvaer/2/90 genome total length was found to be 13227 nt and very similar to that of influenza virus A/PR/8/34 isolate for which 11 of the 12 first nucleotides at the 3’ termini were common to all the segments, contrasting with influenza A virus (IAV; A/PR/8/34 isolate) for which 11 of the 12 first nucleotides at the 3’ termini are common to all the segments. A moderate sequence identity could be found between ISAV 3’ UTR and those from other orthomyxoviruses. Moreover, ISAV 3’ UTRs were found to be extremely variable in length (7–48 nt) with three of the eight segments displaying only 7–9 nt upstream of the start codon. For comparison, the IAV 3’ UTR of each segment varies in length from 20 to 45 nt. In contrast, the ISAV 5’ UTR were extremely long with 67–147 nt in length compared with 20–58 nt for IAV. Only 10 of the 21 extreme nucleotides at the 5’ termini were common to all ISAV segments compared with 17 nt for all IAV segments. These 21 extreme nucleotides of the 5’ termini present a uridine-rich region that serves as polyadenylation signal, which was shorter for ISAV segments than those of IAV (3–5 versus 5–6 uridines, respectively). Despite the shorter size of the uridine-rich region in ISAV segments, the position of these regions correlates with the beginning of the polyadenosine tail found in the 3’ ends of the mRNA sequences available in GenBank. The most interesting characteristic of the ISAV 5’ UTR was the presence of a conserved motif in all segments upstream of the polyadenylation signal, which was not found in other orthomyxoviruses: C(N)3AACU(N)3A. Finally, as described for IAV, the 5’- and 3’-terminal sequences of each segment showed partial and inverted complementarity (data not shown).

To generate mAbs specific for ISAV, six-week-old BALB/c mice were immunized with sucrose-gradient purified virus from a pool of infected-TO cell supernatants (approx. 120 ml) as described previously (Mori et al., 2005). Four specific anti-ISAV secreting hybridomas was selected by testing their culture supernatants by indirect fluorescent antibody test (IFAT) against mock- and ISAV-infected cells. After subcloning these four positive hybridomas, ascite fluids were produced. The reactivity of these mAbs was tested by IFAT on ISAV-infected cells. TO cells were infected at a low m.o.i., and 3 days post-infection cells were incubated either after fixation with alcohol/acetone (Fig. 2a; fixed cells) or directly (Fig. 2b; live cells) with the mAbs. On mock- and ISAV-infected TO cells fixed and permeabilized, the four mAbs, namely B1, B23, B24 and E14, were found to be highly specific for ISAV proteins with optimal dilutions of 1:2500, 1:10000, 1:5000 and 1:25000, respectively (Fig. 2a). Only the B23 mAb was reactive on infected live cells (Fig. 2b) and thus was supposed to recognize an ISAV protein exposed on the
external side of the cell membrane. B23 mAb displays a strong neutralizing activity (neutralizing titre of 1:10 000 not shown). Depending on the mAb used the fluorescence pattern observed differed. B23 mAb exhibited staining at the cellular membrane that clearly underlined the cellular projections together with a diffuse staining of the cytoplasm. In contrast, B1 mAb recognized a viral protein located both in the nucleus and in the cytoplasm of the infected cells. B24 and E14 mAbs exhibited staining mainly in the cytoplasm and to a lesser extent compared with B1 in some nucleus. In the cytoplasm, B24 mAb detected a viral protein strongly accumulated around the nucleus and in some infected cells, round perinuclear structures could be observed (Fig. 2c). In contrast, E14 mAb displayed staining throughout the cytoplasm and on the interior side of the cellular membrane that seems to co-localize in part with the cellular microskeleton (Fig. 2c). Finally, to characterize the viral protein recognized by each mAb, TO cells were transfected with eukaryotic expression vectors pcDNA1.1/Amp (Invitrogen) encoding the full set of ISAV proteins cloned from individual RT-PCR products. The overlapping ORFs of segment 8 encoding M1 and M2 were amplified using specific primers in which the ATG start codon of the alternative ORF was removed by a silent mutation (Supplementary Table S2, available in JGV Online). As shown in Fig. 2(c), a similar fluorescence pattern could be observed after transfection of the targeted viral protein alone compared with that obtained on ISAV-infected cells (Fig. 2a) for each mAb. As expected from the above observations, B23 mAb recognized a viral envelope protein (HE). B1 mAb was specific to the NP protein, which is in agreement with its nuclear and cytoplasmic staining. Surprisingly, B24 and E14 mAbs were directed against the matrix M protein despite exhibiting a different staining.

To determine if E14 mAb recognizes a cytoskeletal-associated form of M protein, mock- (Fig. 2d) or ISAV-infected TO cells (Fig. 2e) at 3 days post-infection were stained for actin filaments as well as for M. M and actin filaments exhibited strong co-localization, especially at the interior side of the cellular membrane. Similar distribution of M could be observed in cells transfected with M alone (Fig. 2c, panel E14/M1 and data not shown), excluding any participation of other viral proteins for M-actin filament co-localization. Thus, these results suggest an interaction of the M protein with actin filaments.

The reactivity of the four ISAV mAbs was evaluated through various assays. The four mAbs were tested first for their reactivity by Western blot against either sucrose-purified ISAV or total lysates from mock- or ISAV-infected TO cells. The left panel of Fig. 3(a) illustrates the protein profile of sucrose-gradient purified virion directly visualized by Coomassie blue staining after separation on a polyacrylamide gel. This profile is composed of four major structural proteins: the NP (68 kDa), the matrix M (22 kDa) and the two envelope glycoproteins HE (42 kDa) and F (49 kDa), as described previously (Clouthier et al., 2002; Falk et al., 2004). It can be seen by the Western blot analysis that the B1, E14 and B24 mAbs were reactive against sucrose-gradient purified virion and ISAV-infected cell lysates as well, and recognized NP and M proteins, respectively, as previously characterized by IFAT. No background was detected in mock-infected lysates. The B23 mAb failed to detect HE in Western blot under reducing and denaturing conditions. However, in non-reducing and non-denaturing conditions, an HE-specific band was detected by B23 mAb at least with the sucrose-gradient purified virion sample (Fig. 3b). Notably, B23 mAb only reacts with the monomeric form of HE and does not recognize the multimeric forms of HE as previously visualized after immunoprecipitation of radiolabelled-ISAV virions with an anti-HE mAb (Falk et al., 2004).
These mAbs were then tested for their ability to immunoprecipitate ISAV proteins from \[^{35}\text{S}\text{]}	ext{methionine radiolabelled virus-infected cell lysates (Fig. 3c). B1 and B23 mAbs against NP and HE, respectively, recognized proteins of expected molecular mass (approx. 68 and 43 kDa, respectively). For the two mAbs against M, only E14 was reactive in an immunoprecipitation assay. No protein could be detected with any mAbs when mock-infected radiolabelled cell lysates were used.

In this study, we have presented for the first time the complete genome nucleotide sequence for an ISAV isolate. Knowledge of the non-coding 3’ and 5’ end sequences is an absolute prerequisite for the elaboration of a functional reverse genetics system for this atypical orthomyxovirus. In addition, we have generated and characterized mAbs against some of the ISAV proteins that might be very useful for the development of diagnostic tools for this salmonid virus.

Fig. 2. Reactivity of the mAbs in IFAT on ISAV-infected or ISAV-derived protein transfected TO cells. (a, b) Cells were infected with ISAV Glesvaer/2/90 isolate at an m.o.i. of 0.05 p.f.u. per cell. At 3 days post-infection, cells were incubated with the indicated mAbs after fixation with alcohol/acetone (a, fixed cells) or directly (b, live cells). (c) Cells were transfected with 2 μg expression vector encoding the ISAV proteins as indicated. At 2 days post-transfection, cells were incubated with the indicated mAbs after fixation. (d, e) Cells were either mock- (d) or infected with an m.o.i. of 0.01 p.f.u. per cell (e). At 3 days post-infection, cells were fixed and F-actin was stained with phalloidin (red) before incubation with the anti-ISAV M E14 mAb (green). In all experiments, nucleus staining (blue) was performed with Hoechst 33342 for live cells and DAPI for fixed cells. Bars, 10 μm.
were reacted with a peroxidase-conjugated goat anti-mouse IgG antibody to a membrane (right panels of a and b). The membranes were stained with Coomassie blue staining (left panel of a) or electrotransferred onto nitrocellulose under reducing (b) or non-reducing and non-denaturing conditions (a). The proteins were either directly visualized by Coomassie blue staining or immunoprecipitated using anti-ISAV mAbs as indicated and separated on a 4–20% polyacrylamide gel. The electrophoretic profile of the total ISAV-infected cell lysates is shown on the right together with the molecular mass of the complete unmodified protein as calculated from the nucleotide sequence. Molecular marker (M) is shown, with molecular masses in kilodaltons indicated at the left.

Fig. 3. Reactivity of ISAV mAbs in Western blot and immunoprecipitation assays. Sucrose gradient-purified virions of ISAV (V) and mock-infected (NI) or ISAV-infected (I) total cell lysates were analysed on 4–20% polyacrylamide gels under reducing and denaturing conditions (a) or non-reducing and non-denaturing conditions (b). The proteins were either directly visualized by Coomassie blue staining (left panel of a) or electrotransferred onto a membrane (right panels of a and b). The membranes were incubated with anti-ISAV mAbs as indicated. Bound antibodies were reacted with a peroxidase-conjugated goat anti-mouse IgG antibody and visualized by chemiluminescence. (c) [35S]Methionine radio-labelled lysates of NI or I cells were immunoprecipitated using anti-ISAV mAbs as indicated and separated on a 4–20% polyacrylamide gel. The electrophoretic profile of the total ISAV-infected cell lysates is shown on the right. The ISAV proteins are indicated on the right together with the molecular mass of the complete unmodified protein as calculated from the nucleotide sequence. Molecular marker (M) is shown, with molecular masses in kilodaltons indicated at the left.

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