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Establishment of an infectious RNA transcription system for *Striped jack nervous necrosis virus*, the type species of the betanodaviruses

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A system has been established to produce infectious RNA transcripts for *Striped jack nervous necrosis virus* (SJNNV), the type species of the betanodaviruses, which infect fish. An enzymological analysis suggested that both RNA1 and RNA2 of SJNNV have a 5′ cap. Both RNAs were largely resistant to 3′ polyadenylation and ligation, suggesting the presence of an interfering 3′ structure, while a small quantity of viral RNAs were polyadenylated *in vitro*. The complete 5′ and 3′ non-coding sequences of both segments were determined using the rapid amplification of cDNA ends method. Based on the terminal sequences obtained, RT–PCR was carried out and plasmid clones containing full-length cDNA copies of both RNAs, positioned downstream of a T7 promoter, were constructed. These plasmids were cleaved at a unique restriction site just downstream of the 3′ terminus of each SJNNV sequence and were transcribed *in vitro* into RNA with a cap structure analogue. A mixture of the transcripts was transfected into the fish cell line E-11. Using indirect immunofluorescence staining with anti-SJNNV serum, fluorescence was observed specifically in these transfected cells; this culture supernatant exhibited pathogenicity to striped jack larvae. Northern blot analysis of E-11 cells infected with the recombinant virus or SJNNV showed small RNA (ca. 0·4 kb) that was newly synthesized and corresponded to the 3′-terminal region of RNA1. Finally, the complete nucleotide sequences of these functional cDNAs (RNA1, 3107 nt; RNA2, 1421 nt) were determined. This is the first report of betanodavirus cDNA clones from which infectious genomic RNAs can be transcribed.

Introduction

Betanodaviruses, members of the family *Nodaviridae*, cause highly destructive diseases in hatchery-reared larvae and juveniles of a variety of marine fish species. Since its first description in 1990 (Glazebrook *et al.*, 1990; Yoshikoshi & Inoue, 1990), the disease, named viral nervous necrosis or viral encephalopathy and retinopathy, has spread to 19 or more marine fish species of 10 families in the Indo-Pacific region, Mediterranean and Scandinavia (Munday & Nakai, 1997; Office International des Epizooties, 2000). Recently, the virus has been proven to have existed in North America (Curtis *et al.*, 2001). Affected fish exhibit a range of neurological abnormalities, which are characterized by vacuolization and cellular necrosis in the central nervous system and retina.

Nodaviruses have a bipartite genome of positive-sense RNA, with RNA1 encoding the RNA-dependent RNA polymerase and RNA2 encoding the coat protein (CP). Both RNAs are capped, but not polyadenylated. During RNA replication, a subgenomic RNA3, which is co-terminal with RNA1 and encodes small proteins, is synthesized. The family *Nodaviridae* comprise two genera: *Alphanodavirus* and *Beta-*

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novirus, members of which primarily infect insects and fish, respectively (van Regenmortel *et al*., 2000). *Striped jack nervous necrosis virus* (SJNNV), which had been purified from diseased larvae of the striped jack *Pseudocaranx dentex*, was first identified as a betanodavirus (Mori *et al*., 1992). RNA1 (3·1 kb) and RNA2 (1·4 kb) of SJNNV encode a 100 kDa protein (presumably RNA-dependent RNA polymerase) and a major CP of 42 kDa, respectively (Mori *et al*., 1992; Nagai & Nishizawa, 1999). The sequence similarities of RNA2, about 870 bases in open reading frame (ORF), were less than 29% at the nucleotide level and less than 11% at the amino acid level between SJNNV and four representative insect nodaviruses, *Nodamura virus* (NoV), *Black beetle virus* (BBV), *Flock house virus* (FHV) and *Boolarra virus* (Dasgupta *et al*., 1984; Dasgupta & Sgro, 1989), whereas they were 70% or higher among four piscine nodavirus isolates (Nishizawa *et al*., 1995). On the other hand, the RNA1 sequence similarities between SJNNV and the alphanodaviruses were 28% at the nucleotide and amino acid levels (Nagai & Nishizawa, 1999).

With the progress of recombinant DNA technology, single- or double-stranded RNA viruses have been genetically analysed and infectious RNA transcripts or cDNA clones have been produced from a variety of RNA viruses (Boyer & Haenni, 1994). The alphanodaviruses can be propagated in a wide range of cultured cells, such as insect, plant, vertebrate and yeast cells, and their infectious RNA transcripts have been used frequently for RNA transfection into these permissive cells. This has led to studies of their RNA replication, gene expression and virion assembly (reviewed by Ball & Johnson, 1998). In contrast, the establishment of such a reverse genetics system for betanodaviruses has long been hampered by the lack of an appropriate cell culture system (Breuil *et al*., 1991; Mori *et al*., 1991; Munday *et al*., 1992; Nguyen *et al*., 1994; Grotmol *et al*., 1995). The studies of Frerichs *et al*. (1996) and our own group (Iwamoto *et al*., 1999, 2000) have revealed, however, that the striped snakehead cell line (SSN-1) (Frerichs *et al*., 1991) and the clonal cell line E-11 from the SSN-1 cells are useful for qualitative and quantitative analyses for all of the betanodaviruses, including SJNNV. Furthermore, it has been confirmed that the SJNNV genomic RNA is infectious when transfected into E-11 cells and that the progeny virus recovered from the cells is virulent to striped jack larvae (Iwamoto *et al*., 2001).

Recently, a reverse genetics system was developed for *Infectious pancreatic necrosis virus*, a double-stranded RNA virus of fish (Yao & Vakharia, 1998). To date, however, there is no reverse genetics system for positive-sense single-stranded RNA viruses that infect fish or aquatic animals. In general, cDNA containing entire viral genome sequences is required to obtain infectious *in vitro* RNA transcripts. Although the nucleotide sequences for RNA1 and RNA2 of SJNNV and other betanodaviruses have been published (Nishizawa *et al*., 1995, 1997; Delsert *et al*., 1997*a*; Sideris, 1997; Aspehaug *et al*., 1999; Nagai & Nishizawa, 1999; Thiery *et al*., 1999; Grotmol *et al*., 2000; Starkey *et al*., 2000), precise sequences of their 5′ and 3′ non-coding regions have not been determined. In this report, we describe the construction and sequencing of full-length cDNA clones of SJNNV and the recovery of infectious SJNNV from E-11 cells transfected with RNA transcripts that, in turn, were synthesized *in vitro* from their cDNAs. This is the first report of the production of cDNA clones of a betanodavirus from which infectious genomic RNAs can be transcribed.

Methods

■ **Cells.** The E-11 cell line (Iwamoto *et al*., 2000) was grown at 25 °C in Leibovit's L-15 medium (Gibco BRL) supplemented with 5% foetal bovine serum.

■ **SJNNV purification.** Naturally infected striped jack larvae that had been collected at the Nagasaki prefecture in Japan in 1993 were used as the source of SJNNV. SJNNV was purified as described by Mori *et al*. (1992) and stored at −80 °C.

■ **Confirmation of the 5′ and 3′ end structures of the SJNNV genome.** SJNNV virion RNA was extracted from the purified virus as described previously (Kroner & Ahlquist, 1992). The 5′ terminus of the virion RNA was treated with bacterial alkaline phosphatase from *Escherichia coli* C75 (Takara) and then labelled with T4 polynucleotide kinase (Toyobo) in the presence of [γ-^{32}P]ATP (Amersham) either with or without prior decapping treatment with tobacco acid pyrophosphatase (TAP) (Nippon gene) under the conditions recommended by the manufacturer. The 3′ terminus of each strand was treated with T4 RNA ligase (Takara) and poly(A) polymerase (Takara) in the presence of [^{32}P]pCp (Amersham) and [α-^{32}P]ATP (Amersham), respectively, according to the manufacturer's recommendations. The treated RNAs were separated by electrophoresis in 1% agarose gels and the signal was then detected by autoradiography.

■ **Determination of the 5′- and 3′-terminal sequences of the SJNNV genome.** The rapid amplification of cDNA ends (RACE) method (Frohman *et al*., 1988) was used to determine the complete nucleotide sequences of the 5′ and 3′ termini of the SJNNV genome. For 5′ RACE, virion RNA was reverse-transcribed with SuperScript II (Gibco BRL) using the synthetic oligonucleotide primers SJ1R1 or SJ2R1 (Table 1). The first-strand cDNAs were polyadenylated with terminal deoxynucleotidyltransferase (Takara) and then the second-strand cDNAs were synthesized using the primer ANCH (Table 1), after purification through the GLASS MAX Column (Pharmacia), according to the manufacturer's instructions. The double-stranded cDNAs were amplified using the primers AUAP and either SJ1R2 or SJ2R2 (Table 1). Decapped RNA, prepared as described above, was also used in 5′ RACE for the detection of cap structure. For 3′ RACE, viral RNA was polyadenylated with poly(A) polymerase in the presence of ATP and reverse-transcribed using the primer ANCH, as described above, and then amplified with the primers AUAP and either SJ1F1 or SJ2F1 (Table 1). These amplified products were purified by 1% low-melting-point (LMP) agarose gel electrophoresis and then further amplified by nested PCR using the primers AUAP and either SJ1F2 or SJ2F2. The nested PCR products were purified and used to determine the terminal sequences. Sequencing reactions were performed using the BigDye Deoxy Terminator Cycle Sequencing kit (PE Applied Biosystems), according to the manufacturer's instruction, and nucleotide sequences were determined using the automated sequencer ABI Prism 310 (PE Applied Biosystems).

Table 1. Oligonucleotide primers for the construction and determination of full-length cDNA clones of SJNNV RNAs

Oligonucleotide sequences used for both 5′ and 3′ RACE and for the synthesis of full-length SJNNV cDNA are shown. SJNNV-specific nucleotides are shown in bold and the T7 promoter sequence is underlined. Restriction sites are indicated in lowercase. The oligonucleotide positions correspond to the SJNNV full-length cDNA within pSJ1TK19 and pSJ2TK30, the sequences of which were deposited into the DNA Data Bank of Japan (DDBJ) under accession nos AB056571 and AB056572, respectively.

Purpose	Primer	Sequence (5′ → 3′)	Position
	ANCH	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT	–
	AUAP*	pGGCCACGCGTCGACTAGTAC	–
5′ RACE	SJ1R1†	**GAGATAATGTATGGCTCGTAGCC**	370–392
	SJ1R2	**AAATGCGCACTAGTTCCATCTG**	262–283
	SJ2R1†	**CACATTGGCTGAATTTCGAACTC**	349–371
	SJ2R2	**GAACGATTGTGGAATCGACGAC**	262–283
3′ RACE	SJ1F1	**GAGCTAGAACAGCTCTTCAAG**	2795–2815
	SJ1F2	**GTAGTCCAGGTAAACGAGATG**	2855–2875
	SJ2F1	**TTGATTACGGCACTAACCACT**	1101–1121
	SJ2F2	**CAACAAGAGCGAAATTGAAGC**	1142–1162
SJNNV cDNA synthesis	SJ1-5HdT7	CCCCaagcttAATACGACTCACTATAG**TAACATCAGCTCTTGCTCTG**	1–20
	SJ1-3Ec	ACCGgaatt**cGCCGAAGCGTAGGACAGCA**	3088–3107
	SJ2-5HdT7	CCCCaagcttAATACGACTCACTATAG**TAATCTAACACCGCTTTGCA**	1–20
	SJ2-3Ec	ACCGgaatt**cGCCGAGTAATGTGGCGATC**	1402–1421

* Primer AUAP is phosphorylated at the 5′ end.
† These primers were also used for direct sequencing.

■ **Construction of full-length cDNA and cloning into a transcription vector.** Full-length cDNA copies of genomic RNA1 and RNA2 were synthesized by RT–PCR using specific primers for the 5′ and 3′ termini of each RNA. First-strand cDNA copies of RNA1 and RNA2 were synthesized, as described above, using virion RNA as a template and the oligonucleotide primers SJ1-3Ec and SJ2-3Ec, respectively (Table 1). These oligonucleotides were complementary to the 3′ end sequences of the SJNNV RNA1 and RNA2 and contained an *Eco*RI site to facilitate cloning and to linearize the plasmids. The single-stranded cDNAs were then used as templates for PCR using the above-mentioned oligonucleotides as reverse primers and SJ1-5HdT7 (for RNA1) or SJ2-5HdT7 (for RNA2) as forward primers (Table 1). These primer sequences correspond to the 5′ ends of RNA1 and RNA2 and contain the T7 promoter sequence and a *Hin*dIII site to facilitate cloning. PCR was performed using the high fidelity DNA polymerase KOD plus (Toyobo). Thermocycling was carried out for 20 (RNA1) or 15 (RNA2) cycles of 40 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C, with a final extension of 10 min at 72 °C. Amplified full-length cDNAs of each RNA were purified using 1% LMP agarose gel electrophoresis and digested with *Eco*RI/*Hin*dIII. These fragments were cloned into the *Hin*dIII and *Eco*RI sites of pUC118 (Takara), according to the standard protocol (Sambrook *et al*., 1989). Plasmid DNA was maintained in *E. coli* DH5α cells and extracted using the Plasmid Midi kit (Qiagen), according to the manufacturer's protocol. The resulting plasmids were named according to the format pSJ*x*TK*y*, where *x* is the SJNNV component number and *y* is an arbitrary isolation number of the pUC118 recombinant plasmid containing the SJNNV cDNA insert. Full-length cDNA inserts within the representative plasmids pSJ1TK19 and pSJ2TK30 were sequenced using synthetic oligonucleotides designed after the known sequences (Nishizawa *et al*., 1995; Nagai & Nishizawa, 1999).

■ ***In vitro* transcription.** Plasmids containing full-length SJNNV cDNA were linearized with *Eco*RI and then used for *in vitro* transcription with T7 RNA polymerase (Takara) in the presence of synthetic cap analogue [$m^7G(5')ppp(5')G$] (New England Biolabs), as described previously (Kroner & Ahlquist, 1992). After being treated with RQ1 DNase I (Promega), transcripts were purified through a Sephadex G-50 column (Pharmacia) and their concentrations were quantified spectrophotometrically before transfection into cells. The RNA products were analysed by agarose gel electrophoresis in TBE buffer (Sambrook *et al*., 1989).

■ **Inoculation of cultured cells and infection assay.** The infectivity of transcripts was examined by transfection with lipofectin reagent (Gibco BRL) into E-11 cells followed by 24 h of incubation at 25 °C, as described previously (Iwamoto *et al*., 2001). For infection analysis of progeny virus, media cultured in the same manner as above for 72 h were collected, inoculated onto fresh E-11 cells and incubated at 25 °C for 24 h. Cell infectivity of the transcripts and their progeny was examined by immunofluorescence staining using anti-SJNNV rabbit polyclonal antibody and FITC-conjugated swine immunoglobulin (Ig) to rabbit Ig (Dako), as described previously (Nguyen *et al*., 1996). The fluorescent cells were then counted.

■ **Northern blot analysis.** Total RNA was extracted from E-11 cells infected with progeny viruses using ISOGEN (Nippon gene), according to the manufacturer's instructions. The RNA was subjected to Northern blot analysis, essentially as described by Damayanti *et al*. (1999), except that the DIG Labelling and Detection kit (Roche) was used, according to the supplier's instructions.

DIG-labelled RNA probes specific for the positive- and negative-sense strand of SJNNV RNA1 and RNA2 were prepared as follows. For RNA1, a PCR product was amplified from pSJ1TK19 using the M13

primers M4 and RV (Takara) and digested with *Cla*I/*Eco*RI and the resulting 0·3 kb fragment was inserted into the transcription vector pBluescript II KS(−) (Stratagene) to create pSJ1BS1. For RNA2, pSJ2TK30 was digested with *Bam*HI/*Eco*RI and the resulting 0·4 kb fragment was ligated into pBluescript II SK(−) to create pSJ2BS2. To prepare probes for positive-sense RNA1 and RNA2, pSJ1BS1 and pSJ2BS2 were linearized with *Sal*I and *Bam*HI, respectively, and transcribed with T7 RNA polymerase (Takara). To prepare probes for negative-sense RNA1 or RNA2, either pSJ1BS1 or pSJ2BS2 was linearized with *Eco*RI and then transcribed with T3 RNA polymerase (Gibco BRL).

■ **Western blot analysis.** Infected fish cells were suspended in Laemmli's sample buffer and subjected to SDS–PAGE. Western blot analysis was carried out as described previously (Damayanti *et al.*, 1999) using an Immobilon-P transfer membrane (Millipore). SJNNV CP was detected using anti-SJNNV rabbit polyclonal antibody and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) followed by incubation with BCIP/NBT for colour development.

■ **Electron microscopy of progeny.** Cell culture supernatants containing progeny virus were layered onto 10–40% sucrose gradients in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7·2) and centrifuged at 80000 ***g*** for 2 h at 16 °C. Each fraction was collected, analysed by Western blotting and concentrated using the Centricon centrifugal filter unit (Millipore), according to the manufacturer's instructions. Virus suspensions were stained with 1% uranyl acetate. Simultaneously, progeny virus was inoculated onto freshly prepared E-11 cells and incubated at 25 °C. After incubation for 3 days, the cells were fixed in 2·5% glutaraldehyde and post-fixed with 1% osmium tetroxide. Ultra-thin sections were stained with 1% uranyl acetate and 1% lead citrate. These samples were examined under a Hitachi (Model H-7100FA) electron microscope.

■ **Virulence assay for fish larvae.** One-day-old striped jack larvae reared at Kamiura Station of the Japan Sea-Farming Association were used for a virulence assay of the progeny virus produced in cells infected with the *in vitro* RNA transcripts. Before the infection experiment, these larvae were confirmed to be SJNNV-free by RT–PCR (Nishizawa *et al.*, 1994). Duplicate groups of 250 larvae were kept at 23 °C in glass beakers containing 1 litre of sea water to which kanamycin was added to a final concentration of 5 μg/ml. The 96 h culture supernatant of E-11 cells (50 μl) infected with progeny viruses was inoculated into the beakers and moribund fish were collected daily. The homogenate (progenitor virus) of striped jack larvae naturally infected with SJNNV and the culture supernatant of uninfected E-11 cells were used as positive and negative controls, respectively. Virus titres of the inocula used were determined, as described previously (Iwamoto *et al.*, 2000), to be $10^{9·6}$ $TCID_{50}$/ml for the progeny and $10^{10·6}$ $TCID_{50}$/ml for the progenitor. Fish were fixed with 10% formalin and embedded in paraffin. Sections were subjected to immunofluorescence staining as described above.

Results

Terminal sequences of SJNNV RNA1 and RNA2

SJNNV RNAs were successfully labelled with [γ-^{32}P]ATP at the 5′ end only after decapping treatment with TAP (data not shown). When we performed 5′ RACE using virion RNAs of *Brome mosaic virus* (BMV), a cytidine residue complementary to the m^7G in the cap structure was incorporated into the RACE products (K. Mise, unpublished data). Consistent with this observation, when SJNNV RNAs were used as a template for 5′ RACE after prior treatment with TAP, the sequence of the RACE products showed that the signal for such a cytidine residue was reduced compared with that of the capped, untreated RNA (data not shown). Polyadenylation of the 3′ end of alphanodavirus RNAs was unsuccessful, probably because of the modification of their ends by an unidentified 'blocking group' (Dasmahapatra *et al.*, 1985). Although the 3′ end of SJNNV RNAs does not have a poly(A) structure (Mori *et al.*, 1992), it remains unknown whether such a blocking structure exists. This was examined by labelling SJNNV RNAs with poly(A) polymerase or T4 RNA ligase; BMV RNAs were used as a positive control as these RNAs have 3′-OH groups that are reactive with these particular enzymes (Ahlquist *et al.*, 1981). Autoradiography showed that SJNNV RNAs were labelled at their 3′ ends, but the efficiency of labelling was considerably less than that of BMV RNAs (data not shown). These results indicate that the 5′ ends of both SJNNV RNAs are capped and that a blocking group rather than a hydroxyl group would thus probably modify most of their 3′ ends.

We then used the sample containing a small quantity of *in vitro* polyadenylated virion RNAs for the determination of the 3′-terminal sequence. Both the 5′ and 3′ termini of SJNNV RNA1 or RNA2 were amplified by RACE using SJNNV-specific primers that were synthesized according to the known sequence (Nishizawa *et al.*, 1995; Nagai & Nishizawa, 1999). We found 14 additional bases at the 5′ terminus and 12 additional bases at the 3′ terminus on RNA1 (Fig. 1A). RNA2 had another 11 bases at the 5′ terminus and the 3′-terminal sequence corresponded to the published sequence, although one nucleotide had been substituted (Fig. 1A).

Construction and sequence of full-length cDNA clones and *in vitro* transcription

To synthesize full-length cDNA copies of SJNNV RNA1 and RNA2, we designed synthetic oligonucleotides based on the 5′- and 3′-terminal sequences of the SJNNV RNAs determined in this study (Fig. 1B, C). The reverse primers included a unique *Eco*RI site for efficient cloning and subsequent plasmid linearization before *in vitro* transcription. The forward primers contained both a *Hin*dIII site and a T7 promoter sequence. *Hin*dIII and *Eco*RI sites were chosen because these recognition sites were not found in the known internal or terminal sequences of the SJNNV genome. PCR amplification was carried out with few (15 or 20) cycles to avoid the incorporation of undesired mutations into the RT–PCR products. cDNA clones containing the entire coding and non-coding regions of SJNNV RNA1 and RNA2 were prepared, the terminal regions of these complete cDNA copies were sequenced and the clones containing full-length sequences were selected. Finally, seven clones were obtained: one (pSJ1TK19) for RNA1 and the others (pSJ2TK9, -10, -22, -28, -29 and -30) for RNA2. Plasmids containing full-length SJNNV cDNA were linearized by cleavage with *Eco*RI and transcribed

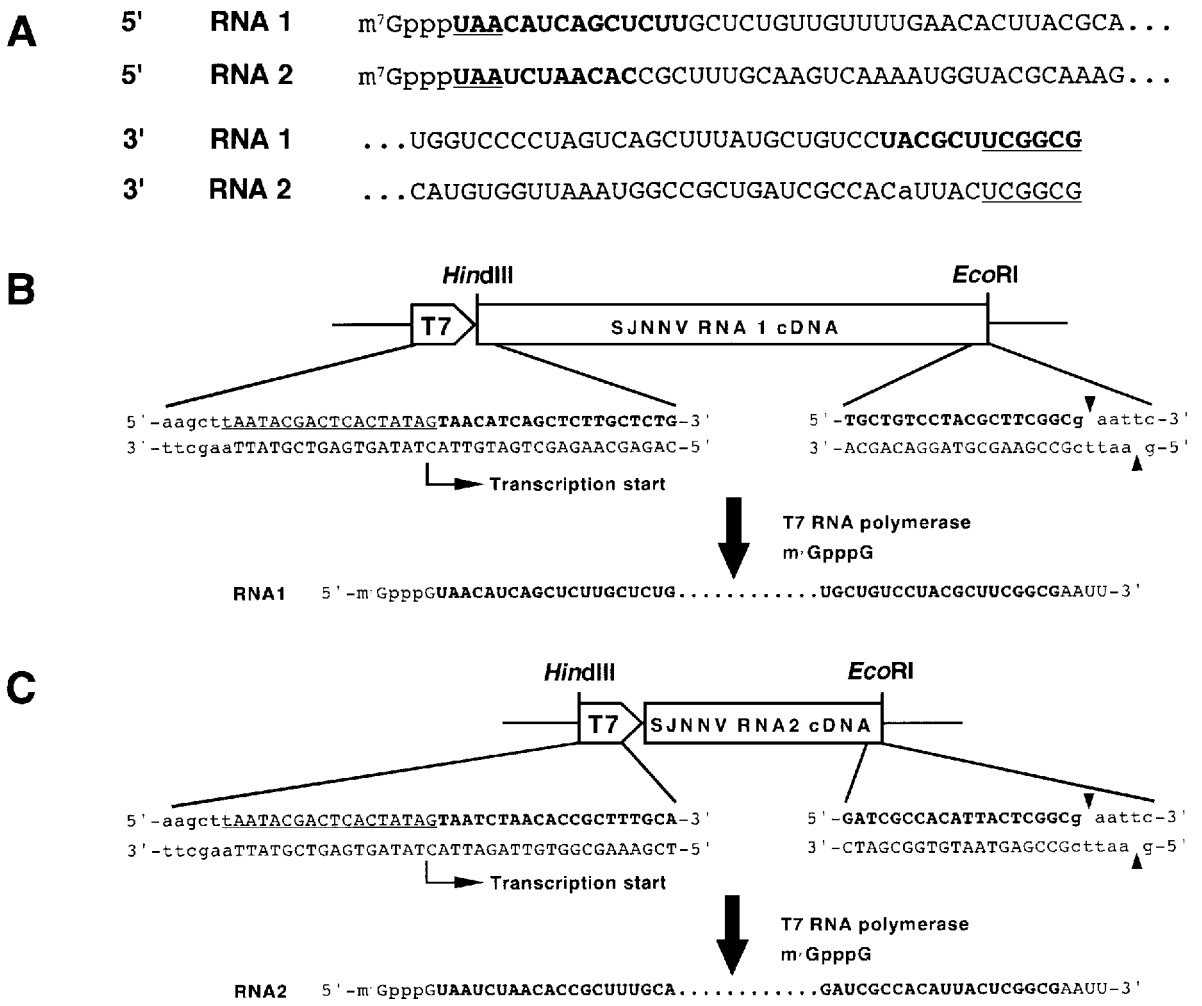

Fig. 1. (A) The 5′- and 3′-terminal sequences of SJNNV RNA1 and RNA2, as determined by 5′ and 3′ RACE. The newly determined sequences are shown in bold. A nucleotide in the 3′-terminal region of RNA2 that differs from the published sequence is shown in lowercase. Consensus sequences conserved between RNA1 and RNA2 are underlined. (B, C) Terminal sequences of the cloned full-length cDNAs of SJNNV RNAs within pUC118 and those of RNA1 (B) and RNA2 (C) transcribed from the cDNAs. Full-length cDNA plasmid clones were linearized with *Eco*RI and transcribed with T7 RNA polymerase in the presence of a synthetic cap analogue. Both RNA strands are predicted to have an extra G residue at the 5′ end and four extra residues (AAUU) at the 3′ end. Bold, lowercase and underlined letters indicate the sequence of viral RNA, restriction endonuclease recognition sites and the T7 promoter sequence, respectively.

with T7 RNA polymerase in the presence of a synthetic cap analogue. These transcripts co-migrated with the authentic SJNNV RNA1 and RNA2 by agarose gel electrophoresis (data not shown). The amount of transcripts obtained after Sephadex G-50 treatment corresponded to about 25 and 16 copies of the transcripts per plasmid, in the case of *Eco*RI-linearized pSJ1TK19 and pSJ2TK30, respectively, as estimated by optical density measurements.

Infectivity test of transcripts and progeny virions

To identify infectious transcripts of RNA2, individual transcripts were combined with a preparation of cognate RNA1 (purified twice by 1% LMP agarose gel electrophoresis) derived from virions and the mixtures were tested for their infectivity to cells. The results of indirect immunofluorescence staining showed that all transcripts except for pSJ2TK10 were infectious when transfected into E-11 cells with virion RNA1. From these plasmids, including the full-length cDNA of RNA2, pSJ2TK30 was selected because the transcript showed the highest infectivity in this trial (data not shown). Equimolar mixtures of transcripts from pSJ1TK19 and pSJ2TK30 at two different concentrations were transfected into E-11 cells (ca. 4×10^4 cells). After 24 h of incubation at 25 °C, fluorescent cells (28·7 ± 8·6 cells) were observed in the transfections with the higher concentration (1·5 μg) of transcripts, but not with the lower one (0·15 μg) and the infectivity of the transcripts was significantly less than that following transfection with authentic virion RNAs (1914·0 ± 99·7 fluorescent cells per 0·15 μg of inoculum RNA) (Fig. 2A, B). However, progeny

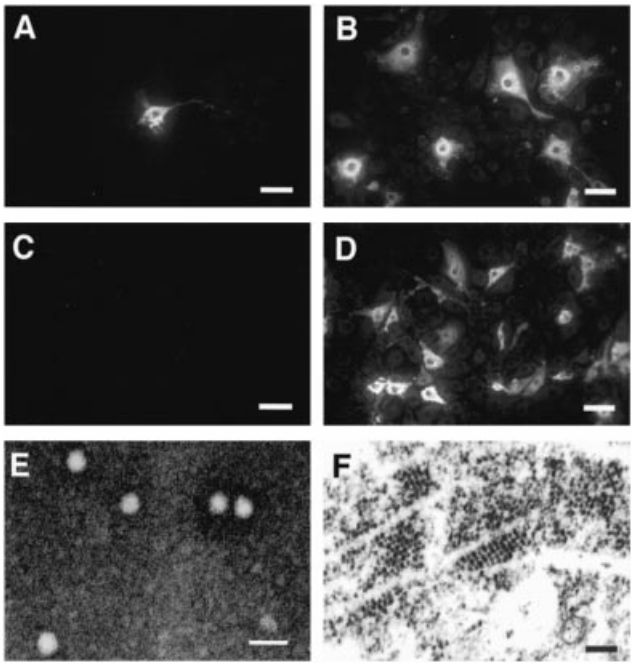

Fig. 2. (A)–(D) Detection of SJNNV antigen in E-11 cells at 24 h post-transfection or -infection by immunofluorescence staining. Specific fluorescent cells transfected with a mixture of transcripts from pSJ1TK19 and pSJ2TK30 (A) or virion RNAs (B), or (C) mock-transfected with DEPC water are shown. Fluorescent cells were detected by an anti-SJNNV rabbit polyclonal antibody. (D) Cells infected by a 72 h culture supernatant from (A). Bar, 50 μm. (E)–(F) Electron micrographs of rSJ. (E) Negatively stained virions purified from the supernatant of cultured cells transfected with a mixture of transcripts from pSJ1TK19 and pSJ2TK30. Bar, 50 nm. (F) Virions assembled in a crystalline array in an E-11 cell infected with rSJ and incubated at 25 °C for 3 days. Bar, 200 nm.

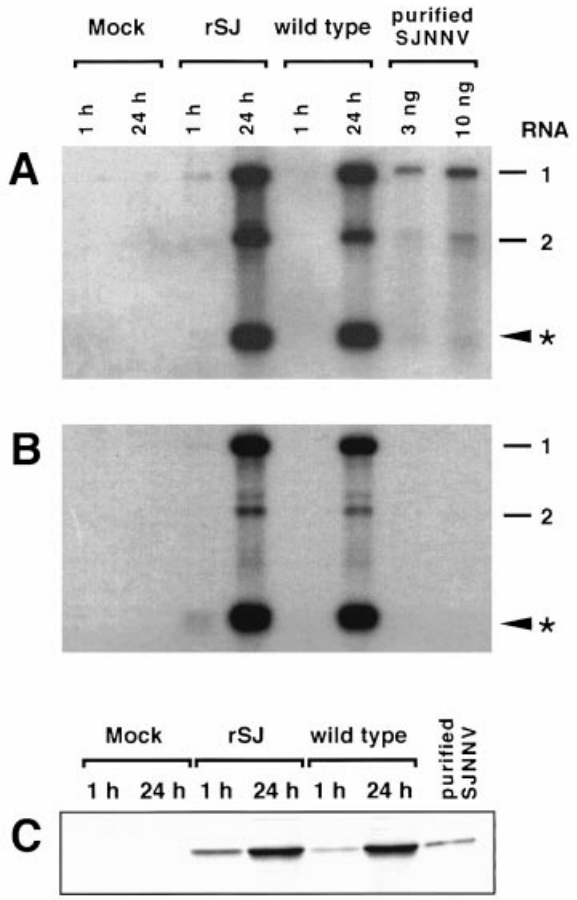

Fig. 3. (A, B) Northern blot analyses of progeny virus RNA in E-11 cells inoculated with rSJ or wild-type SJNNV. Total RNA was extracted from infected cells at 1 and 24 h p.i., separated by electrophoresis in a 1·0% agarose–formaldehyde gel and transferred onto a nylon membrane. (A) Viral RNAs were detected with a mixture of DIG-labelled positive-strand-specific probes, each complementary to the 3′-proximal region of SJNNV RNA1 or RNA2. (B) Viral RNAs were detected with a mixture of DIG-labelled negative-strand-specific probes, each corresponding to the 3′-proximal region of SJNNV RNA1 or RNA2. The mock controls contain cells inoculated with the culture supernatant of uninfected E-11 cells. Note that the RNA2 signals are weaker than those of RNA1, probably because of the low hybridization efficiency of RNA2-specific probes. The positions of RNA1 and RNA2 are indicated at the right. Asterisks indicate bands related to subgenomic RNA3, similar to that of alphanodaviruses. Blots were exposed to films for 10 s (A) and 5 min (B). (C) Western blot analysis of the accumulation of SJNNV CP in E-11 cells inoculated with rSJ or wild-type SJNNV. Proteins were extracted from the infected cells at 1 and 24 h p.i., separated by 12·5% SDS–PAGE and electroblotted onto a PVDF membrane. CP was detected with anti-SJNNV polyclonal antibody. The mock control is as above.

viruses (recombinant SJNNV, rSJ) in the culture supernatants of transfected cells were highly infectious to fresh E-11 cells (Fig. 2D).

To identify rSJ, we performed observations by electron microscopy. From the supernatant of cultured cells transfected with a mixture of transcripts from pSJ1TK19 and pSJ2TK30, we detected rSJ particles approximately 25 nm in diameter (Fig. 2E), similar to the size reported previously (Mori *et al.*, 1992). Furthermore, we also observed a crystalline array of virions in the cytoplasm of E-11 cells infected with rSJ (Fig. 2F).

Northern blot analysis of total RNA extracted from infected E-11 cells was performed using DIG-labelled riboprobes specific for positive- or negative-sense RNA. In the sample taken 24 h post-inoculation (p.i.), we detected strong signals that hybridized to SJNNV positive-sense-specific probes and co-migrated with original virion RNAs (Fig. 3A). Negative-sense RNA1 and RNA2 were also detected from cells at 24 h p.i. (Fig. 3B). However, the signals for RNA2, especially for the negative-sense one, were significantly lower than those of RNA1 (Fig. 3A, B). These differences were due to the difference in hybridization efficiency between the probe for (+) RNA1 and that for (+) RNA2 as well as between the probe for (−) RNA1 and that for (−) RNA2, which was confirmed by hybridizing those probes with known amounts of RNAs transcribed *in vitro* (data not shown). In addition to RNA1 and RNA2, bands showing faster migration were also detected in both positive- and negative-sense hybridizations (Fig. 3A, B). In parallel hybridization analyses using each segment-specific probe, these extra bands reacted with the RNA1-specific probes, but not with the RNA2-specific probes (data not shown). Meanwhile, Western blot analysis of the sample of cells at 24 h p.i. showed an obvious increase in CP

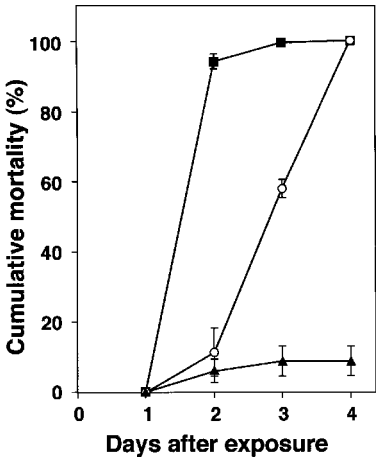

Fig. 4. Cumulative mortality of 1-day-old striped jack larvae exposed to SJNNV. Larvae were inoculated with the homogenate of naturally infected striped jack larvae (■), supernatant of E-11 cells containing rSJ (○) or the culture supernatant of uninfected E-11 cells (▲) in a 1 litre beaker. Data are represented as the mean ± SD from two separate experiments.

accumulation when compared with that of cells at 1 h p.i. (Fig. 3C).

rSJ was virulent to 1-day-old striped jack larvae and the virulence of rSJ was similar to that of the progenitor virus. All the larvae exposed to these virus inocula died at 2–4 days p.i. (Fig. 4) and SJNNV antigen was detected from the brain, spinal cord and retina of affected larvae (Fig. 5). RT–PCR analysis of the dead larvae were all positive (data not shown). Neither significant mortality nor SJNNV antigens were observed in the larvae exposed to the supernatant of uninfected E-11 cell culture.

Sequence of functional cDNA clones

As described above, *in vitro* transcripts synthesized from the plasmid clones pSJ1TK19 and pSJ2TK30 successfully initiated SJNNV amplification in transfected cells. Both cDNA inserts were then fully sequenced to establish definitive, functional nucleotide sequences of the two viral genome segments. Based on the published SJNNV sequences, several oligonucleotide primers were synthesized and used for the determination of full-length cDNA sequences. The cDNAs in pSJ1TK19 and pSJ2TK30 contained 3107 and 1421 nucleotides, respectively. The full-length cDNA sequence of pSJ1TK19 differed from the published sequence (accession no. AB025018) (Nagai & Nishizawa, 1999) by three nucleotides: position 378 was G (A), 1692 was C (T) and 2148 was T (C) in pSJ1TK19 (published nucleotides are in parentheses). The full-length cDNA sequence of pSJ2TK30 also differed from the published sequence (accession no. D30814) (Nishizawa *et al.*, 1995) by four nucleotides: position 766 was G (T), 881 was G (A), 969 was A (G) and 1411 was A (G) in pSJ2TK30 (published nucleotides are in parentheses). Although the nucleotide differences in pSJ1TK19 do not lead to amino acid changes, the

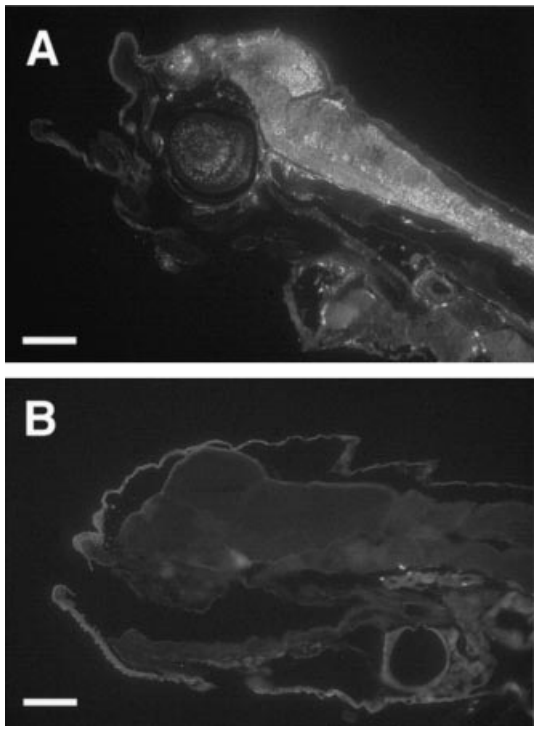

Fig. 5. Virus antigen from striped jack larvae infected with rSJ detected by immunofluorescence staining. (A) Larvae were inoculated with the culture supernatant containing rSJ, fixed at 3 days p.i., sectioned and stained with anti-SJNNV polyclonal antibody. (B) Larvae were inoculated with the culture supernatant of uninfected E-11 cells. Bar, 100 μm.

pSJ2TK30 sequence encodes an A at position 247 and an R at position 285 in the ORF rather than an S and a Q in the published sequence, respectively. These nucleotide differences were also found in the other functional cDNA clones pSJ2TK22 and pSJ2TK29 and were also confirmed by direct sequencing of the RT–PCR products from RNA2 (data not shown).

Discussion

In the present study, we completely determined the 5′- and 3′-terminal sequences of SJNNV genomic RNAs by poly(A)-tailing and RACE and then constructed full-length cDNA clones that can serve as templates for the production of RNA transcripts infectious to cultured fish cells. This is the first report of infectious RNA transcripts derived from cloned cDNA of betanodavirus genomic RNAs.

Single-stranded RNA genomes of many plant and animal viruses have unique structures at their 5′ and 3′ termini: the cap or the VPg structure at the 5′ end and the tRNA-like structure or the poly(A) tract at the 3′ end (Goldbach, 1987). The 3′ ends of alphanodavirus RNAs lack a poly(A) tail (Newman & Brown, 1976; Scotti *et al.*, 1983) and are not reactive with RNA ligase or poly(A) polymerase (Dasgupta *et al.*, 1984; Guarino *et al.*, 1984; Dasmahapatra *et al.*, 1985; Kaesberg *et al.*, 1990), suggesting that the 3′ end is modified by an unidentified blocking group. Our results suggest that SJNNV also has the

cap structure at the 5′ end and the blocking group at the 3′ end. Because the 3′ end of alphanodavirus RNA is blocked in, the double-stranded RNAs or head-to-tail homodimers in infected cultured cells have been used to determine the 3′-terminal sequences (Guarino *et al*., 1984; Johnson *et al*., 2000). Although this was a significant obstacle to conquer for the sequencing of the 3′ end of SJNNV, a small quantity of viral RNAs were, fortunately, polyadenylated *in vitro* and hence we were able to obtain the 3′-terminal cDNA fragments of SJNNV genomic RNAs by 3′ RACE. Those minor unmodified RNAs might exist because nascent viral RNAs were packaged before modification and/or because the blocking structure was eventually removed from the 3′ end during the RNA extraction procedure. Consistent with our results, the 3′-terminal sequences of genomic RNAs of the betanodaviruses *Greasy grouper nervous necrosis virus* (GGNNV) and *Dicentrarchus labrax encephalitis virus* (DlEV) have been determined after *in vitro* genomic RNA self-ligation (Tan *et al*., 2001) and *in vitro* polyadenylation (Delsert *et al*., 1997*a*), respectively. Consequently, we completely determined the 5′ and 3′ non-coding sequences of both RNAs in which 11–14 nucleotides were found at both termini for RNA1 and at the 5′ terminus for RNA2, in addition to the published sequences of SJNNV (Nishizawa *et al*., 1995; Nagai & Nishizawa, 1999). Comparison of the 5′- and 3′-terminal sequences between both RNA species demonstrated that each 5′ terminus begins with 5′ UAA … 3′ and each 3′ terminus ends with 5′ … UCGGCG 3′. The identical sequences are also present at the 5′ end of RNA1 and RNA2 of GGNNV (accession nos AF318942 and AF319555, respectively) (Tan *et al*., 2001) and at the 3′ end of RNA2 of DlEV (accession no. U39876) (Delsert *et al*., 1997*a*). Such sequences, conserved between two genomic RNAs but different from those of SJNNV, were found among the alphanodaviruses BBV [accession nos K02560 (Dasmahapatra *et al*., 1985) and X00956 (Dasgupta *et al*., 1984)], FHV (accession nos X77156 and X15959) (Dasgupta & Sgro, 1989) and NoV (accession nos AF174533 and AF174534), in which the sequence starts with 5′ GU … 3′ and ends with 5′ … GGU 3′. These results might suggest that other betanodaviruses also have consensus sequences such as those found in SJNNV.

The transcript RNAs were less infectious than their SJNNV virion RNA counterparts. This may be caused by the absence of a peculiar, unknown blocking structure at the 3′ end and/or by the presence of extra non-viral nucleotides at both termini of the transcripts. At the 5′ end of the transcripts, a non-viral extra G residue that originated from the T7 promoter sequence was added. It has been reported that transcription efficiency is increased when G residues are inserted between the T7 promoter and viral cDNA sequences, although the amplification efficiency in cells is lost to some extent (Janda *et al*., 1987). Our previous experiments suggest that the poly(A) tract was not entirely present or was not long in SJNNV viral RNAs, because they were not trapped with an oligo(dT) column (Mori *et al*., 1992). However, we could not rule out the possibility that the 3′ ends contain an oligo(A) tract that was short enough for the RNAs to pass through the column and could not be distinguished from the poly(A) added *in vitro* with poly(A) polymerase during 3′ RACE. Alternatively, if the 3′ terminus ending with oligo(A) is important for the infectivity of SJNNV, UU residues within the extra non-viral sequence AAUU at the 3′ end might have lowered the infectivity of these transcripts.

It has been known that subgenomic RNA3 (0·4 kb), derived from RNA1 of alphanodavirus, can only be detected from infected cells (Guarino *et al*., 1984; Dasmahapatra *et al*., 1985; Johnson *et al*., 2000). In this study, we also detected signals for RNA with faster migration by Northern blot analysis from cells infected with wild-type or rSJ. Although a similar phenomenon has been reported for the betanodavirus DlEV (Delsert *et al*., 1997*b*), detailed analysis was not performed. In this study, we detected the extra bands only from infected cells and verified that the bands reacted with both positive- and negative-strand-specific probes for the 3′-proximal region of SJNNV RNA1. Furthermore, the molecular size for these bands was estimated to be approximately 0·4 kb. These results strongly suggest that RNA3 is generated from RNA1 during SJNNV RNA replication.

Previously, we demonstrated the pathogenicity to striped jack larvae of the progeny that was generated by transfection of SJNNV virion RNAs into E-11 cells (Iwamoto *et al*., 2001). rSJ obtained from *in vitro* transcripts in this study was pathogenic to striped jack larvae. In regard to fish viruses, this is the first known instance in which a recombinant virus has the ability to kill the original hosts. As mentioned before, betanodaviruses can be classified into four genotypes, designated SJNNV, *Barfin flounder nervous necrosis virus* (BFNNV), *Tiger puffer nervous necrosis virus* and *Redspotted grouper nervous necrosis virus*, based on the RNA2 partial sequences (Nishizawa *et al*., 1997). We have reported recently that the optimal growth temperature for virus growth in cultured cells differs among the genotypes. Furthermore, because SJNNV genotype virus was not infectious to the Atlantic halibut *Hippoglossus hippoglossus*, which BFNNV genotype virus can infect, it has been suggested that host-specificity might be different among some betanodaviruses (Totland *et al*., 1999). A reverse genetics system for SJNNV, as reported here, will open the way for molecular studies directed at virus multiplication and pathogenesis of the betanodaviruses. In particular, the relationship between genetic variations and host specificities in betanodaviruses and comparative studies with alphanodaviruses will be of great interest.

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