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Characterisation of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut *Hippoglossus hippoglossus* and design of an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay

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ABSTRACT: A 1349 nucleotide fragment of the RNA2 from a nodavirus affecting Atlantic halibut *Hippoglossus hippoglossus* was characterised and the nuclotide sequence (accession no. AJ245641) was employed to develop an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. The sequenced part of the RNA2 of Atlantic halibut nodavirus (strain AH95NorA) was highly similar in organisation to that of the RNA2 of striped jack nervous necrosis virus (SJNNV), and comprised features common to all nodaviruses. These characteristics confirmed that the virus that causes viral encephalopathy and retinopathy (VER) in Atlantic halibut is a nodavirus. The nucleotide sequence of the 1349 nucleotide fragment of Atlantic halibut nodavirus RNA2 was 80% identical to the RNA2 of SJNNV. The T2 region (830 nucleotides) of the RNA2 of Atlantic halibut nodavirus shared 98% of the nucleotide sequence when compared with the homologous region of barfin flounder nervous necrosis virus (BFNNV), while the nucleotide sequence identity to SJNNV in this region was 76%. Phylogenetic analysis based on the nucleotide sequences of the T4 region (421 nucleotides) of Atlantic halibut nodavirus and of other fish nodaviruses revealed a close relationship to the nodaviruses of the barfin flounder clad that have been found in other cold-water species (Pacific cod *Gadus macrocephalus* and barfin flounder *Verasper moseri*). The nucleotide sequence of the RNA2 of Atlantic halibut nodavirus included some features that differ from that of SJNNV. The ORF of the RNA2 of Atlantic halibut nodavirus lacked 6 nucleotides through a single deletion and a 5-nucleotide deletion, separated by 4 nucleotides. The 3'-non-encoding region contained a 21 nucleotide insert and a 3 nucleotide deletion when compared with SJNNV. In comparison with the RNA2 of SJNNV, the 3'-non-encoding region showed a nucleotide sequence identity of 84.5%. A primer set based on the Atlantic halibut nodavirus nucleotide sequence was employed in order to design an optimal RT-PCR. The detection limit of the PCR was 10 to 100 copies of plasmid, while the detection limit of the RT-PCR assay was 100 to 1000 copies of *in vitro* transcribed viral RNA.

KEY WORDS: Fish nodavirus · Atlantic halibut · RNA2 · Capsid protein gene · RT-PCR

INTRODUCTION

The viruses of the Nodaviridae are pathogenic to insects and fish. Fish nodaviruses cause the disease viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), which affects a variety of farmed marine teleosts (Munday & Nakai 1997) and often results in high mortality, particularly in the larval and juvenile stages. VER is characterised by the high neurovirulence of the fish nodaviruses, and

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lesions comprised of cellular vacuolation and neuronal degeneration may be found in the brain, retina, spinal cord and ganglia.

Most of our knowledge of the structure and molecular biology of the Nodaviridae has been obtained from studies of viruses isolated from insects (for review see Schneeman et al. 1998). The virions are unenveloped and have icosahedral capsids with diameters ranging from 25 to 30 nm. Their genomes consist of 2 molecules of messenger sense RNA, both of which are encapsidated in the same virion. RNA1 (3.1 kb) carries a gene that encodes the putative RNA-dependent RNA polymerase (Protein A). RNA2 (1.4 kb) contains a main open reading frame encoding the capsid protein precursor α, which is cleaved to the capsid proteins β and γ during viral maturation in insect nodaviruses. In addition, a subgenomic transcript of RNA1, RNA3, which encodes a non-structural protein (Protein B), is present in the infected cell.

The nucleic acids and structural proteins of the nodaviruses affecting striped jack *Pseudocaranx dentex* (striped jack nervous necrosis virus [SJNNV]), European sea bass *Dicentrarhcus labrax* (*D. labrax* encephalitis virus [*Dl*EV]) and barramundi *L. calcarifer* (*Lates calcarifer* encephalitis virus [*Lc*EV]) have been partially characterised (Mori et al. 1992, Comps et al. 1994) and these viruses belong to the Nodaviridae. The complete RNA2 of SJNNV and *Dl*EV have been sequenced, and like insect nodaviruses they contain a main open reading frame that encodes the capsid protein (Nishizawa et al. 1995, Delsert et al. 1997). Although fish nodaviruses seem to be highly similar to insect nodaviruses in the organisation of genomic RNA and in other physical properties, the RNA2 of insect and fish nodaviruses share a low nucleotide sequence identity (Nishizawa et al. 1995, Delsert et al. 1997). Furthermore, capsid protein processing seems to differ between the groups, supporting the notion that fish nodaviruses possess unique features and constitute a group distinct from the insect nodaviruses (Delsert et al. 1997). Comparisons between the nucleotide sequences of a variable region (T4) of the viral capsid protein gene of more than 20 nodavirus strains showed that these may, according to this criterion, be divided into 4 major clads: the striped jack clad, the redspotted grouper clad, the tiger puffer clad and the barfin flounder clad (Nishizawa et al. 1997). The RNA2 of a nodavirus from European sea bass from the Mediterranean was found to have a sequence 99.5% identical to that of viruses of the redspotted grouper clad found in Japan (Sideris 1997), while a nodavirus affecting European sea bass from the Atlantic coast of France (Thiéry et al. 1999) could not be assigned to any of these clads. The significance of the genetic diversity among fish nodaviruses is unclear. Although it has been demonstrated that nodaviruses with distinct genomes may infect the same teleost species (Nishizawa et al. 1997, Thiéry et al. 1999), evidence of a restricted host range or adaptation to different temperature optima has been presented (Totland et al. 1999).

Since 1995, incidences of high mortality associated with a nodavirus-like agent have been reported from juvenile rearing facilities for Atlantic halibut *Hippoglossus hippoglossus* in Norway. The virus detected in Atlantic halibut is morphologically indistinguishable from other fish nodaviruses, and it cross-reacts with polyclonal antisera raised against SJNNV and *Dl*EV (Grotmol et al. 1997). The primary aim of the present study was to further characterise the virus by investigating its genetic relationship to other fish nodaviruses by comparing the sequences of their capsid protein genes, and by characterising a recombinant partial capsid protein.

In diagnostic, epidemiologic and scientific work sensitive and reliable detection methods for nodavirus are essential. A reverse-transcriptase polymerase chain reaction (RT-PCR) (Nishizawa et al. 1994) has been designed to detect SJNNV, but this method has proved to be suboptimal for other nodavirus strains (Thiéry et al. 1999). In addition, it is possible to detect certain nodavirus strains by means of a fish cell line (SSN-1) derived from striped snakehead *Channa striatus* (Frerichs et al. 1996), but the Atlantic halibut nodavirus does not replicate efficiently in these cells (Grotmol unpubl. results). The second aim of our study was to utilise the RNA2 sequence acquired to develop a sensitive RT-PCR for detection of the nodavirus affecting Atlantic halibut, thus expanding the repertoire of diagnostic tools available.

MATERIALS AND METHODS

Virus source. Dead and moribund Atlantic halibut juveniles were collected from a commercial rearing facility in the western part of Norway during an outbreak of high acute mortality. The fish were stored at –80°C. Histopathological and immunohistochemical examination revealed lesions typical of VER as described by Grotmol et al. (1997). Eyes and brains were dissected free and used as source of viral RNA. The nodavirus strain from this outbreak was denoted AH95NorA. As a positive control, striped jack larvae infected with SJNNV strain SJ93Nag (Nishizawa et al. 1997) were utilised.

RT-PCR amplification and cloning. Total RNA was extracted from the brains and eyes of the Atlantic halibut and from whole striped jack larvae (75 mg of tissue) using Trizol® Reagent (Life Technologies, New York, USA) according to the protocol provided by the manufacturer. Target region 2 (T2) of the capsid pro-

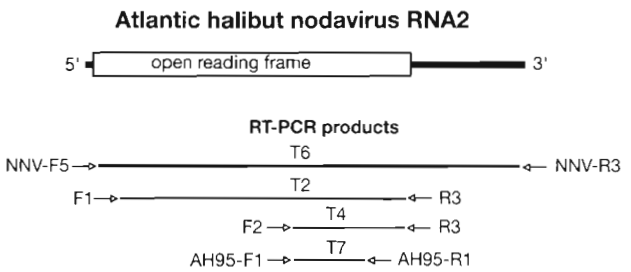


Fig. 1 Schematic map of the nodavirus RNA2, RT-PCR products and corresponding primers

tein genes (Fig. 1) of the 2 nodaviruses were amplified using an RNA-PCR kit (Perkin Elmer, CT, USA) as described by Nishizawa et al. (1994) using the modified primers F1 exp (5'-aaacatatg-GGATTTGGACGT-GCGACCAA-3') and R3 exp (5'gctaagcttca-CGAGT-CAACACGGGTGAAGA-3'). The sense primer F1 exp (nt 155–174) included 6 additional nucleotides as a linker composing the *Nde* I recognition site in addition to the initiation codon (atg), while the antisense primer, R3 exp, had 11 additional nucleotides of linker containing the *Hin*d III recognition site and termination codon. Target region 6 (T6) (Fig. 1) was amplified using the sense primer VNN-F5 (5'-ATGGTACGCAA-AGGTGA-3') and antisense primer VNN-R3 (5'GGC-CATTTAACCACATG-3') (Nakai et al. 1995). The RT-PCR procedure was performed as above with the exception of a higher concentration of $MgCl_2$ (2.5 mM) and an annealing temperature of 50°C. The amplified products were analysed by 1.5% agarose gel electrophoresis. The T2 and T6 regions were cloned into the pCR-Script SK(+) vector using the pCR-Script cloning kit (Stratagene Inc., CA, USA) utilising the manufacturer's protocol, and the plasmids were denoted pAHT6 and pAHT2, respectively.

Sequence determination. The cloned PCR products were sequenced by means of the ABI dye primer cycle sequencing kit (Perkin Elmer), an automatic thermal cycler and the ABI auto sequencer A737-36, used according to the manufacturer's recommended procedures. The sequences were obtained by sequencing serveral cDNA clones in both directions according to standard procedures and the sequences were assembled and analysed with the Mac Dnasis program (Hitachi Software Engineering, CA, USA).

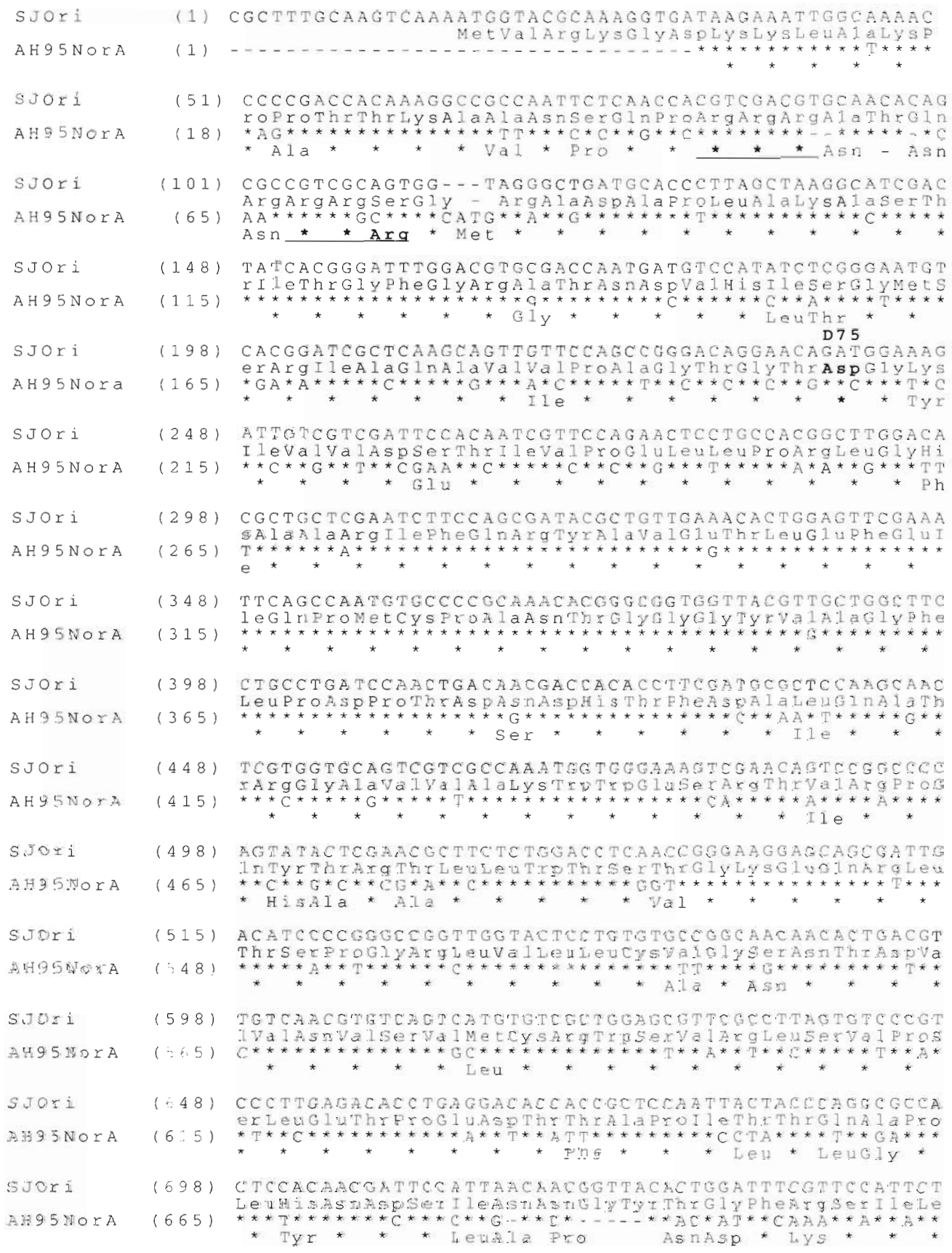
Sequence alignment and phylogenetic analysis. The sequence of the T2 region was compared with previously published homologous sequences of SJNNV, the tiger puffer nervous necrosis virus (TPNNV), red-spotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous necrosis virus (BFNNV) (Nishizawa et al. 1995). A dendrogram based on the T4 sequence (Fig. 1) of the nodavirus from Atlantic halibut and the T4 sequences of 25 other fish nodaviruses (Nishizawa et al. 1997) was generated using the Clustral W (Thompson et al. 1994) and the TreeView (Roderic 1996) computer programs.

Design of RT-PCR detection. Based on the sequence of the RNA2 from AH95NorA, new primers were designed using the VectorNTI software (InforMax, Bethesda, USA). These primers, designated AH95-F1 (5'-AGTGCTGTGTCGCTGGAGTG-3') and AH95-R1 (5'-CGCCCTGTGTGAATGTTTTG-3'), generate a PCR fragment of 341 bp (T7) (Fig. 1). The PCR Optimisation Kit from Boehringer Mannheim (Ingelheim, Germany) was used to optimise the reaction regarding $MgCl_2$ concentration and pH. The result was the following procedure for the RT reaction: 1 µl of RNA isolated by the TriZol method described above was added to 9 µl RT-reaction mix containing (final concentrations) 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 10 µM Random Hexamer primers (Perkin Elmer), 2 mM each of dATP, dCTP, dGTP, dTTP, 1 unit RNase inhibitor (Perkin Elmer) and 2.5 units MuLV RT (Perkin Elmer). The mixture was incubated at 22°C for 10 min, at 42°C for 5 min, and finally at 95°C for 10 min.

For the PCR, 10 µl of the RT reaction fluid was added to 40 µl PCR reaction mix containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.25 mM $MgCl_2$, 0.25 µM of each of the primers AH95-F1/AH95-R1, and 1.25 units AmpliTaq DNA polymerase (Perkin Elmer). The mixture was heated to 95°C for 2 min, and then submitted to 30 cycles each of 50 s at 95, 56 and 72°C, respectively. Finally, the reaction was heated to 72°C for 5 min and then cooled to 4°C. An aliquot of 7 µl of the reaction volume was added to sample buffer and run on a 1% agarose gel in TBE buffer using standard methods (Sambrook et al. 1989).

Sensitivity of the PCR amplification. From a starting concentration of 1 µg $µl^{-1}$, the plasmid pAHT6 was 10-fold serial diluted. Samples containing from 10^{10} to less than 1 copy of the plasmid molecule were subjected to PCR, employing similar conditions as for the RT-PCR described above. Samples of 7 µl of the 50 µl reaction volumes were then subjected to 1% agarose gel electrophoresis in TBE buffer.

***In vitro* transcription of viral RNA.** The plasmid pAHT6 was linearised by *Bgl* II digestion (Fig. 2) and purified by means of a Qiaquick Nucleotide Removal kit (Qiagen, Basel, Switzerland). RNA was *in vitro* transcribed using T7 polymerase and the RiboMAX Large Scale Production System from Promega (Madison, WI, USA) according to the procedure supplied by the manufacturer, except that the RNA at the final step was purified using TriZol. The RNA was treated with DNase and the presence of template DNA was controlled by PCR. The concentration of RNA was determined by measuring the optical density at 260 nm


SJOri (1) CGCTTTGCAAGTCAAAATGGTACGCAAAGGTGATAAGAAATTGGCAAAAC
MetValArgLysGlyAspLysLysLeuAlaLysP
AH95NorA (1)
SJOri (51) CCCCGACCACAAAGGCCGCCAATTCTCAACCACGTCGACGTGCAACACAG
roProThrThrLysAlaAlaAsnSerGlnProArgArgArgAlaThrGln
AH95NorA (18)
Ala Val Pro Asn - Asn
SJOri (101) CGCCGTCGCAGTGG---TAGGGCTGATGCACCCTTAGCTAAGGCATCGAC
ArgArgArgSerGly - ArgAlaAspAlaProLeuAlaLysAlaSerTh
AH95NorA (65)
Asn Arg Met
SJOri (148) TATCACGGGATTTGGACGTGCGACCAATGATGTCCATATCTCGGGAATGT
rIleThrGlyPheGlyArgAlaThrAsnAspValHisIleSerGlyMetS
AH95NorA (115)
Gly LeuThr
D75
SJOri (198) CACGGATCGCTCAAGCAGTTGTTCCAGCCGGGACAGGAACAGATGGAAAG
erArgIleAlaGlnAlaValValProAlaGlyThrGlyThrAspGlyLys
AH95Nora (165)
Ile Tyr
SJOri (248) ATTGTCGTCGATTCCACAATCGTTCCAGAACTCCTGCCACGGCTTGGACA
IleValValAspSerThrIleValProGluLeuLeuProArgLeuGlyHi
AH95NorA (215)
Glu Ph
SJOri (298) CGCTGCTCGAATCTTCCAGCGATACGCTGTTGAAACACTGGAGTTCGAAA
sAlaAlaArgIlePheGlnArgTyrAlaValGluThrLeuGluPheGluI
AH95NorA (265)
e
SJOri (348) TTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC
leGlnProMetCysProAlaAsnThrGlyGlyGlyTyrValAlaGlyPhe
AH95NorA (315)
SJOri (398) CTGCCTGATCCAACTGACAACGACCACACCTTCGATGCGCTCCAAGCAAC
LeuProAspProThrAspAsnAspHisThrPheAspAlaLeuGlnAlaTh
AH95NorA (365)
Ser Ile
SJOri (448) TCGTGGTGCAGTCGTCGCCAAATGGTGGGAAAGTCGAACAGTCCGGCCCC
rArgGlyAlaValValAlaLysTrpTrpGluSerArgThrValArgProG
AH95NorA (415)
Ile
SJOri (498) AGTATACTCGAACGCTTCTCTGGACCTCAACCGGGAAGGAGCAGCGATTG
lnTyrThrArgThrLeuLeuTrpThrSerThrGlyLysGluGlnArgLeu
AH95NorA (465)
HisAla Ala Val
SJOri (515) ACATCCCCGGGCCGGTTGGTACTCCTGTGTGCCGGCAACAACACTGACGT
ThrSerProGlyArgLeuValLeuLeuCysValGlySerAsnThrAspVa
AH95NorA (515)
Ala Asn
SJOri (598) TGTCAACGTGTCAGTCATGTGTCGCTGGAGCGTTCGCCTTAGTGTCCCGT
lValAsnValSerValMetCysArgTrpSerValArgLeuSerValProS
AH95NorA (565)
Leu
SJOri (648) CCCTTGAGACACCTGAGGACACCACCGCTCCAATTACTACCCAGGCGCCA
erLeuGluThrProGluAspThrThrAlaProIleThrThrGlnAlaPro
AH95NorA (615)
Phe Leu LeuGly
SJOri (698) CTCCACAACGATTCCATTAACAACGGTTACACTGGATTTCGTTCCATTCT
LeuHisAsnAspSerIleAsnAsnGlyTyrThrGlyPheArgSerIleLe
AH95NorA (665)
Tyr LeuAla Pro AsnAsp Lys

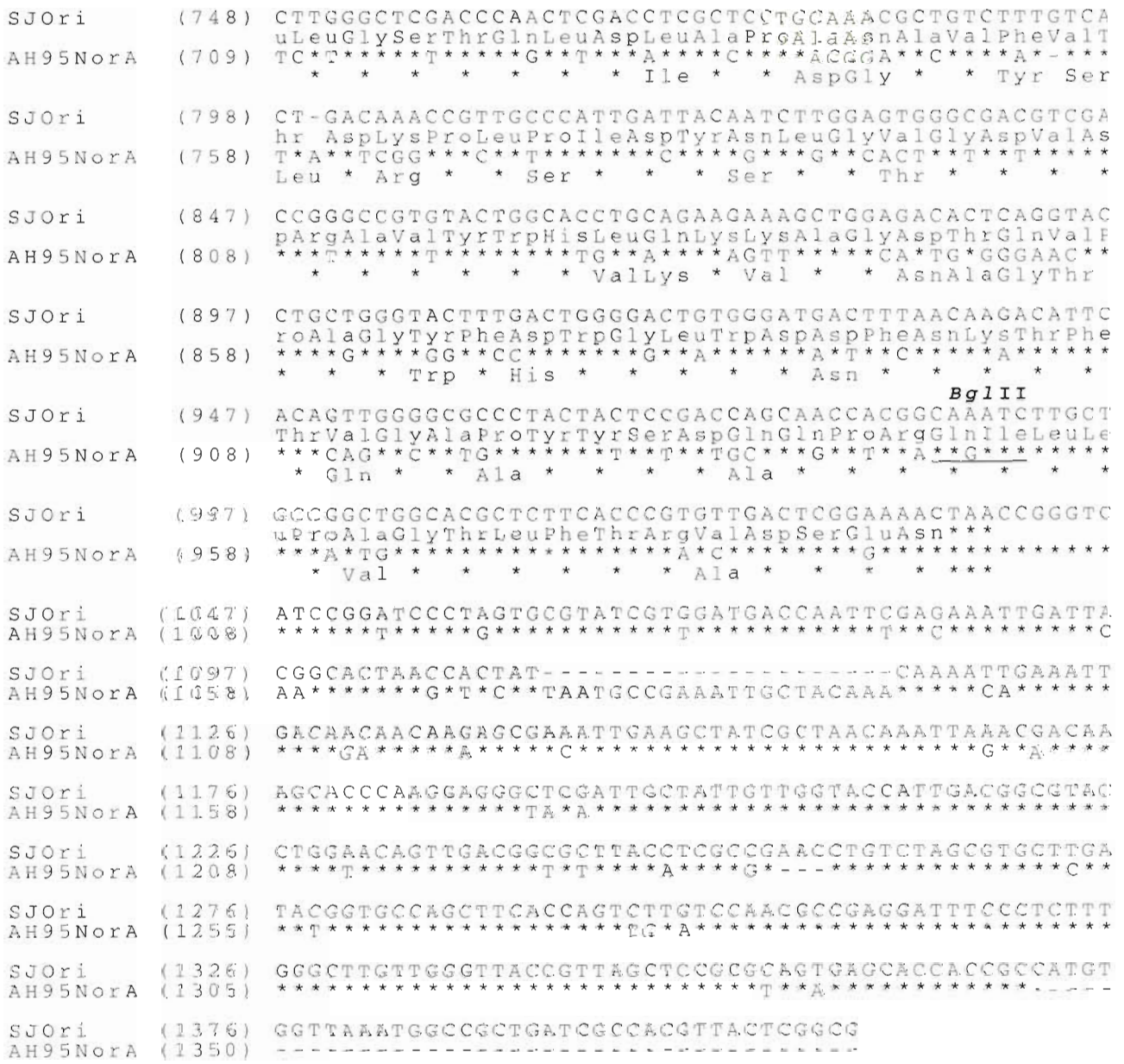


Fig. 2. (above and facing page) Alignment of the cDNA nucleotide sequence and the deduced amino acid sequence of the RNA2 from the nodavirus strains SJOri (Nishizawa et al. 1995) and AH95NorA constructed by the Clustral W program. *Nucleotides or amino acids identical to those in the same positions in SJOri. (-) Nucleotide gaps. Asp75 (D75) is in **bold** and represents a catalytic residue conserved among all nodaviruses. Two stretches of Arg residues also common to all nodaviruses are underlined and in **bold**, and are assumed to participate in the binding of the genomic RNA to the internal capsid wall. The *Bgl* II site where the *in vitro* RNA transcription terminated is indicated and underlined. The Genbank accession number is AJ245641

and the number of RNA molecules ml^{-1} was calculated. The RNA was stored in TE buffer (pH 8.0) at -80°C.

Sensitivity of the RT-PCR assay. Ten-fold dilutions of *in vitro* transcribed RNA, starting with 10^8 copies of RNA molecules $sample^{-1}$, were subjected to the RT-PCR amplification as described above. Random primers were used for the RT reaction and AH95-F1/AH95-R1 for the PCR amplification. Samples of 7 µl of the 50 µl reaction volumes were subjected to 1% agarose gel electrophoresis in TBE buffer.

Expression of recombinant T2 protein. The T2 insert of the pAH95T2 was exerted by applying *Nde* I and *Hind* III restriction enzymes with recognition sites in the linker sequences of the forward and reverse PCR primers. T2 exerts were ligated into an expression vector

plasmid, pET-25b (+) (Novagen, WI, USA), using the DNA Ligation High Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The resulting plasmid construct was denoted pAHT2 exp. The plasmid was used to transform *Escherichia coli* (BL21) and clones containing the expression plasmid with the partial capsid protein gene ORF (T2 region) were cultured in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.4) with 50 µg ml^{-1} ampicillin. The cells were induced by resuspension in fresh LB medium containing 50 µg ml^{-1} ampicillin and 1 mM isopropylthio β-D-galactoside (IPTG). Following incubation at 37°C for 3 h, the cells were washed and resuspended in 50 mM Tris-HCl (pH 8.0) – 2mM EDTA solution. After addition to 100 µg ml^{-1} lysozyme and 0.1% (V/V) Triton X-100, the cell suspension was incubated at 30°C for 15 min and then sonicated until the solution lost its viscosity. The insoluble fraction containing the induced T2 expression protein was washed twice by centrifugation (12 000 × *g*, 15 min, 4°C) and resuspended into 50 mM Tris-HCl (pH 8.0) – 2mM EDTA solution.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The insoluble protein fraction from the induced BL21 cells and uninduced controls was submitted to SDS-PAGE (12% gel) under the reducing conditions of Laemmli (1970) and the relative amount of recombinant protein from both SJ93Nag and AH95NorA was analysed by gel scanning and the Molecular Analysis Software (Bio-Rad, CA, USA), and found to be nearly identical. The Western blot was performed by electroblotting the proteins to nitrocellulose membranes as described by Towbin et al. (1979). The membranes were incubated at room temperature for 2 h with a polyclonal rabbit antiserum against SJNNV and positive protein bands were visualised by alkaline phosphatase-conjugated antibodies, bromochloroindolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT).

RESULTS

Nucleotide sequence

The sequenced fragment of the RNA2 from the Atlantic halibut nodavirus (strain AH95NorA) was highly similar in organisation to that of the SJNNV RNA2, and seemed to contain 1 main open reading frame (ORF) (Figs. 1 & 2). Comparison with the nucleotide sequence of the 1349 base fragment from the Atlantic halibut nodavirus indicated an 80% identity to the nucleotide sequence of the RNA2 of the SJNNV (strain SJOri, Nishizawa et al. 1996) (Fig. 2). In the ORF a nucleotide sequence identity of 78.7% to SJOri was found. In the deduced amino acid sequence an aspartic acid residue in position 75 (D75) that is common to all nodaviruses was identified (Fig. 2). This residue represents part of a catalytic site which is involved in capsid protein cleavage in insect nodaviruses. Two stretches of arginine residues common to all nodaviruses were also identified. These basic residues are assumed to participate in the binding of the genomic RNA to the internal capsid wall. Adjacent to these arginines a number of other negatively charged amino acid residues were present. The nucleotide sequence of the RNA2 of Atlantic halibut nodavirus contained a number of features that differ from that of SJOri. From nucleotide 716 (referring to SJOri, Fig. 2), the Atlantic halibut nodavirus lacked 6 nucleotides, consisting of a single deletion and a 5-nucleotide deletion, separated by 4 nucleotides (Fig. 2). In the codons between nucleotide 225 and nucleotide 260, 11 out of 12 codons had base substitutions in the third position which resulted in only 1 alteration in the amino acid sequence. The 3'-non-encoding region contained an additional 21 nucleotides from position 1112 and a 3-nucleotide deletion from position 1255 when compared to SJOri. In comparison with the RNA2 of SJOri the 3'-non-encoding region showed a nucleotide sequence identity of 84.5%.

The T2 region (830 nucleotides) of the Atlantic halibut nodavirus RNA2 had a nucleotide sequence that was practically identical (98.4%) to that of the T2 region of the BFNNV, while the nucleotide sequence identity to the SJNNV in this region was 76% (Table 1). When the T4 region (421 nucleotides) of the Atlantic halibut nodavirus was compared with 25 other nodavirus strains, the nucleotide sequence identity ranged from 66 to 98%, while the identity in the deduced amino acid sequence ranged from 70 to 97% (Table 2). The T4 nucleotide sequence from the Atlantic halibut strain showed identities of 97.9 and 98.2% to nodavirus strains from barfin flounder and Pacific cod, respectively, while the identity to the strains of the striped jack group was approximately 66% (Table 2). In the molecular dendrogram the Atlantic halibut nodavirus formed a clad together with the nodaviruses from barfin flounder and Pacific cod (Fig. 3).

Table 1. Nucleotide and amino acid sequence similarities of the T2 region of the Atlantic halibut nodavirus (AH95NorA) and nodavirus strains from other teleosts (data from Nishizawa et al. 1995). (SJNNV = striped jack nervous necrosis virus; TP = tiger puffer; BF = barfin flounder; JF = Japanese flounder; RG = red spotted grouper)

	AH95NorA	
	Nucleotide level	Amino acid level
SJNNV	75.51	88.85
TPNNV	75.96	89.57
BFNNV	98.43	98.55
JFNNV	82.77	95.65
RGNNV	82.29	95.65

Table 2. Nucleotide and predicted amino acid sequence identity of the T4 region of the Atlantic halibut nodavirus to other fish nodaviruses (data from Nishizawa et al. 1997). The different nodavirus strains are shown in the same order as in Fig. 3 from top to bottom

Virus strain	Source	Sequence identity (%) to AH95NorA at: Nucleotide level	Amino acid level
TP93Kag	Tiger puffer *Takifugu rubripes* (Japan)	68.7	73.4
JF95Hok	Japanese flounder *Paralichthys olivaceus* (Japan)	68.7	71.9
SJOri	Striped jack *Pseudocaranx dentex* (Japan)	66.7	71.1
SJ91Nag	Striped jack *Pseudocaranx dentex* (Japan)	66.4	71.1
RS95Hir	Red sea bream *Pargus major* (Japan)	66.1	70.3
SJ92Nag	Striped jack *Pseudocaranx dentex* (Japan)	66.1	70.3
SJ93Nag	Striped jack *Pseudocaranx dentex* (Japan)	66.1	70.3
SJ94Nag	Striped jack *Pseudocaranx dentex* (Japan)	66.1	70.3
BF93Hok	Barfin flounder *Verasper moseri* (Japan)	97.9	96.0
PC96Hok	Pacific cod *Gadus macrocephalus* (Japan)	98.2	96.8
Ba94Aus	Barramundi *Lates calcarifer* (Australia)	74.5	84.1
JF93Hir	Japanese flounder *Paralichthys olivaceus* (Japan)	75.9	85.7
MR94Tha	Malabar reef cod *Epinephelus malabaricus* (Thailand)	75.3	86.5
RG94Oka	Redspotted grouper *Epinephelus akaara* (Japan)	76.9	86.5
JF94Wak	Japanese flounder *Paralichthys olivaceus* (Japan)	76.9	86.5
JF95Oit	Japanese flounder *Paralichthys olivaceus* (Japan)	76.9	86.5
RG91Tok	Redspotted grouper *Epinephelus akaara* (Japan)	76.9	86.5
SB95Ita	Sea bass *Dicentrarchus labrax* (Italy)	76.6	85.7
Umb95Ita	Umbrina *Umbrina* sp. (Italy)	76.9	86.5
SG94Oit	Sevenband grouper *Epinephelus septemfasciatus* (Japan)	76.9	85.7
JF95Tok	Japanese flounder *Paralichthys olivaceus* (Japan)	76.9	85.7
KG95Oit	Kelp grouper *Epinephelus moara* (Japan)	77.7	86.5
JF95Sag	Japanese flounder *Paralichthys olivaceus* (Japan)	77.2	86.5
PA94Oit	Purplish amberjack *Seriola dumerili* (Japan)	77.4	86.5
JS95Shi	Japanese sea perch *Lateolabrax japonicus* (Japan)	77.2	86.5

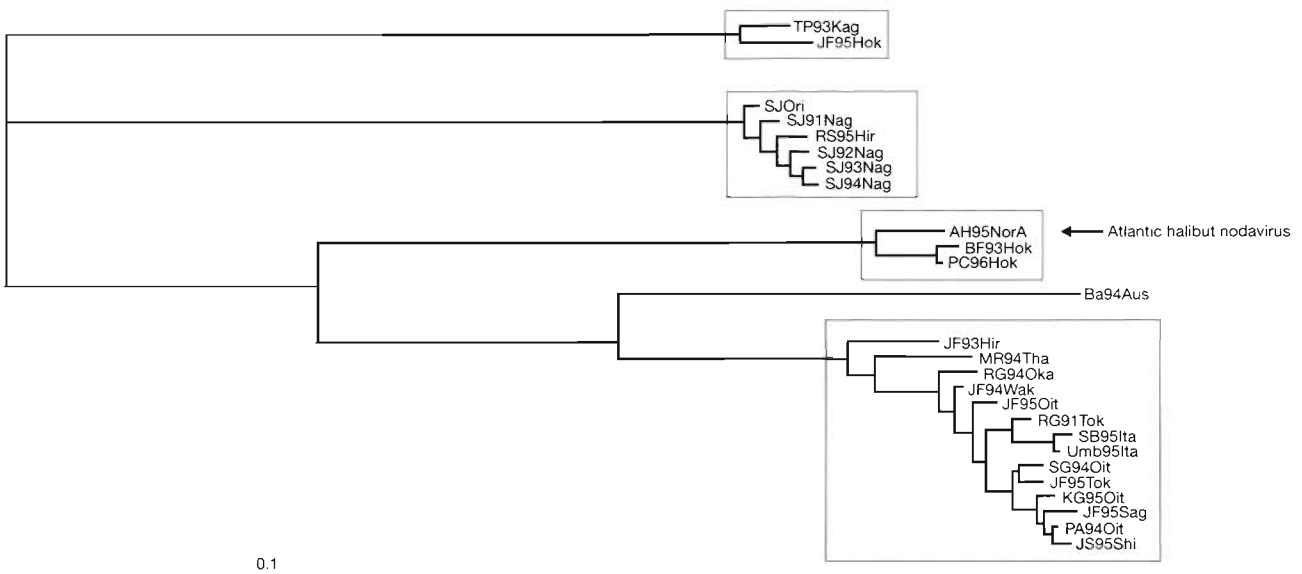


Fig. 3. The molecular dendrogram deduced from analysis of the T4 nucleotide sequence of 26 fish nodavirus strains based on data from Nishizawa et al. (1997). The dendrogram was built by the neighbour joint criteria with the Clustral W and TreeView 1.0 programs. The lengths of horizontal branches are proportional to the number of nucleotide substitutions, and the scale bar length indicates 0.1 nucleotide replacements site^{-1}. Boxes indicate the major clads. From above: tiger puffer clad, striped jack clad, barfin flounder clad and red-spotted grouper clad. Note the Atlantic halibut nodavirus in the clad with strains from the barfin flounder and the Pacific cod

RT-PCR detection assay

Serial dilution of the plasmid pAHT6 showed that 10 to 100 copies sample^{-1} could be detected by the PCR amplification (Fig. 4A). Agarose gel electrophoresis of *in vitro* transcribed viral RNA revealed 1 major band with the expected molecular weight (Fig. 4B). Serial dilution of the *in vitro* transcribed viral RNA revealed that the lower detection limit of the RT-PCR detection assay was 100 to 1000 RNA copies sample^{-1} (Fig. 4C).

SDS-PAGE and Western blot

SDS-PAGE analysis showed that the expression proteins from the T2 region of both the Atlantic halibut and the SJNNV (SJ93Nag) had a molecular mass of approximately 32 kDa (Fig. 5A). In Western blotting the anti-SJNNV serum reacted with the striped jack virus T2 expression protein and showed a weaker cross-reaction with the Atlantic halibut virus T2 expression protein (Fig. 5B). Uninduced controls did not react in the Western blot (data not shown).

DISCUSSION

Sequence analysis

Viral encephalopathy and retinopathy (VER) in larval and juvenile Atlantic halibut associated with a nodavirus-like agent has been reported previously (Grotmol et al. 1997, 1999). The significant level of nucleotide sequence identity between the RNA2 of the Atlantic halibut nodavirus and SJNNV confirms that these virus strains are closely related and that the causative agent of VER in the Atlantic halibut, as in other teleost species (Nishizawa et al. 1995, 1997, Delsert et al 1997, Munday & Nakai 1997, Sideris 1997, Thiéry et al. 1999) is a nodavirus.

Phylogenetic analysis based on the nucleotide sequence of the T4 region, which has high nucleotide sequence variability between strains, indicates that the Atlantic halibut nodavirus is closely related to the nodaviruses of the barfin flounder clad (Nishizawa et al. 1997). This clad comprises nodaviruses found in Pacific cod *Gadus macrocephalus* and barfin flounder *Verasper moseri*, which are also cold-water species. The nodaviruses within this clad are found in Pacific and Atlantic Oceans, respectively. One possible explanation of this phenomenon is that the nodavirus strains that belong to the barfin flounder clad may be adapted to replication at low temperature through selection and may have spread naturally among cold-water species in the oceans of the northern hemisphere. Alternatively, the nodavirus may have moved from the Pacific to the Atlantic Ocean or vice versa in the process of fish exports or through other human activities. Although it cannot be excluded, it is not probable that the BFNNV and AH95NorA, which have nearly identical nucleotide sequences (98.4 %) in the T2 region, have evolved independently in Japan and Norway. Further characterisation of the complete genomes and mutation rates, but also possible host ranges or temperature adapta-

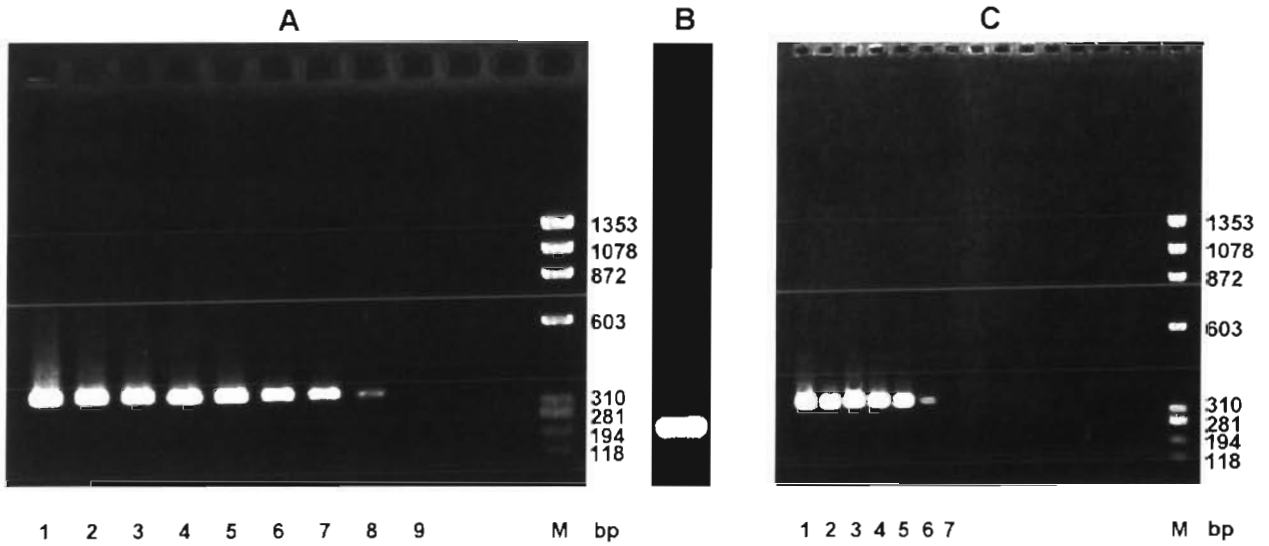


Fig. 4. Agarose gel electrophoresis. (A) Sensitivity of PCR using serial dilutions of the plasmid pAHT6 as template. The number of plasmid copies per reaction for each lane was as follows: (1) 10^{10}, (2) 10^{9}, (3) 10^{8}, (4) 10^{7}, (5) 10^{6}, (6):10^{5}, (7) 10^{4}, (8) 10^{3}, (9) 10^{2} M: DNA marker ϕx174 *Hae* III. (B) *In vitro* transcribed viral RNA. (C) Sensitivity of the RT-PCR assay using *in vitro* transcribed viral RNA as template. The number of RNA copies per reaction for each lane was as follows: (1) 10^{8}, (2) 10^{7}, (3) 10^{6}, (4) 10^{5}, (5) 10^{4}, (6) 10^{3}, (7) 10^{2}. M: DNA marker ϕx174 *Hae* III

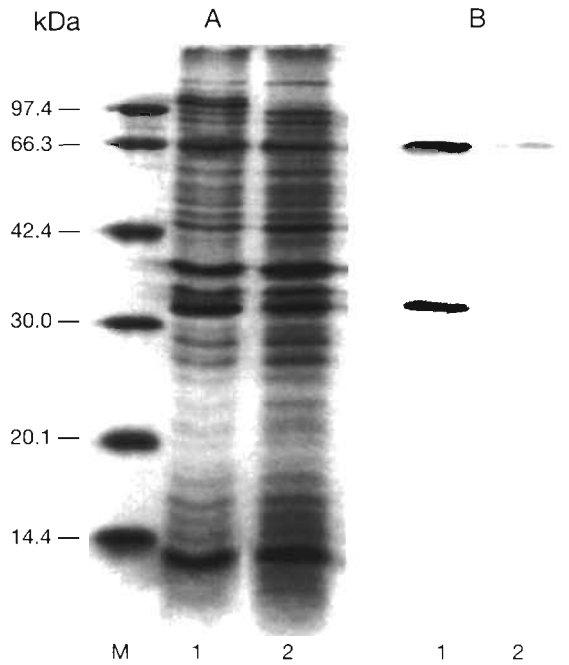


Fig. 5. (A) 12% SDS-polyacrylamide gel stained with Coomassy brilliant blue (CBB). (B) Western blot using anti-SJNNV rabbit serum. M: molecular size marker, 1: expressed T2 protein of SJNNV, 2: expressed T2 protein of Atlantic halibut nodavirus. Note the 2 bands in the Western blot, which probably represent a monomer and a dimer form of the T2 expression proteins

tions of the fish nodaviruses is necessary for a more thorough understanding of their relationship and epidemiologies.

As in the SJNNV, the RNA2 of Atlantic halibut nodavirus possesses a long 3'-non-encoding tail which, when compared to the sequence of the SJOri, is conserved and may imply an important functional or structural role of this region in fish nodaviruses.

RT-PCR assay

At present nodaviruses may be detected using RT-PCR (Nishizawa et al. 1994, Thiéry et al. 1999), immunological methods such as immunohistochemistry (Grotmol et al. 1999) or ELISA (Arimoto et al. 1992) and the striped snake head (SSN-1) cell line (Frerichs et al. 1996). The latter method may detect some nodavirus strains, but the Atlantic halibut nodavirus, for instance, does not replicate efficiently in these cells (Grotmol unpubl. results). The detection of viral genomes using RT-PCR may be hampered by the high degree of specificity of the method. The presence of sequence variation within fish nodaviruses may result in mismatches between the primers and the sequences of their putative binding sites, causing variation in sensitivity or even failure of detection (Nishizawa et al. 1996, Thiéry et al. 1999). Established RT-PCR methods (Nishizawa et al. 1994, Thiery et al. 1999) are based on specific nodaviral genomes and may be suboptimal for other nodaviruses. Our results indicate that there are mismatches between published primers and the genome of the Atlantic halibut nodavirus, necessitating a more specific RT-PCR assay. The problems associated with the specificity of RT-PCR detection of fish nodaviruses may be solved in the future if primers can be constructed that match highly conserved regions of the genomes, for instance if these are present within RNA1.

SDS-PAGE and Western blot

The recombinant proteins encoded by the T2 region reacted with antiserum raised against SJNNV but the protein derived from the Atlantic halibut nodavirus seemed to have a lower affinity to the antiserum. The positive reaction with the T2 expression protein in the Western blot experimentally confirms the putative ORF of the capsid protein gene within the RNA2. Current knowledge of the nodavirus genome does not permit the identification of genetic components relating to specific viral phenotypical features, but the existence of phenotypical differences between the 2 strains (SJ93Nag and AH93NorA) has been experimentally demonstrated in infection trials (Totland et al. 1999). The difference in staining intensity between the recombinant proteins of the 2 nodavirus strains is most likely due to differences in epitopes and may suggest the existence of serotypes within the Nodaviridae. Further studies are needed to determine whether differences between the capsid proteins of individual nodavirus strains are related to host range or other nodaviral phenotypical features.

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