Characterization of Striped jack nervous necrosis virus subgenomic RNA3 and biological activities of its encoded protein B2

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19 January 2005
22 July 2005

Striped jack nervous necrosis virus (SJNNV), which infects fish, is the type species of the genus Betanodavirus. This virus has a bipartite genome of positive-strand RNAs, designated RNAs 1 and 2. A small RNA (ca. 0.4 kb) has been detected from SJNNV-infected cells, which was newly synthesized and corresponded to the 3′-terminal region of RNA1. Rapid amplification of cDNA ends analysis showed that the 5′ end of this small RNA (designated RNA3) initiated at nt 2730 of the corresponding RNA1 sequence and contained a 5′ cap structure. Substitution of the first nucleotide of the subgenomic RNA sequence within RNA1 selectively inhibited production of the positive-strand RNA3 but not of the negative-strand RNA3, which suggests that RNA3 may be synthesized via a premature termination model. The single RNA3-encoded protein (designated protein B2) was expressed in Escherichia coli, purified and used to immunize a rabbit to obtain an anti-protein B2 polyclonal antibody. An immunological test showed that the antigen was specifically detected in the central nervous system and retina of infected striped jack larvae (Pseudocaranx dentex), and in the cytoplasm of infected cultured E-11 cells. These results indicate that SJNNV produces subgenomic RNA3 from RNA1 and synthesizes protein B2 during virus multiplication, as reported for alphanodaviruses. In addition, an Agrobacterium co-infiltration assay established in transgenic plants that express green fluorescent protein showed that SJNNV protein B2 has a potent RNA silencing-suppression activity, as discovered for the protein B2 of insect-infecting alphanodaviruses.

INTRODUCTION

Striped jack nervous necrosis virus (SJNNV) is the type species of the genus Betanodavirus in the family Nodaviridae. This virus is a causative agent of viral nervous necrosis (VNN) in larvae of striped jack (Pseudocaranx dentex), and has been highly detrimental to aquaculture industries (Arimoto et al., 1993; Mori et al., 1992). The bipartite SJNNV genome consists of two positive-strand RNA molecules with 5′ cap structures and without 3′ poly(A) tails (Iwamoto et al., 2001). RNA1 (3107 nt) encodes a 100 kDa non-structural protein (protein A), which is almost certainly the catalytic subunit of the viral RNA-dependent RNA polymerase (RdRp). RNA2 (1421 nt) encodes a 42 kDa major coat protein (CP) (Mori et al., 1992; Nagai & Nishizawa, 1999). Full-length cDNA clones of SJNNV RNAs 1 and 2 can serve as templates for the production of infectious RNA transcripts and have been previously described (Iwamoto et al., 2001).

In Flock house virus (FHV), the most extensively studied virus among the alphanodaviruses, which naturally infect insects, a single subgenomic RNA3 is synthesized from RNA1 during RNA replication. This subgenomic RNA3 encodes two non-structural proteins, B1 and B2 (Friesen & Rueckert, 1982; Guarino et al., 1984). The crucial roles of FHV proteins B1 and B2 in virus multiplication, as reported for alphanodaviruses. In addition, an Agrobacterium co-infiltration assay established in transgenic plants that express green fluorescent protein showed that SJNNV protein B2 has a potent RNA silencing-suppression activity, as discovered for the protein B2 of insect-infecting alphanodaviruses.
have been preliminary reports on subgenomic RNA3 and its encoded protein (protein B) for *Dicentrarchus labrax encephalitis virus* (Delsert et al., 1997) and SJNNV (Nagai & Nishizawa, 1999). RNA3 of Atlantic halibut nodavirus (AHNV) was recently identified in infected fish cells (Sommerset & Nerland, 2004). Some previous reports for betanodaviruses have designated the RNA3-encoded protein as ‘protein B’ because RNA3s of betanodaviruses have only one open reading frame (ORF), whereas those of most alphanodaviruses contain two ORFs. However, to avoid future confusion in the literature, ‘protein B’ 2 in this report is used for SJNNV protein B, following Johnson et al. (2001). We previously reported that a subgenomic 0.4 kb RNA was observed in SJNNV-infected fish cells. This RNA hybridized with both positive and negative strand-specific riboprobes for the 3′-proximal region of SJNNV RNA1, but not for that of RNA2 (Iwamoto et al., 2001). These results prompted an investigation into the production of SJNNV RNA3 and protein B2 during viral multiplication, as described for alphanodaviruses. In this study, we have determined the primary structure of SJNNV RNA3 and detected its encoded protein B2 in infected fish cells using an antiserum raised against recombinant protein B2. Moreover, we have shown that protein B2 can suppress RNA silencing in plants, using an *Agrobacterium*-mediated transient system.

**METHODS**

**Cells and virus.** The E-11 cells (ECACC no. 01110916; Iwamoto et al., 2000) were grown at 25°C in Leibovitz’s L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum. SJNNV, derived from naturally infected striped jack larvae, was grown in E-11 cells and stored at ~80°C until use.

**Determination of the 5′ end of the SJNNV subgenomic RNA.** E-11 cells were inoculated with SJNNV and cultured at 25°C for 16 h. Total cellular RNA was extracted from the infected cells using Isogen (Nippon Gene) according to the manufacturer’s instructions, and then separated by electrophoresis on 2% agarose gels (NuSieve 3:1 agarose; Cambrex), and stained with ethidium bromide. The subgenomic RNA (ca. 0.4 kb) was isolated from the gel using Gene-Capsule (Geno Technology) according to the manufacturer’s instructions, and was used as a template for the rapid amplification of cDNA ends (RACE) method (Frohman et al., 1988) to determine the 5′-terminal sequences. Prior to 5′ RACE, the recovered RNA was treated with tobacco acid pyrophosphatase (TAP) (Nippon Gene), according to the supplier’s instructions, to remove the 5′ cap structure or left untreated. 5′ RACE was carried out as previously described (Iwamoto et al., 2001) by using the synthetic oligonucleotide primers SJ1-3Ec, ANCH, SJ1R11 and AUAP (Table 1).

**Plasmid construction, in vitro transcription and RNA transfection.** The plasmid pSJ1TK19 contains the full-length cDNA of SJNNV RNA1 (Iwamoto et al., 2001). All SJNNV RNA1 cDNA mutants, namely pSJ1U2730A, pSJ1U2730C, pSJ1U2730G, pSJ1A2B2, pSJ1A2B2C and pSJ1A2B2NC, were constructed by PCR-mediated site-directed mutagenesis (Barik, 1993) using plasmid pSJ1TK19 as a template and appropriate primers (Table 1). The cDNA regions amplified by PCR were verified by sequencing. The plasmids were linearized with EcoRI and used as templates for in vitro transcription using T7 RNA polymerase (Takara) as previously described (Iwamoto et al., 2001), which resulted in capped full-length transcripts. RNA1 transcripts are designated according to their plasmid templates. For example, the transcript produced from pSJ1TK19 is designated SJ1TK19. Transcripts were purified using Nick column (Pharmacia) and were quantified by measuring the absorbance value at 260 nm. E-11 cells were transfected with each

![](https://www.microbiologyresearch.org/heres/FIG.jpg)

**Table 1. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer</th>
<th>Sequence* (5′-3′)</th>
<th>Position†</th>
</tr>
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<tbody>
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<td>5′-RACE</td>
<td>ANCH</td>
<td>GGCCACGCCTGACATGACATTTTTTTTTTTTTTTTT</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>AUAP†</td>
<td>pGGCCACGCCTGACATG1AC</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>SJ1-3Ec</td>
<td>ACCGgaattGCGCAAGGCTAGGACAGCA</td>
<td>3088–3107</td>
</tr>
<tr>
<td></td>
<td>SJ1R11</td>
<td>CCTCTCAGCTCAGTCGCTCCAT</td>
<td>2968–2987</td>
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<td>Mutagenesis</td>
<td>SJ1T2730A</td>
<td>TCACACCGTTCCAAGTCAAACTCTCAAA</td>
<td>2717–2746</td>
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<td>SJ1T2730C</td>
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</tr>
<tr>
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<td>SJ1T2730G</td>
<td>TCACACCGTTCCAAGTCAAACTCTCAAA</td>
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<td>SJ2dN</td>
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<td>SJ2dC</td>
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<td>2870–2899</td>
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<td>Expression</td>
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<td>2756–2774</td>
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<td></td>
<td>SJ3B2-Bar</td>
<td>CCACAGTCCCGTCATGCCTCCTCAT</td>
<td>2968–2983</td>
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<td>Suppression</td>
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<td>CGGGAATTCATGAAAGGCTAGTAAAGC</td>
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<td>EcoRI-SJ2B3</td>
<td>CCACAGTCCCGTCATGCCTCCTCAT</td>
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<td>BamHI-SJCP5</td>
<td>TTCCACCAGTCCCGTCATGCCTCCTCAT</td>
<td>28–48</td>
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* SJNNV-specific nucleotides are shown in bold. Substituted nucleotides in *in vitro* mutagenesis are underlined. Lower-case letters indicate restriction endonuclease sites.
† The oligonucleotide positions correspond to the SJNNV full-length cDNA within pSJ1TK19 (except for BamHI-SJCP5) and pSJ2TK30 (used only with the primer BamHI-SJCP5). The viral cDNA sequences were deposited (Iwamoto et al., 2001) in the GenBank/DDBJ database with the accession numbers AB056571 and AB056572, respectively.
‡ The primer AUAP is phosphorylated at the 5′ end.
RNA1 transcript as previously described (Iwamoto et al., 2001). At 24 h post-transfection, total RNA was extracted with Isogen and subjected to Northern blot analysis using digoxigenin-labelled RNA probes, which are specific for the 3'-proximal region of the positive- or negative-strand of SJNNV RNA1, as previously described (Iwamoto et al., 2001).

**Preparation of Histagged protein B2 (SJpB2) and anti-SJpB2 antisem.** A putative SJNNV ORF B2 was amplified by PCR using pSJ1T1K9 as a template and with forward primer SJ3B2-Nde and reverse primer SJ3B2-Bam (Table 1), which contain NdeI and BamHI recognition sites, respectively, to facilitate cloning. Amplified products were digested with NdeI and BamHI and the ORF B2 fragment was isolated from a 2 % agarose gel. This fragment was cloned into the NdeI and BamHI sites of the pET16b vector (Novagen) according to standard protocols (Sambrook & Russell, 2001), to obtain pETSpB2. pETSpB2 was transformed into the Escherichia coli BL21(DE3) (Novagen). Recombinant protein B2 (SpB2) was expressed as an N-terminal-polyhistidine-tagged protein and was purified using Ni-NTA resin (Qiagen) according to the manufacturer's procedure. Anti-SpB2 antisem was raised in a New Zealand White rabbit, immunized with a mixture of the SpB2 and complete Freund's adjuvant (Difco) as previously described (Nguyen et al., 1996).

**Inoculation of E-11 cells and host fish.** E-11 cells grown in eight-chamber slides (Nunc and 12-well plates (Iwaki) were inoculated with wild-type SJNNV virions. After incubation for 24 h at 25 °C, the cells in eight-chamber slides were fixed with methanol and those in the 12-well plates were suspended in 50 μL Laemmli sample buffer (Laemmli, 1970). SJNNV-free striped jack larvae hatched at the Japan Fisheries Research Agency (formerly the Japan Sea-farming Association) were inoculated with wild-type SJNNV virions as previously described (Iwamoto et al., 2001). At 3 days post-inoculation, 50 mg moribund fish (fresh weight) was collected and suspended in 50 μL Laemmli sample buffer. A portion of the inoculated fish was fixed with 10 % formalin, embedded in paraffin and sectioned.

**Immunological detection of SJNNV protein B2.** Immunofluorescence staining of fixed samples was performed as previously described (Iwamoto et al., 1999). Nine microlitre samples from the E-11 cells and striped jack larvae were resolved by Tricine-SDS-PAGE. The gels were essentially prepared according to the method of Schägger & von Jagow (1987) and consisted of a stacking gel [4 % acrylamide (acrylamide:bisacrylamide=29:1)], a spacer gel (10 % acrylamide) and a separating gel (16-5 % acrylamide). Western blot analysis was performed as described by Damayanti et al. (1999), except that electroblotting was done in Towbin buffer (10 mM Tris base, 96 mM glycine in 40 % methanol) and anti-SpB2 antisem was diluted 1000-fold for use as the primary antibody.

**Assay for silencing-suppression activity.** All primers for the following PCR contained an appropriate restriction endonuclease recognition sequence for cloning (Table 1). PCR products containing a 5' leader (5'-AGAGGAGATATAA-3') and SJNNV ORF B2 were amplified from pSJ1T1K9 and pSJ1AB2NC, digested with BamHI and EcoRI and cloned into the BamHI/EcoRI sites of pBICP35 (Mori et al., 1991) to create pBICSJb2 and pBICSJAB2NC, respectively. A PCR product containing the same 5' leader sequence and the SJNNV CP gene was amplified from pSJ2T3K0, digested with BamHI (within the primer sequence and at nt 1062 of pSJ2T3K0) and cloned into the BamHI site of pBICP35 to obtain pBICSJCP. The Agrobacterium-mediated transient assay, established in transgenic plants (Nicotiana benthamiana line 16c; Voinnet et al., 2000) that express green fluorescent protein (GFP), GFP imaging under UV light and RNA analyses were carried out as previously described (Takeda et al., 2002).

**RESULTS**

**Structure of SJNNV subgenomic RNA3**

When SJNNV RNA1 and RNA2 were treated with TAP and used as templates for 5' RACE, nucleotide sequencing of the RACE products showed that the signal for a 5'-terminal cytidine residue was reduced compared with that of the capped, untreated RNA (Iwamoto et al., 2001). When gel-purified small RNA extracted from SJNNV-infected E-11 cells was amplified by 5' RACE, the resulting products also showed a decreasing 5'-terminal cytidine signal after TAP treatment (data not shown). This result suggests that the small RNA had a cap structure at its 5' end. In addition, the small RNA started with 5'-UAGUCAA-3', which corresponded to the sequence immediately downstream of nt 2730 in RNA1, suggesting that the small RNA would contain 378 nt if it shared its 3' terminus with RNA1 (Fig. 1a). Furthermore, the dinucleotide 'UA' at the 5' terminus of the small RNA corresponded to those of RNA1 and RNA2. These results indicate that the small SJNNV RNA should be identified as subgenomic RNA3 by analogy with the RNA species synthesized by the alphanodaviruses. Although there are two ORFs reported in FHV RNA3, only one ORF exists in SJNNV RNA3, which extends from nt 27 to 254. The ORF encodes putative protein B2 of 75 aa (predicted molecular mass 8.3 kDa) and is encoded in a reading frame that is +1 with respect to ORF A (Fig. 1b), as reported previously (Nagai & Nishizawa, 1999). This ORF corresponds to the alphanodavirus protein B2.

**Effects of single-nucleotide substitutions on RNA3 synthesis**

In FHV, RNA2 and RNA3 are counter-regulatory. FHV RNA2 replication depends on RNA3 synthesis (Eckerle & Ball, 2002), and RNA3 synthesis is suppressed by the replication of RNA2 (Zhong & Rueckert, 1993). Such a sophisticated strategy has not yet been reported for SJNNV. However, we have observed that SJNNV RNA1 alone can...
direct its own replication and synthesize RNA3 from the 3’-proximal region of RNA1 in transfected host cells (data not shown). Therefore, in this study, we used RNA1 alone as an inoculum and examined the synthesis of RNA3 as well as of RNA1. To test the potential for the synthesis of subgenomic RNA3 from RNA1, the uridine residue at nt 2730 of SJ1TK19 that corresponds to the 5’ uridine residue of RNA3 (Fig. 1a) was changed to A (SJ1U2730A), C (SJ1U2730C) or G (SJ1U2730G). Amino acid sequences encoded by ORF A were not affected by these mutations (Table 2). On transfection of capped in vitro transcripts of these mutants into E-11 cells, Northern blot analysis of their RNA replication products revealed that negative-strand RNA3 accumulated to levels similar to those of the wild-type for each of the mutants tested (Fig. 2b, lanes 1–3 and 7). In contrast, positive-strand RNA3 was undetectable for all of the mutants tested (Fig. 2a, lanes 1–3 and 7). Nonetheless, after raising anti-B2 rabbit antiserum (see below), we demonstrated by immunofluorescence staining that small numbers of protein B2-positive cells were detected in cells transfected with SJ1U2730C or SJ1U2730G (data not shown), suggesting that low levels of positive-strand RNA3 must be synthesized. These results suggest that the first nucleotide of SJNNV RNA3 must be U, rather than A, C or G, for optimal synthesis of positive-strand RNA3.

Table 2. Summary of SJNNV RNA1 mutant plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation*</th>
<th>Change in ORF A</th>
<th>Change in ORF B2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Codon†</td>
<td>Amino acid</td>
</tr>
<tr>
<td>pSJ1U2730A</td>
<td>T2730A</td>
<td>CCT884CCA</td>
<td>Pro→Pro</td>
</tr>
<tr>
<td>pSJ1U2730C</td>
<td>T2730C</td>
<td>CCT884CCC</td>
<td>Pro→Pro</td>
</tr>
<tr>
<td>pSJ1U2730G</td>
<td>T2730G</td>
<td>CCT884CCG</td>
<td>Pro→Pro</td>
</tr>
<tr>
<td>pSJ1ΔB2N</td>
<td>T2757C</td>
<td>GAT893GAC</td>
<td>Asp→Asp</td>
</tr>
<tr>
<td>pSJ1ΔB2C</td>
<td>T2883A</td>
<td>GTT935GTA</td>
<td>Val→Val</td>
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<td></td>
<td>C2884A</td>
<td>CGT936AGA</td>
<td>Arg→Arg</td>
</tr>
<tr>
<td>pSJ1ΔB2NC</td>
<td>T2757C</td>
<td>GAT893GAC</td>
<td>Asp→Asp</td>
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<td>GTT935GTA</td>
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<td></td>
<td>C2884A</td>
<td>CGT936AGA</td>
<td>Arg→Arg</td>
</tr>
</tbody>
</table>

*The numbers indicate the nucleotide positions substituted in SJNNV RNA1 cDNA.
†The numbers indicate the amino acid positions substituted in SJNNV protein A.
‡The numbers indicate the amino acid positions substituted in SJNNV protein B2.

Fig. 2. Northern blot analysis of progeny viral RNAs in E-11 cells transfected with SJNNV RNA1 mutants. Total RNA was extracted at 24 h post-transfection, separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred to a nylon membrane. Viral RNAs were detected with a positive-strand RNA1-specific riboprobe (a) or a negative-strand RNA1-specific riboprobe (b). Positions of RNA1 and RNA3 are indicated to the right. The blots were exposed to films for 10 s (a) and 5 min (b). Inocula used were SJ1U2730A (lane 1), SJ1U2730C (lane 2), SJ1U2730G (lane 3), SJ1ΔB2N (lane 4), SJ1ΔB2C (lane 5), SJ1ΔB2NC (lane 6), SJ1TK19 (wild-type; lane 7) and water (Mock; lane 8).
Detection of protein B2 expression in infected cells and fish

To detect the expression of protein B2 in SJNNV-infected E-11 cells and striped jack larvae, anti-SJpB2 antiserum was obtained from a rabbit immunized with recombinant SJpB2 tagged with polyhistidine. The protein B2 antigen was detected predominantly in the cytoplasm when this antiserum was used for immunofluorescence staining of E-11 cells 24 h post-inoculation. In these cells, the immunofluorescence-staining pattern was heavily punctate (Fig. 3a), whereas when anti-SJNNV antiserum was used as the primary antibody the cells showed diffuse cytoplasmic staining (Fig. 3e). In striped jack larvae, the protein B2 antigen was only detectable in the brain, spinal cord and retina of moribund fish (Fig. 3b), which is a pattern similar to the tissue distribution detected for CP antigen stained with anti-SJNNV antiserum (Fig. 3f). Protein B2 antigen was not detected in SJNNV-infected E-11 cells or affected striped jack larvae stained with pre-immune serum (Fig. 3c and d), or without primary antibody (data not shown). Consistent with the immunofluorescence data, Western blot analysis showed protein B2 accumulation only in infected E-11 cells and striped jack larvae during SJNNV multiplication (Fig. 3g). When E-11 cells were inoculated with SJNNV at an m.o.i. of 10, protein B2 accumulation was observed at 12 h post-inoculation by Western blot analysis (data not shown). Protein B2 was undetectable in purified SJNNV virion fractions by Western blot analysis, even when 300 ng of the SJNNV was tested (data not shown). These results suggest that SJNNV protein B2 is expressed in host cells but may not be encapsidated as a structural component of virions.

Effect of protein B2 expression on the synthesis of RNAs 1 and 3

To examine the effect of protein B2 expression on the multiplication of SJNNV RNAs 1 and 3, we constructed three mutants, SJ1ΔB2N, SJ1ΔB2C and SJΔB2NC, that express truncated B2 proteins but wild-type protein A (Table 2). The primary structures of the truncated B2 proteins are summarized in Fig. 4. Capped mutant and wild-type (SJ1TK19) transcripts were synthesized in vitro and transfected into E-11 cells (ca. 5 × 10⁴ cells), and total RNA was subjected to Northern blot analysis. Compared
with the wild-type, accumulation levels of negative-strand RNAs 1 and 3 were moderately reduced in the transfection with SJ1ΔB2N transcripts and further reduced with SJ1ΔB2C and SJ1ΔB2NC transcripts (Fig. 2b, lanes 4–7). In the transfection with SJ1ΔB2C or SJ1ΔB2NC transcripts, positive-strand RNA1 accumulated to lower levels compared with the wild-type, which could be a direct consequence of low-level accumulation of negative-strand RNA1. Low levels of positive-strand RNA3 were detected in the transfection with SJ1ΔB2N transcripts, but not with SJ1ΔB2C or SJ1ΔB2NC RNA (Fig. 2a, lanes 4–6). Immunofluorescence staining of protein B2 with anti-SJpB2 antisera demonstrated that fluorescent cells were observed in the transfection with the wild-type transcripts but not with SJ1ΔB2C or SJ1ΔB2NC RNA (data not shown).

**Silencing-suppression activity of protein B2**

The function of SJNNV protein B2 has not been characterized. A recent study of the corresponding alphanodavirus non-structural protein B2 showed that FHV protein B2 has an ability to suppress RNA silencing in plants and in insect cells (Li et al., 2002). In a more recent study, it has been demonstrated that NoV protein B2 also suppresses RNA silencing in fruit fly and mosquito cells (Li et al., 2004). Although alphanodavirus B2 and SJNNV B2 proteins share low sequence identity (Johnson et al., 2001), we speculated that SJNNV protein B2 may also function as an RNA silencing suppressor. To examine this hypothesis, we used a transient silencing-suppression assay based on the *Agrobacterium* co-infiltration method, established in a GFP-expressing transgenic *N. benthamiana* (line 16c) (Takeda et al., 2002; Voinnet et al., 2000). In this system, GFP expression is normally silenced on introduction into the GFP-expressing plant of a plasmid that expresses GFP, resulting in low or undetectable GFP fluorescence. However, if a suppressor blocks the onset of RNA silencing, GFP fluorescence is easily detected under UV light.

We used an *Agrobacterium* strain carrying pBICGFP as an RNA silencing inducer, together with a second *Agrobacterium* strain bearing another plasmid that expresses one of several proteins to be tested. A mixture of *Agrobacterium* carrying pBICSJB2 and *Agrobacterium* carrying pBICGFP was co-infiltrated into line 16c leaves. As controls, *Agrobacterium* carrying either pBICP35 (empty vector) or pBICNSs was co-infiltrated with that carrying pBICGFP. pBICNSs contains the *Tomato spotted wilt virus* (TSWV) NSs protein, which has been shown to suppress RNA silencing in a similar assay (Takeda et al., 2002). For simplicity, each *Agrobacterium* strain will hereafter be referred to by the name of the plasmid it carries. The leaf patches receiving pBICGFP plus pBICP35 did not show strong green fluorescence (Fig. 5b) at 6 days post-infiltration (p.i.). In contrast, the patches that received pBICGFP plus pBICSJB2 showed bright green fluorescence (Fig. 5a). Patches that received pBICGFP plus pBICNSs showed even stronger green fluorescence (Fig. 5d). These results indicate that SJNNV protein B2 suppressed RNA silencing of GFP, although the suppression was not as potent as that mediated by the TSWV NSs protein. Infiltration with pBICGFP plus pBICSJB2NC did not demonstrate strong green fluorescence, indicating that RNA silencing had not been suppressed in the patch (Fig. 5c).

To verify the visual observations, we analysed the accumulation of GFP mRNA in each infiltrated patch at 6 days p.i. by Northern blot analysis. In accordance with the relative intensities of GFP fluorescence (Fig. 5), GFP mRNA accumulation was higher in the patches that received pBICNSs than in those that received pBICSJB2 (Fig. 5e, lanes 1 and 4). In contrast, GFP mRNA accumulation in the patches that received pBICGFP plus either pBICP35 or pBICSJB2NC (Fig. 5e, lanes 2 and 3) was significantly less than in that suppressor-infiltrated patches. GFP-specific small-interfering RNAs (siRNAs), a hallmark of RNA silencing (Hamilton & Baulcombe, 1999), were readily detected in the patches that received pBICGFP plus either pBICP35 or pBICSJB2NC (Fig. 5f, lanes 2 and 3). However, we detected substantially less accumulation of siRNAs in the patches that received pBICGFP plus either pBICSJB2 or pBICNSs (Fig. 5f, lanes 1 and 4).

Interestingly, at 12 days p.i., necrosis was observed in the patches infiltrated with pBICGFP plus pBICSJB2 in the *N. benthamiana* line 16c expressing GFP (data not shown). To further explore this observation, we tested the necrosis-inducing activity of several constructs in non-transgenic wild-type *N. benthamiana* plants. Although necrosis was observed in the patches infiltrated with pBICSJB2 alone (Fig. 6b), no necrosis was observed in the patches that received pBICP35, pBICSJB2NC or pBICNSs (Fig. 6a, c and d). The patches that received pBICSCP, and therefore express SJNNV CP, did not show necrosis (Fig. 6e).

**DISCUSSION**

In this study, we have determined the 5' end of SJNNV subgenomic RNA3 by 5' RACE and have detected protein
B2 expression in SJNNV-infected E-11 cells and SJNNV-infected striped jack larvae. This is the first evidence that betanodaviruses express a non-structural protein other than the RdRp.

The sequence ‘UAA’ is conserved at the 5’ end of SJNNV RNAs 1 and 2 (Iwamoto et al., 2001). This study found that, in SJNNV RNA3, the dinucleotide ‘UA’ is conserved at the 5’ terminus and that this RNA contained a 5’ cap structure. Thus, SJNNV RNAs 1, 2 and 3 share the initial dinucleotide ‘UA’, a situation similar to that seen for the alphanodavirus FHV whereby RNAs 1, 2 and 3 share ‘GU’ at the 5’ end (Ball, 1995). According to the report by Nishizawa et al. (1997), betanodaviruses can be divided into four genotypes: SJNNV, Redspotted grouper nervous necrosis virus (RGNNV), Tiger puffer nervous necrosis virus and Barfin flounder nervous necrosis virus (BFNNV). The sequence ‘UAA’ is also present at the 5’ ends of RNAs 1 and 2 of Greasy grouper nervous necrosis virus (RGNNV-type), Sevenband grouper nervous necrosis virus (RGNNV-type) and AHNV (BFNNV-type) (Grotmol et al., 2000; Iwamoto et al., 2004; Sommerset & Nerland, 2004; Tan et al., 2001). Further, these viruses share the 5’-terminal sequence of SJNNV RNA3, 5’-UAGUCAA-3’, at nucleotide positions 2730–2736 in RNA1. These results suggest that this sequence may contain important determinants of subgenomic RNA3 synthesis and that these viruses may synthesize RNA3 by a common mechanism.

For FHV, RNA3 synthesis is greatly reduced in baby hamster kidney BHK21 cells when the first nucleotide of the RNA3 sequence in RNA1 is changed from G to A. In addition, RNA3 synthesis is essentially eliminated by a G-to-T or G-to-C substitution at the same position (Ball, 1995; Eckerle & Ball, 2002). In a more recent study in yeast cells, the same G-to-T substitution greatly inhibited positive-strand RNA3 accumulation, although negative-strand RNA3 accumulation was at wild-type levels (Price et al., 2000). In our current study, the transfection experiments using three RNA1 mutants that had substitutions at the first nucleotide of RNA3 from U to A, C or G, showed that negative-strand RNA3 accumulation was not significantly reduced compared to the wild-type (Fig. 2b). In contrast, positive-strand RNA3 accumulation for the mutants was essentially

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**Fig. 6.** Expression of SJNNV protein B2 in a wild-type *N. benthamiana* leaf using the *Agrobacterium*-mediated transient expression method described in the legend to Fig. 5. Leaves were infiltrated with *Agrobacterium* carrying either pBICP35 (a), pBICSJB2 (b), pBICSJAB2NC (c), pBICNSs (d) or pBICSJCP (e) and were photographed at 12 days p.i.

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**Fig. 5.** Assay for suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. (a–d) Infiltrated leaves of *N. benthamiana* line 16c with *Agrobacterium* carrying pBICGFP plus *Agrobacterium* carrying either pBICSJB2 (a), pBICP35 (b), pBICSJAB2NC (c) or pBICNSs (d) were photographed under UV light at 6 days p.i. (e–g) Northern analysis of GFP mRNA (e) and GFP-specific siRNAs (f) extracted from the patches infiltrated with *Agrobacterium* carrying pBICGFP plus *Agrobacterium* carrying either pBICSJB2 (lane 1), pBICP35 (lane 2), pBICSJAB2NC (lane 3) or pBICNSs (lane 4). (g) rRNA stained with ethidium bromide indicates similar loading of samples in (e) and (f).
abolished (Fig. 2a). Altogether, changing the first nucleotide of the SJNNV subgenomic RNA selectively inhibited production of positive- but not of negative-strand RNA3. This result suggests that synthesis of negative-strand subgenomic RNA3 may precede synthesis of positive-strand RNA3. This result also suggests that SJNNV subgenomic RNA3 may be synthesized not by internal initiation but by premature termination as proposed for FHV and some plant positive-strand RNA viruses (Price et al., 2000; White, 2002).

There are two ORFs found in RNA3 of FHV, Black beetle virus, Pariacato virus and NoV (Ball, 1995; Guarino et al., 1984; Johnson et al., 2000, 2003). However, SJNNV RNA3 has a single ORF from nt 27 to 254, encoding a predicted protein (designated protein B2) of 8.3 kDa, like RNA3 of the alphanodavirus Boolarra virus (Harper, 1994). SJNNV protein B2 (75 aa) is the smallest amongst those identified from known nodaviruses. Moreover, SJNNV protein B2 differs from alphanodavirus B2 proteins in its amino acid sequence (Johnson et al., 2001). These differences between SJNNV protein B2 and alphanodavirus B2 proteins prevented prediction of SJNNV protein B2 function, which prompted us to further examine SJNNV protein B2. Immunofluorescence staining of SJNNV-infected E-11 cells and striped jack larvae showed that protein B2 was specifically detected in the cytoplasm of E-11 cells, as well as in the central nervous system and retina of striped jack larvae. Similar localization was observed when CP and/or virions were detected with anti-SJNNV antiserum. However, localization of protein B2 and CP were different to some extent in E-11 cells (Fig. 3). This result suggests that protein B2 was localized at limited sites in infected cells. The mutant SJ1ΔB2N lacks the N-terminal 20 aa of protein B2 (Fig. 4). The truncated protein B2 could barely be detected in the transfected cells (T. Iwamoto & K. Misawa, unpublished data). However, no fluorescent cells were observed in the transfection with mutant SJ1ΔB2C or SJ1ΔB2NC (data not shown), both of which were designed to express a truncated protein B2 lacking the C-terminal 33 aa (Fig. 4). These results suggest that the anti-SlP2 antisera may recognize the C-terminal region of protein B2. Alternatively, the lack of the C-terminal region could affect the stability of protein B2.

RNA silencing is a conserved biological response to double-stranded RNA (dsRNA) that regulates gene expression and has evolved in plants as a defence against viruses (Guiltin & Andino, 2003; Hannon, 2002; Voinnet, 2001). As a counter-defence, many viruses encode proteins called ‘RNA silencing suppressors’ that specifically inhibit the RNA silencing machinery (Li & Ding, 2001). RNA silencing also appears to contribute to antiviral defence in invertebrates. Importantly, a recent study showed that FHV RNAs can be targeted by RNA silencing in Drosophila cells, and that productive FHV infection requires suppression of RNA silencing by FHV-encoded protein B2 (Li et al., 2002), a counterpart of SJNNV protein B2. So far, there has been no report of RNA silencing suppressor activity of any fish virus protein. To obtain some insights into the functions of SJNNV protein B2, we used an Agrobacterium-mediated assay to show that SJNNV protein B2 has suppressor activity against RNA silencing, at least in plants. Similar to this result, recent studies have shown that a dsRNA-binding protein from a mammalian reovirus (Lichner et al., 2003) and the NS1 protein of influenza virus (Bucher et al., 2004; Delgadillo et al., 2004) could suppress RNA silencing in plants. In addition, Li et al. (2004) demonstrated cross-kingdom suppression of RNA silencing in an animal system by the plant viral suppressor, tombusvirus p19. These results and a previous report (Li et al., 2002) show that the RNA silencing mechanism is conserved between the plant and animal kingdoms and suggest that SJNNV protein B2 may have a role in counteracting RNA silencing in natural infections in fish. The adaptive immune system is a major defence mechanism for vertebrates to protect against viral infection. Generally in fish the adaptive immune system has not fully developed at the larval stage. If SJNNV protein B2 also plays a role in suppression of RNA silencing in fish, RNA silencing may also be an important defence to prevent virus attack in these animals.

There is still a need to investigate whether SJNNV RNAs can be targets of RNA silencing, and whether productive SJNNV infection requires suppression of RNA silencing by an SJNNV-encoded protein B2 in fish cells or in whole fish. Interestingly, the latter possibility could be supported by the observation that SJNNV-related RNAs accumulated to lower levels in fish cells transfected with protein B2-minus mutants (SJ1ΔB2N, SJ1ΔB2C and SJ1ΔB2NC) (Fig. 2). Johnson et al. (2004) recently reported that B2-minus mutants of NoV accumulate to different levels in transfected cells depending on the cell type, implying that the efficacy of RNA silencing differs between different host cells. Our data suggest that E-11 cells have detectable activity of RNA silencing against viral RNA multiplication. On the other hand, for the SJNNV RNA3-minus mutants (SJ1U2730A, SJ1U2730C and SJ1U2730G), all SJNNV-related RNAs, except for positive-strand RNA3, accumulated to levels similar to those in wild-type-transfected cells. This could be due to the suppressing activity of small amounts of protein B2 barely detected by immunofluorescence.

SJNNV-infected fish exhibit a range of neurological abnormalities, which are characterized by vacuolization and cellular necrosis in the central nervous system and retina (Yoshikoshi & Inoue, 1990). Recently, Guo et al. (2003) showed that the capsid protein of a betanodavirus, Greasy grouper nervous necrosis virus, induces apoptotic cell death in cultured fish and mammalian cells. In the present study, it was found that SJNNV protein B2, but not its capsid protein, induced necrotic cell death in plants. It is of great interest to test whether protein B2 has necrosis-inducing activity in the nervous system of fish. This may lead to the interesting hypothesis that SJNNV protein B2 could bind to 21 nt siRNAs, and thereby suppress RNA silencing. Then, the function of a putative Drosophila
bantam-like microRNA, which suppresses programmed cell death (Brennecke et al., 2003), could be inhibited, leading to necrosis of the nervous tissue. Detailed analysis of the infectivity of protein B2-minus mutants in fish may shed light on the role of protein B2 in the ability of betanodaviruses to cause nervous necrosis in fish, and on a possible correlation between suppression of RNA silencing and induction of nervous necrosis.

ACKNOWLEDGEMENTS

We are grateful to David Baulcombe for *N. benthamiana* line 16c. We wish to thank L. Andrew Ball, Kyle L. Johnson, Lance D. Eckerle and Karyn N. Johnson for critical review of the manuscript and helpful suggestions. This work was supported by a grant from the Japan Fisheries Research Agency (formerly the Japan Sea-Farming Association) and by a Grant-in-Aid (14366110) for Scientific Research (B), a Grant-in-Aid (13580035) for Scientific Research (B), a Grant-in-Aid (11306005) for Scientific Research (A) from the Japan Society for the Promotion of Science, and a Grant-in-Aid (12052201) for Scientific Research on Priority Area (A) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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