Determination of the complete nucleotide sequences of RNA1 and RNA2 from greasy grouper (Epinephelus tauvina) nervous necrosis virus, Singapore strain

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The complete nucleotide sequences of RNA1 and RNA2 from grey grouper (Epinephelus tauvina) nervous necrosis virus (GGNNV), Singapore strain, were determined. 5′ RACE and RNA ligation were used to obtain the complete nucleotide sequences of the 5′ and 3′ non-coding regions (NCRs). GGNNV RNA1 was determined to be 3103 nt long, containing an ORF of 982 aa, while GGNNV RNA2 was determined to be 1433 nt long, containing an ORF of 338 aa. Both GGNNV RNAs are longer than those of other published betanodavirus sequences and the additional nucleotides were located within the NCRs. Analysis of GGNNV RNA2 revealed that it is closely related to redspotted grouper nervous necrosis virus and that both grouper viruses share the same neutralization epitope. Predicted domains for six RNA-dependent RNA polymerase motifs and two putative ORFs (proteins B1 and B2) were confirmed by sequence analysis of GGNNV RNA1.

Betanodaviruses, members of the Nodaviridae family, are the causative agents of viral nervous necrosis in fish. These viruses result in high mortality in hatchery-reared larvae and juveniles of a wide range of marine finfish species in Asia, Europe, Australia, Martinique and Tahiti (Bloch et al., 1991; Bovo et al., 1999; Breuil et al., 1991; Comps et al., 1994; Ferriehs et al., 1996; Le Breton et al., 1997; Mori et al., 1992; Munday & Nakai, 1997; Renault et al., 1991; Yoshikoshi & Inoue, 1990). In recent years, losses have also been reported in adult and mature fish (Le Breton et al., 1997). The family Nodaviridae contains two genera, betanodaviruses, which predominantly infect fish, and alphanodaviruses, which predominantly infect insects. Alphanodaviruses are the most characterized nodavirus genus to date (Ball & Johnson, 1999; Schneemann et al., 1998; Van Regenmortel et al., 2000). Nodaviruses are small, non-enveloped, spherical viruses with bipartite positive-sense RNA genomes, which are capped but not polyadenylated (Ball & Johnson, 1999; Schneemann et al., 1998). The larger genomic segment, RNA1, encodes protein A, which is the viral component of the viral RNA-dependent RNA polymerase (RdRp). The smaller genomic segment, RNA2, encodes the capsid protein (Nagai & Nishizawa, 1999; Nishizawa et al., 1995). In the alphanodaviruses, a subgenomic RNA3 is synthesized during RNA replication from the 3′ terminus of RNA1 and encodes one or two small proteins (B1 and B2) with unknown functions (Ball & Johnson, 1999; Johnson et al., 2000; Schneemann et al., 1998).

To date, a limited number of full-length betanodavirus RNA2 sequences have been determined and the only RNA1 sequence known is that from striped jack (Pseudocaranx dentex) nervous necrosis virus (SJNNV) (Aspehaug et al., 1999; Delsert et al., 1997a; Nagai & Nishizawa, 1999; Nishizawa et al., 1995, 1997, 1999). Based on the sequence analysis of RNA1 and RNA2, betanodaviruses are clearly distinguished from alphanodaviruses (Delsert et al., 1997a; Nagai & Nishizawa, 1999; Nishizawa et al., 1995). In this study, the complete nucleotide sequences of RNA1 and RNA2 from grey grouper (Epinephelus tauvina) NNV (GGNNV), Singapore strain were determined and compared to other known betanodavirus sequences. The sequences presented in this report are longer than any nodavirus sequence listed in GenBank and the additional nucleotides in our determined sequences are located within the non-coding region (NCR).

Moribund grouper showing signs of clinical neuro-pathology were collected at a marine net-cage hatchery on the
northern coast of Singapore. Virus was purified from the brain tissue and propagated on a sea bass cell line (Chong et al., 1987, 1990). When virus-induced cytopathic effect reached 90%, cells were freeze–thawed three times before tissue culture fluid was harvested, filtered through a 0.45 µm membrane and centrifuged at 100000 g for 2.5 h at 4 °C. TRIzol reagent (Life Technologies) was used to extract RNA from the virus-enriched pellet. Extracted viral RNA was then used as a template for hexamer-primed cDNA synthesis using the Expand RT (reverse transcription) system (Boehringer). A partial RNA2 fragment was amplified by Expand High Fidelity PCR (Boehringer) using degenerate primers RNA2-F1 and RNA2-R1, which were designed on the basis of the consensus RNA2 sequence from SJNNV (Nishizawa et al., 1995) and Dicentrarchus labrax (European sea bass) encephalitis virus (DLEV) (Delsert et al., 1997a). RNA2, amplified by RT–PCR, was ligated into plasmid pGEX-5X-1 and transformed into Escherichia coli strain JM105. Restriction digestion analysis and agarose gel electrophoresis confirmed the insertion of the RNA2 fragments into pGEX-5X-1. The RNA2 sequence obtained was sequenced in at least three independent clones and was further confirmed by direct sequencing of at least two separate RT–PCR products. To determine the sequence of RNA1, 16 degenerate primers were designed on the basis of the consensus sequence of RNA1 from SJNNV, black beetle virus (BBV) and flock house virus (FHV) (R. Dasgupta, personal communication; Dasmahapatra et al., 1985; Nagai & Nishizawa, 1999). A partial RNA1 fragment was successfully amplified by RT–PCR using degenerate primers RNA1-F1 and RNA1-R1 using Expand High Fidelity PCR. Agarose gel electrophoresis confirmed amplification of the RNA1 fragment by RT–PCR. The amplified RNA1 fragment was sequencing by walk-through with the degenerate primer RNA1-F1 and specific primers RNA1-F3, RNA1-F4, RNA1-F5, RNA1-F6, RNA1-F7, RNA1-F8, RNA1-F9 and RNA1-F10. At least four separate RT–PCR reactions were used to obtain the RNA1

Fig. 1. ORFs deduced from GGNNV RNA1 (a) and RNA2 (b) nucleotide sequences. The nucleotide sequence and location of the primers used in this study are shown.
sequence and each partial sequence was confirmed in at least two independent and overlapping sequences.

To determine the 5'NCR sequence and nucleotide substitution by degenerate primers used for amplification of RNA1 and RNA2, 5'RACE (5' rapid amplification of cDNA ends) was performed on both poly(A)- and poly(C)-tailed cDNAs with 5'/3'RACE (Boehringer) and specific primers RNA2-R2 and RNA1-R2. RACE products were ligated into plasmid pGEX-5x-1 and transformed into E. coli strain JM105. Restriction digestion analysis confirmed that the RACE products were inserted into the plasmid. At least five individual clones were used to determine the 5'NCR sequence, which was further confirmed by direct sequencing of at least four separate 5'RACE products. To determine the 3'NCR sequence and nucleotide substitution by degenerate primers, viral RNA was circularized with T4 RNA ligase and purified using the RNase Mini kit (Qiagen). The sequence of the ligated junction region was determined by RT–PCR using specific primers RNA1-F2 and RNA1-R2 (for RNA1), and RNA2-F2 and RNA2-R2 (for RNA2). At least four separate RT–PCR reactions were used to determine the sequence of the 3'NCR.

Automated sequencing was performed on an Applied Biosystems sequencer using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Sequence analysis was carried out using the Lasergene analysis software package (DNASTAR). A map of GGNNV RNA1 and RNA2 and the primers used for amplification and sequence analyses are shown in Fig. 1. Sequence data were deposited into GenBank under accession numbers AF319555 (RNA1) and AF318942 (RNA2).

The nucleotide and deduced amino acid sequences of GGNNV RNA1 and RNA2 are shown in Figs 2 and 3, respectively. GGNNV RNA2 is 1433 nt in length, encodes the capsid protein (nt 27–1043) and is flanked by a 26 nt 5’NCR and a 390 nt 3’NCR. The predicted molecular mass of GGNNV RNA2, as calculated from the sequence, is 459,025 Da and the encoded capsid protein (338 aa) has a calculated molecular mass of 37,004 Da. GGNNV RNA1 is 3103 nt in length, contains an ORF encoding protein A (nt 79–3027) and is flanked by a 78 nt 5’NCR and a 76 nt 3’NCR. The predicted molecular mass of GGNNV RNA1, as calculated from the sequence, is 992,735 Da. The ORF encoding protein A (982 aa) has a predicted molecular mass of 110,420 Da. Among the 30 RNA1 5’RACE clones, two were confirmed to have a guanine residue at the 5’ terminus. Assuming that GGNNV RNAs are capped like those of alphanodaviruses, then these two clones contained the 5’RACE fragment most likely to correspond to the capped form of RNA1.

Nucleotide and deduced amino acid sequences for GGNNV RNA2 were aligned with those of SJNNV (1140 nt), DIEV (1400 nt), tiger puffer (Takifugu rubripes) NNV (TPNNV) (836 nt), Japanese flounder (Paralichthys olivaceus) NNV (JFNNV) (830 nt), redspotted grouper (Epinephelus akaara) NNV (RGNNV) (830 nt), barfin flounder (Verasper moseri) NNV (BFNNV) (830 nt) and halibut (Hippoglossus hippoglossus) NNV (HHNNV) (836 nt) (Aspehaug et al., 1999; Delsert et al., 1997a; Nishizawa et al., 1995). The length of GGNNV RNA2 was 23 nt longer than SJNNV RNA2 and 27 nt longer than DIEV RNA2, although the encoded capsid protein was 2 aa shorter than SJNNV capsid protein and the same length as DIEV capsid protein. The sequence similarities between RNA2 from GGNNV and that from other known betanodaviruses was between 75.7 and 98.6% at the nucleotide level, and 80.5 and 98.9% at the amino acid level. GGNNV RNA2 was the most similar to RGNNV RNA2 at the level of both nucleotide and amino acid sequence. The increased length of GGNNV RNA2 was attributed to additional nucleotides at both the 5’ and 3’NCRs. To date, the majority of the available RNA2 sequences of betanodaviruses have been deduced from cDNA libraries (Aspehaug et al., 1999; Delsert et al., 1997a; Nishizawa et al., 1995). cDNA molecules, however, commonly lack a few nucleotides corresponding to the 5’ end of the mRNA due to the inability of reverse transcriptase to read through the entire gene sequence and due to the 3’→5’ exonuclease activity of RNA polymerase I, which removes the terminal nucleotides of the first-strand cDNA. In order to overcome these drawbacks, 5'RACE was employed to identify the extreme nucleotide at the 5’ end of the RNA, whereas the extreme 3’ nucleotide was identified following RNA self-ligation and RT–PCR amplification of the ligated region. Interestingly, both the ability to ligate betanodavirus RNAs and a previous report of DIEV RNA2 polyadenylation suggested a difference in structure from the 3’ end of the alphanodaviruses, which are reported to be resistant to in vitro ligation and polyadenylation (Dasgupta et al., 1984; Dasmahapatra et al., 1985; Delsert et al., 1997a; Kaesberg et al., 1990).

Phylogenetic comparisons of GGNNV against SJNNV, DIEV, TPNNV, JFNNV, RGNNV, BFNNV and HHNNV (Aspehaug et al., 1999; Delsert et al., 1997a; Nishizawa et al., 1995) were performed according to Nishizawa et al. (1997). Analyses revealed that the GGNNV sequence was most closely related to an RGNNV strain isolated from Japan (Tokushima), with an amino acid identity of 98.5% (results not shown). Indeed, the neutralization epitope of GGNNV was identical to that of RGNNV, as mapped by Nishizawa et al. (1997). Sequence comparison of RNA2 from barramundi (Lates calcarifer) NNV, Taiwan strain demonstrated 99.2% amino acid identity to GGNNV (Singapore strain), 99.0% amino acid identity to RGNNV (Japanese strain) and shared the same neutralization epitope (results not shown). As the host species of these viruses live in Pacific waters, it is probable that the viruses evolved from a common ancestor. However, whether this is the result of a natural, widespread distribution of nodavirus strains from a common progenitor or promiscuous translocation of fish for aquacultural purposes is not clear.

Pair-wise alignments of the nucleotide and deduced amino acid sequences of GGNNV RNA1 against those of SJNNV (Nagai & Nishizawa, 1999) revealed that GGNNV RNA1 was

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Fig. 2. Full-length nucleotide and deduced amino acid sequences of GGNNV RNA1. Protein A and the putative proteins B1 and B2 are shown. The start codon of protein B1 is indicated by an arrow. Stop codons are denoted by asterisks. The conserved RdRp motifs are underlined.
Nodavirus RNA1 and RNA2 full-length sequence

Fig. 3. Full-length nucleotide and deduced amino acid sequences of GGNNV RNA2. The stop codon is denoted by asterisks.

The neutralization epitope is underlined.

22 nt longer than SJNNV RNA1, although the encoded protein was 1 aa shorter. The sequence similarity between RNA1 from GGNNV and SJNNV was 80% at the nucleotide level and 87–4% at the amino acid level. Similar to GGNNV RNA2, the longer GGNNV RNA1 sequence resulted from additional nucleotides at both the 5'NCR and the 3'NCR.

In this study, six polymerase motifs, including a GDD motif, which is conserved in positive-strand RNA viruses, were identified in GGNNV protein A from the amino acid sequence alignment of viral RdRps (Delarue et al., 1990; Poch et al., 1989). Specifically, these motifs were the acidic motif (aa 585–590), the SG.T motif (aa 646–651), the GDD motif (aa 686–688), the basic motif (aa 712), a basic sequence preceded by an aromatic residue (aa 788) and an aromatic residue preceded by a basic sequence (aa 808). Similar motifs were also found at almost the same positions in protein A of alphanodaviruses and in exactly the same position in protein A of SJNNV (Nagai & Nishizawa, 1999). Although no study has been made concerning the existence of proteins B1 and B2 of betanodaviruses, an RNA3 of approximately 400 nt was detected in cells of sea bass larva infected with DIEV (Delsert et al., 1997b).

Two potential ORFs conforming to the Kozak consensus sequence (Kozak, 1999) were found in the sequence of GGNNV RNA1. The ORF for the putative B1 protein was translated in the same reading frame as protein A and represented the C-terminal portion (nt 2692–3027) of GGNNV protein A. The ORF for the putative B2 protein overlapped the C terminus of GGNNV protein A (nt 2753–2980) and was translated in the +1 reading frame with respect to protein A. The putative B1 protein is 111 aa long, with a molecular mass of 12068 Da, while the putative B2 protein is 75 aa long, with a molecular mass of 8524 Da. Between these two GGNNV ORFs, the B2 protein ORF has a more favourable Kozak sequence. Indeed, the ORF for protein B2 of GGNNV was the same length and at the same position as that suggested for the putative B protein of SJNNV (Nagai & Nishizawa, 1999). The amino acid identity between the B2 proteins of GGNNV and SJNNV was 74–6%. Further studies will be required to prove that these are the potential ORFs for the betanodavirus B proteins.

From previous studies of different RNA viruses, it is evident that the NCRs are functionally important. For picornaviruses, the 5'NCR contains a structural internal ribosomal entry site that mediates cap-independent translation initiation (Belsham & Sonenberg, 2000). Cellular proteins that might be involved in virus replication were reported to bind to the 3'NCR of simian haemorrhagic fever virus (Hwang & Margo, 1998). Studies with alphanodaviruses suggested that the 3'NCR of RNA2 contains primary and secondary structures that are conserved among the alphanodaviruses and might be
necessary for the replication and template recognition of RdRp (Kaesberg et al., 1990). Additionally, the 5’NCR of RNA1 was predicted to fold into stem–loop structures common to BBV and FHV, which might interfere with translational initiation (Dasmahapatra et al., 1985; Kaesberg et al., 1990). In contrast, the 5’NCR of FHV RNA2 is considered to be a single-stranded region that serves as an efficient entry site for ribosomes (Kaesberg et al., 1990). Although most of these characteristics of alphanodavirus NCRs were not found in betanodaviruses, the 5’ and 3’NCRs are likely to play important role(s) in regulating critical virus function(s) in betanodaviruses. Indeed, deletion analysis of the alphanodavirus RNA2 cDNA demonstrated that both the 5’ and the 3’NCRs were necessary for efficient replication (Ball, 1994). Although nodaviruses have been described previously in a number of fish species, little is known about the biology of these viruses. On the basis of the sequences reported here, construction of full-length recombinant GGNNV (RNA1 and RNA2) infectious clones is under way. With these infectious clones, the biological function of these viruses can be further elucidated.

We thank Dr Andrew Lucy for critical reading of the manuscript.

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Received 13 July 2000; Accepted 24 November 2000