1   *Identification of RNA regions that determine temperature sensitivities in betanodaviruses*

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1 **Summary**

2   Betanodaviruses, the causative agents of viral nervous necrosis in marine fish, have

3 bipartite positive-sense RNA genomes. The larger genomic segment RNA1 (~3.1 kb)

4 encodes an RNA-dependent RNA polymerase (protein A) and the smaller genomic

5 segment RNA2 (~1.4 kb) codes for the coat protein. The viruses can be classified

6 basically into four genotypes, designated striped jack nervous necrosis virus (SJNNV),

7 redspotted grouper nervous necrosis virus (RGNNV), tiger puffer nervous necrosis virus

8 (TPNNV), and barfin flounder nervous necrosis virus (BFNNV), based on similarities

9 in the partial RNA2 sequences. The optimal temperatures for the growth of these viruses

10 are 20–25 °C (SJNNV), 25–30 °C (RGNNV), 20 °C (TPNNV), and 15–20 °C

11 (BFNNV). However, little is known about the mechanisms underlying temperature

12 sensitivities of these viruses. We first constructed two reassortants between SJNNV and

13 RGNNV to test their temperature sensitivity. The levels of viral growth and RNA

14 replication of these reassortants and parental viruses in cultured fish cells were similar

15 at 25 °C. However, the levels of all the viruses but RGNNV were markedly reduced at

16 30 °C. These results indicate that both RNA1 and RNA2 control temperature

17 sensitivities of betanodaviruses via modulating RNA replication or earlier viral growth

18 processes. We then constructed 10 mutated RGNNV, the RNA1 segment of which was

19 chimeric between SJNNV and RGNNV, and showed that only chimeric viruses bearing

20 the RGNNV RNA1 region, encoding 1–445 th amino acid residues, grew similarly to

21 the parental RGNNV at 30 °C. The portion of protein A is known to serve a

22 mitochondrial-targeting signal rather than functions as an enzymatic domain.

23

1   **Introduction**

2   Betanodaviruses cause a highly destructive disease of hatchery-reared larvae and

3   juveniles of a variety of marine fish. Adult and mature fish have also sufferred from the

4   disease in some species in aquaculture facilities. The disease, designated as viral

5   nervous necrosis (VNN) or viral encephalopathy and retinopathy, has spread worldwide

6   to a large munber of marine fish species [37, 40]. The viruses multiply in the brains,

7   spinal cords, and retinas of the affected fish. Recently, the viruses were detected in the

8   organs of apparently healthy wild marine fish [14, 48].

9   Betanodavirus is comprised in the family *Nodaviridae* along with the other member

10   alphanodavirus that infects insects [4]. Betanodaviruses are nonenveloped, spherical

11   viruses (~25 nm in diameter) with a bipartite positive-sense RNA genome RNA1 (~3.1

12   kb) and RNA2 (~1.4 kb), which encode an RNA-dependent RNA polymerase (protein

13   A) and the coat protein (CP), respectively [36, 51]. Recently, we characterized a

14   subgenomic RNA3 (0.4 kb), which encodes protein B2 being a suppressor for

15   post-transcriptional gene silencing [27]. The viruses can be classified basically into four

16   genotypes, designated striped jack nervous necrosis virus (SJNNV), barfin flounder

17   nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), and

18   redspotted grouper nervous necrosis virus (RGNNV), based on similarities in the partial

19   RNA2 sequences [39, 43]. Recently, a betanodavirus isolate from turbot *Scophthalmus*

20   *maximus* (TNV) was suggested to belong to a fifth genotype [29]. The host ranges of

21   TPNNV are limited to tiger puffer (*Takifugu rubripes*). SJNNV was isolated from only

22   striped jack (*Pseudocaranx dentex*) with some exceptions [11, 53]. BFNNV has been

23   isolated from some coldwater species, such as barfin flounder (*Verasper moseri*),

24   Pacific cod (*Gadus macrocephalus*) and Atlantic halibut (*Hippoglossus hippoglossus*).

1   In contrast, RGNNV has a broad host range and causes the disease among a variety of

2   warm water fish species, particularly groupers and sea bass [37]. RNA2 is known to be

3   a determinant for host-specificity of the viruses [26]. Recently, the partial RNA2 region,

4   used to classify betanodaviruses [39], was shown to be suffucuent to control

5   host-specificity [22]. The optimal temperatures for the growth of RGNNV are 25–30 °C

6   which are relatively higher than those of SJNNV (20–25 °C), TPNNV (20 °C), and

7   BFNNV (15–20 °C) [24]. Some RGNNV isolates were shown to multiply at up to 35 °C

8   [17]. However, little is known about the mechanisms underlying temperature

9   sensitivities of these viruses [7, 5, 10, 17]. In this study, we constructed reassortants and

10   chimeric viruses between SJNNV and RGNNV to investigate their temperature

11   sensitivities in some viral growth steps. Base on the obtained data, we also refer to

12   possible temperature sensitivity mechanisms in betanodaviruses.

13

14   **Materials and methods**

15   **Cells.** The E-11 cell line [24], cloned from the striped snakehead (*Ophicephalus*

16   *striatus*) cell line (SSN-1) [13], was grown at 25 °C in Leibovit's L-15 medium

17   (Invitrogen) supplemented with 5% foetal bovine serum.

18   **Plasmids.** The plasmids used or constructed in this study are listed in Table 1.

19   These plasmids contain the cDNAs of SJNNV (SJNag93 strain), RGNNV (SGWak97

20   strain) [23], and the chimeric viruses constructed from SJNNV and RGNNV. Identities

21   of RNA1 and RNA2 sequences between SJNag93A and SGWak97 are 82% and 80%,

22   respectively [43]. A series of chimeric viral cDNAs was constructed by replacing the

23   original cDNA sequences with corresponding heterologous viral cDNAs synthesized

24   using a polymerase chain reaction (PCR)-based method (Table 1). All the recombinant

1   DNA techniques used in this study are described in Sambrook *et al*. [49]. The nucleotide

2   sequences of the constructed viral cDNAs were verified by DNA sequencing.

3   **Preparation of chimeric viruses.** The synthesis of infectious transcripts from the

4   viral cDNAs (Table 1) and the transfection of E-11 cells with these transcripts were

5   performed to produce mature viruses (Table 2), as described previously [22]. Viral

6   growth then was confirmed by the appearance of cytopathic effects. The culture

7   supernatants containing infectious virus particles were stored at –80 °C before use.

8   **Viral growth test.** E-11 cells grown in a 6-well plate (Iwaki) were inoculated with

9   each of the viruses (Table 2) at a multiplicity of infection (MOI) of 1.0. After incubation

10   at 15, 20, 25, or 30 °C for 1 h, the inoculum was replaced by Leibovitz's L-15

11   medium (Invitrogen) supplemented with 5 % fetal bovine serum (2 ml per well) and the

12   cells were cultured at the same temperatures. Fifty µl culture supernatant was collected

13   periodically and its viral titer was measured by determining the 50 % tissue culture

14   infectious dose ($TCID_{50}$)/ml as described in Reed and Muench [47].

15   **RNA replication and encapsidation tests.** E-11 cells were inoculated with the

16   viruses (Table 2) and cultured under the same condition as used for the viral growth test.

17   Periodically, the inoculated cells were washed once with Hanks' balanced salt solution

18   (HBSS) (Nissui) and were homogenized with 250 µl ISOGEN (Nippon gene) to prepare

19   total RNA according to the manufacturer's instruction. Obtained RNA was dissolved in

20   diethylpyrocarbonate (DEPC)-treated water and used for Northern hybridization

21   experiments. Northern hybridization was performed as described previously [17, 25]

22   using DIG-Labelling and Detection Kit (Roche) according to the supplier's instructions.

23   Briefly, to prepare probes for positive-sense RNA1 and RNA3, pSJ1BS1 and pRG1BS1

24   were linearized with *Sal*I, followed by transcription with T7 RNA polymerase (Takara).

1   Similarly, to prepare probes for positive-sense RNA2, pSJ2BS2 and pRG2BS2 were

2   linearized with *Eco*RI before transcription with the polymerase. Hybridization signals

3   were detected by exposing hybridization blots to X-ray films (RX-U; Fuji film). For the

4   encapsidation competence test, virion fractions were prepared periodically from the

5   inoculated cells as described previously [42]. Virion RNA was prepared from the virion

6   fractions using ISOGEN, dissolved in DEPC-treated water, and used for Northern

7   hybridization experiments as described avobe.

8   **RNA1 transfection.** Viral RNA was prepared from SJNNV or RGNNV virions as

9   described previously [26] and separated on a 1 % agarose gel (SeqPlaque; FMC). RNA1

10   was recovered from the gel, purified using RNeasy Mini Kit (Qiagen), and dissolved in

11   DEPC-treated water. E-11 cells grown in a 6-well plate (Iwaki) were transfected with

12   the purified RNA1 (50 ng/well) as described previously [22] and cultured at 25 or 30 °C.

13   Total RNA was prepared periodically from the transfected cells and used for Northern

14   hybridization experiments as described above.

15   **Binding assay.** E-11 cells grown in a 6-well plate (Iwaki) were washed once with 1

16   mL HBSS and inoculated with the reassortant or parental virus resuspended in 1 ml

17   HBSS. The inoculated cells were incubated at 25 or 30 °C for 30 min, washed four

18   times with 1 ml HBSS, and sacrificed to prepared total RNA from them using RNeasy

19   Mini Kit. First strand cDNAs were synthesized from the SJRG-3 primer

20   (5′-CGCCGAAGCGTAGGACAGCA-3′) using the SuperScript Chioce System for

21   cDNA Synthesis kit (Invitrogen) and used as the templates for PCR. PCR was

22   performed using the SJ1RG1-F (5′-AGTTCCACTGAGCTGGTTGA-3′) and

23   SJ1RG1-R (5′-AGCTCGACGAGGTGCTGATC-3′) primers with 28 cycles of

24   denaturation at 94 ˚C for 40 sec, annealing at 65 ˚C for 60 sec, and extension at 72 ˚C

1 for 90 sec. SJNNV and RGNNV RNA1s shared these three primers sequences and gave

2 approximately 1-kb PCR products.

3

4 **Results**

5 **Effects of temperatures on the growth of reassortant and parental viruses.** To

6 identify viral segments that control temperature sensitivities of betanodaviruses, the

7 growth of SJ1/SJ2, RG1/RG2, SJ1/RG2, and RG1/SJ2 were examined at different

8 temperatures. At 25 °C, all the four viruses grew well, showing similar growth rates

9 throughout the experimental periods (Fig. 1). Similarly, no significant difference in viral

10 growth rates was observed among the four viruses at 15 or 20 °C though the rates at 15

11 and 20 °C were lower and slightly lower than those at 25 °C, respectively. In contrast, at

12 30 °C, RG1/RG2 exclusively grew to the similar levels occurred at 25 °C (Fig. 1).

13 RG1/SJ2 slightly produced progeny viruses of up to ca. $10^{5.3}$ TCID$_{50}$/ml at 30 °C. While

14 SJ1/SJ2 and SJ1/RG2 barely grew at this temperature from one to three days after

15 inoculations, their titers finally reduced to the background levels. These data indicate

16 that both RNA1 and RNA2 control temperature sensitivity of these viruses.

17 **Effects of temperatures on the RNA replication, encapsidation competence,**

18 **and CP-expression.** Involvement of both RNA1 and RNA2 in the determination of

19 temperature sensitivity encouraged us to identify the mechanism underlying it. RNA

20 replication of the reassortants and parental viruses was first examined by detecting their

21 progeny viral RNA in the inoculated cultured cells at 25 or 30 °C. At 25 °C, all the four

22 viruses showed similar RNA replication levels at five days after inoculations. However,

23 at 30 °C, only RG1/RG2 exhibited RNA replication similar to the levels at 25 °C (Fig.

24 2). No RNA replication was detected at 30 °C for SJ1/SJ2 or SJ1/RG2. RG1/SJ2 gave a

7

1   small amount of progeny viral RNA at 30 °C (Fig. 2). These RNA replication data of the

2   four viruses well correlated with the viral growth shown in Fig. 1. The kinetics of virion

3   RNA production was closely similar to that of total viral RNA production (Fig. 2).

4   Moreover, the levels of CP expression for the viruses were also correlated to those of

5   RNA replication and viral growth (Fig. 2). At 25 °C, the signals of SJNNV CP were

6   slightly stronger than those of RGNNV despite the similar levels of RNA replication

7   between SJNNV and RGNNV because the anti-SJNNV antibody used in this study

8   cross-reacts SJNNV CP higher than RGNNV CP (26). Collectively, temperature seemed

9   to affect RNA replication or earlier viral growth processes.

10   **Virion stability of reassortant and parental viruses.** Possible differences in the

11   stability of the inocula and progeny viruses at 30 °C might have affected the viral

12   growth and RNA replication shown in Figs. 1-2. Hence, the reassortant and parental

13   viruses were exposed at 30 °C for 5 days to examine their stability at the temperature.

14   The viral titers of the incubated viral solutions showed that the survival rates of SJ1/SJ2,

15   SJ1/RG2, RG1/SJ2, and RG1/RG2 were 24.7, 39.2, 20.3, and 53.4 %, respectively.

16   These small differences in virion stability do not seem to explain the drastic differences

17   in the viral growth or RNA replication in Figs. 1-2. Nevertheless, the higher virion

18   stability of RG1/RG2 may contribute to some extent to its adaptation at 30 °C.

19   **Effects of temperatures on the bindings of reassortant and parental viruses to**

20   **E-11 cells.** One of the viral growth steps that could be affected by temperature is virion

21   attachment to the host cell surface. Binding of SJ1/SJ2, RG1/RG2, SJ1/RG2, and

22   RG1/SJ2 virions to E-11 cells at 25 or 30 °C were evaluated using an RT-PCR based

23   method. At 25 °C, SJ1/RG2 bound the most abundantly to E-11 cells among the four

24   viruses (Fig. 3). Binding was slightly reduced for RG1/RG2 and was further reduced for

1   RG1/SJ2 and SJ1SJ2. This tendency was true of their bindings with E-11 cells at 30 °C

2   (Fig. 3). These results imply that elevating the incubation temperature from 25 to 30 °C

3   does not affect markedly the levels of binding between the four viruses and E-11 cells.

4   **Effects of temperatures on RNA1 replication.** One plausible scenario of the

5   RNA1-dependent control of temperature sensitivity (Figs. 1 and 2) is that an enzyme

6   activity of protein A, encoded by RNA1, is regulated by temperatures. To test this

7   possibility, RNA1 purified from the virions of SJNNV or RGNNV was transfected into

8   E-11 cells and their replication were examined by Northern hybridization. At 25 °C,

9   both the RNA1 replicated and produced RNA3 similarly (Fig. 4). However, at 30 °C,

10   only RGNNV RNA1 replicated and produced RNA3 as occurred at 25 °C. No RNA1

11   replication and RNA3 production was obtained for SJNNV RNA1 at 30 °C throughout

12   the experiment periods (Fig. 4). These results indicate that RNA1 can control

13   temperature sensitivity of betanodaviruses, at least in part, independent of RNA2.

14   **RNA1 regions that control temperature sensitivity.** Chimeric RGNNV viruses,

15   parts of RGNNV RNA1 of which were replaced by the corresponding parts of SJNNV

16   RNA1, were prepared to identify the regions that determine temperature sensitivity (Fig.

17   5A). RG1/RG2, SJ1/RG2, and all the nine chimeric viruses grew similarly at 25 °C,

18   showing cytopathic effects (CPE). The viruses gave viral titers from $10^{6.7}$ to $10^{8.3}$

19   TCID$_{50}$/ml at this temperature (Fig. 5A). At 30 °C, RGSJ1-Cla/RG2, RGSJ1-Hpa/RG2,

20   RGSJ1-Eco/RG2, RGSJ1-Sty/RG2, and SJRGSJ1-5UTR/RG2 grew, giving CPE and

21   similar levels of viral titers to those obtained at 25 °C. In contrast, RGSJ1-Nhe/RG2,

22   RGSJ1-Bst/RG2, SJRGSJ1-Nde/RG2, SJRGSJ1-Eco/RG2, SJ1/RG2 grew less

23   efficiently, showing the viral titers from less than $10^{2.8}$ to $10^{4.7}$ TCID$_{50}$/ml (Fig. 5A). No

24   CPE was observed in these infections during the experimental periods. These results

1   indicate that the RGNNV RNA1 region from 84 to 1419 nt is important for viral growth

2   at 30 °C. This region encodes 1–445 th amino acid residues of RGNNV protein A.

3   **RNA2 regions that control temperature sensitivity.** To identify an RNA2 region

4   that controls temperature sensitivity, chimeric RGNNV viruses, parts of RGNNV RNA2

5   of which were replaced by the corresponding parts of SJNNV RNA2, were prepared.

6   Seven chimeric viruses were designed to investigate systematically the RNA2 segment.

7   However, unfortunately, of the seven chimeric viruses, only the four chimeric viruses

8   shown in Fig. 6 were grew well at 25 °C (data not shown). RG1/RG2, RG1/SJ2, and the

9   four chimeric viruses grew similarly at 25 °C showing CPE (data not shown). The titers

10   of their progeny viruses at this temperature were from $10^{6.6}$ to $10^{7.7}$ TCID$_{50}$/ml (Fig. 6).

11   In contrast, at 32 °C, RG1/RGSJ2Afl gave a low level of titer, $10^{5.1}$ TCID$_{50}$/ml and

12   RG1/RGSJ2ORF showed a further reduced titer of $10^{4.0}$ TCID$_{50}$/ml. Replacing the

13   entire 5′ UTR or a part of the 3′ UTR gave no significant effect on temperature

14   sensitivity, which was evidenced by the inoculations of RG1/RGSJ2UTR-ORF and

15   RG1/RGSJ2ORF-UTR1360. Nevertheless, all the chimeric viruses produced more

16   progeny viruses at 32 °C than RG1/SJ2. These results indicate that temperature

17   sensitivity determinants are dispersed throughout the RNA2 segment. In this RNA2

18   investigation, the viruses were cultured at 32 °C instead of 30 °C because the low level

19   of RG1/SJ2 growth at 30 °C (Fig. 1) was completely suppressed by elevating the culture

20   temperature to 32 °C, which produced more differences in the titers of the six viruses

21   tested.

22

23   **Discussion**

24   Effects of temperatures on viral growth, RNA replication, virion stability, and

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1   virion binding to host cells were examined using SJNNV, RGNNV, and their reassortant

2   viruses. Obtained data demonstrate that both RNA1 and RNA2 play a role in

3   determining temperature sensitivity, and that temperatures affected viral RNA

4   replication but had no significant influence on virion stability or virion attachment to

5   host cells. Further experiments by transfecting E-11 cells with SJNNV RNA1 or

6   RGNNV RNA1 revealed that RNA1 can control temperature sensitivity without

7   interacting with RNA2, which confirmed the vulnerability of viral RNA replication

8   processes to temperature changes. It remains to be addressed whether RNA1 itself

9   and/or encoded protein A confer the temperature sensitivity. In many plant and animal

10   viruses, temperature sensitivities are controlled by viral RNA replication processes. In

11   those cases, RNA replication of wild type [3, 35, 41] or mutant [2, 19, 21, 28, 45]

12   viruses, the latters of which carry amino acid substitutions in the replicases, were

13   examined at different temperatures. However, direct evidence for the effects of

14   temperatures on viral replicase activities has not yet been studied extensively in those

15   viruses [e. g., 1]. One possible reason is a difficulty to express or purify an active viral

16   replicase [20, 30, 44, 50]. Our attempts to evaluate SJNNV and RGNNV protein A

17   activities at 25 and 30 °C have never been succeeded due to the poor expression of

18   protein A in cultured fish cells or the insoluble nature of the protein A expressed in

19   *Escherichia coli* cells (authors' unpublished data). We need an improved experimental

20   method to evaluate the enzyme activities of SJNNV and RGNNV protein As, which will

21   help us to confirm the temperature sensitivity mechanisms of betanodaviruses.

22   Our temperature sensitivity assay using the RNA1-chimeric viruses revealed that

23   the RNA1 region, which encoded the 1–445 th amino acid residues of protein A,

24   seemed sufficient to control temperature sensitivity in those viruses (Fig. 5A). These

1   445 amino acid residues contain the putative signal to target protein A onto

2   mitochondrial membranes (Fig. 4B) [16, 34]. The remaining C-terminal region of

3   protein A harbors a putative RdRp catalytic domain. No other apparent functional

4   domain was predicted in either of SJNNV or RGNNV protein A (authors' unpublished

5   data). These results suggest that temperatures affect transferring protein A to

6   mitochondrial membranes or the protein A conformation, rather than modulate substrate

7   bindings to the putative RdRp domain. Alternatively, the RNA1 sequence itself may be

8   important to confer the temperature sensitivity [2, 35]. RNA-RNA interactions within or

9   between viral segments are important to control RNA replication and basically occur

10   between loop and/or bulge structures of RNA [9, 15, 31, 46]. Such the secondary

11   structures of RNA are known to change depending on the temperature conditions [32].

12   RGSJ1-Cla/RG2, in which the entire RGNNV RNA3 sequence was replaced by that of

13   SJNNV, still retained strong viral growth at 30 °C. This result demonstrates that either

14   RNA3 or encoded protein B2 does not confer temperature sensitivity in these two

15   viruses used in this study.

16   Our experiments using the RNA2-chimeric viruses did not identify a distinct RNA2

17   region that controls temperature sensitivity though RNA2 as well as RNA1 served as a

18   factor affected by temperature. Conversely, such regions were revealed to be dispersed

19   throughout the 1.4 kb RNA2 segment. A mechanism of the RNA2-dependent control is

20   still not clear since temperatures gave no significant influence on virion stability or

21   attachment of the parental and reassortant viruses onto host cells (Fig. 3). Nevertheless,

22   the low levels of viral growth and RNA replication of RG1/SJ2 at 30 °C (Figs. 1 and 2),

23   which was not correlated with the profile of RGNNV RNA1 and RNA3 production at

24   the same temperature (Fig. 4), indicates that SJNNV RNA2 inhibits the production of

1   RGNNV RNA1 and RNA3 at 30 °C. That is, direct or indirect interactions between

2   RNA2 and RNA1 (and/or RNA2 and RNA3) may occur during RNA replication and

3   RNA3 production in betanodavirus. In the insect alphanodavirus, Flock house virus

4   (FHV), RNA3 produced from RNA1 interacts physically with RNA2 and triggers the

5   replication of RNA2. Increased RNA2 then interacts physically with RNA3 again and

6   inhibits the production of RNA3, which finally terminates the replication of RNA1 [12].

7   If a similar RNA replication system is recruited in betanodavirus, one of the possible

8   temperature-sensitive events for this virus is the interaction between RNA2 and RNA3

9   since a direct interaction between RNA1 and RNA2 do not occur during the regulation

10   process in FHV. Unfortunately, temperature sensitivity of RNA replication has not been

11   well studied in alphanodavirus [4].

12   In this report, we thus far propose that RNA replication is a plausible

13   temperature-sensitive processe in betanodavirus growth, which is evidenced by our

14   experiments using chimeric and reassortant viruses. Nonetheless, we do not deny the

15   existence of other temperature-sensitive processes for this virus. In some animal viruses,

16   temperature sensitivity is controlled by capsid proteins; the temperature sensitive

17   mutant of reovirus (tsA279) is blocked in transmembrane transport of virions, the

18   characteristics of which is determined by its M2 capsid gene [18]. In herpes simplex

19   virus, the VP26 capsid protein fails to localize to nucleus under nonpermissive

20   temperature [8] and uncoating is temperature dependent [38]. In tobamovirus that

21   infects capsicum plants, L-gene-mediated defense systems are elicited by coat protein in

22   temperature dependent manner [33]. Finally, some temperature sensitive phage P22

23   variants, the coat proteins of which bear amino acid substitutions, were incompetent for

24   assembly at the nonpermissive temperature [6, 52]. In our study, an effect of

1 temperature on encapsidation competence was not clear because the temperature

2 sensitive viruses had a serious defect in RNA replication in the first place. Among the

3 viral growth processes, virion assembly, uncoating, and a putative defence-related event

4 may be targets to be investigated in the future.

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23

1   **Figure legends**

2   **Fig. 1. Growth of the reassortant and parental viruses at different temperatures.**

3   E-11 cells were inoculated with SJ1/SJ2, SJ1/RG2, RG1/SJ2, or RG1/RG2, and cultured

4   at the indicated temperatures. Culture supernatants then were collected periodically and

5   their viral titers were examined. Data are represented as means ± standard deviations

6   from two (15 and 20 °C) or three (25 and 30 °C) independent experiments.

7

8   **Fig. 2. RNA replication, encapsidation, and CP expression of the reassortant and**

9   **parental viruses at different temperatures.** E-11 cells were inoculated with each of

10   the viruses and cultured at 25 or 30 °C. Total RNA, virion RNA, and total protein

11   fractions were prepared from the inoculated cells 5 days after inoculation. The RNA

12   samples were used for Northern hybridization experiments to detect positive-sense viral

13   RNAs. Five µg total RNA and 50 ng virion RNA were run on the each lane. Total

14   protein fractions were analysis by Western blotting using anti-SJNNV rabbit serum.

15   Representative data were shown from three independent experiments.

16

17   **Fig. 3. Semi-quantitative RT-PCR for the detection of cell-bound virions.** E-11 cells

18   were incubated with SJ1/SJ2, SJ1/RG2, RG1/SJ2, or RG1/RG2 virions for 30 min at the

19   indicated temperatures, followed by the extraction of total RNA from the cells. The

20   extracted samples were used for the detection of RNA1 by semi-quantitative RT-PCR,

21   which was evidenced by the amplification of approximately 1-kb fragments.

22

23   **Fig. 4. Replication of SJNNV RNA1 and RGNNV RNA1 at different temperatures.**

24   E-11 cells were transfected with SJNNV RNA1 or RGNNV RNA1 and cultured at 25 or

21

1   30 °C. Total RNA was isolated from the cells at 0–120 h post transfections and used for

2   Northern hybridization experiments to detect positive-sense RNA1 and RNA3. Five µg

3   total RNA was run on the each lane. Representative data were shown from three

4   independent experiments. C; No RNA1 was transfected. P; 20 ng of virion RNA from

5   SJNNV or RGNNV were loaded per lane as the positive controls.

6

7   **Fig. 5. RNA1 regions which control temperature sensitivities of SJNNV and**

8   **RGNNV.** Blue and red bars show SJNNV RNA1 and RGNNV RNA1 segments,

9   respectively. Thin and thick bars indicate the untranslated regions and the ORF for

10   protein A, respectively. Numbers on the RNA1 segments indicate the nucleotide

11   positions of chimeric junctions. (A) E-11 cells were inoculated with the viruses, each of

12   which had the chimeric or parental RNA1 along with RGNNV RNA2, and were

13   cultured at 25 or 30°C. Five days after inoculations, culture supernatants were collected

14   and their viral titers were examined. Data are represented as means ± standard

15   deviations from three independent experiments. (B) The putative transmenbrane (TM)

16   and RdRp domains are denoted on the protein A map.

17

18   **Fig. 6. RNA2 regions which control temperature sensitivities of SJNNV and**

19   **RGNNV.** Blue and red bars show SJNNV RNA2 and RGNNV RNA2 segments,

20   respectively. Thin and thick bars indicate the untranslated regions and the ORF for CP,

21   respectively. Numbers on the RNA2 segments indicate the nucleotide positions of

22   chimeric junctions. E-11 cells were inoculated with the viruses, each of which had

23   RGNNV RNA1 and the chimeric or parental RNA2, and were cultured at 25 or 32 °C.

24   Five days after inoculations, culture supernatants were collected and their viral titers

22

1   were examined. Data are represented as means ± standard deviations from three

2   independent experiments.

3

1 **Table 1.** Plasmids used in this study

2 ---

3 Plasmid   Characteristics   Reference

4 ---

5 Wild type viral cDNA

6   pSJ1TK19   full-length cDNA of SJNNV RNA1   Iwamoto et al. [25]

7   pSJ2TK30   full-length cDNA of SJNNV RNA2   Iwamoto et al. [25]

8   pRG1TK5 [a]   full-length cDNA of RGNNV RNA1   Iwamoto et al. [26]

9   pRG2TK13 [a]   full-length cDNA of RGNNV RNA2   Iwamoto et al. [26]

10 pRG2TK13B [a]   1057 th T in the pRG2TK13 cDNA is replaced by A   Ito et al. [22]

11

12 Chimeric viral cDNA

13 pRGSJ1-Cla   ca. 430 bp Cla I–EcoR I fragment in pRG1TK5 is replaced by that from pSJ1TK19   This study

14 pRGSJ1-Hpa   ca. 1180 bp Hpa I–EcoR I fragment in pRG1TK5 is replaced by that from pSJ1TK19   This study

15 pRGSJ1-Eco   ca. 1520 bp EcoR V–EcoR I fragment in pRG1TK5 is replaced by that from pSJ1TK19   This study

16 pRGSJ1-Sty   ca. 1690 bp Sty I–EcoR I fragment in pRG1TK5 is replaced by that from pSJ1TK19   This study

17 pRGSJ1-Nhe   ca. 1220 bp Hind III–Nhe I fragment in pSJ1TK19 is replaced by that from pRG1TK5   This study

18 pRGSJ1-Bst   ca. 1090 bp Hind III-BstBI fragment in pSJ1TK19 is replaced by that from pRG1TK5   This study

19 pSJRGSJ1-5UTR   ca. 50 bp Hind III-BstE II fragment in pRG1SJ1-RV is replaced by that from pSJ1TK19   This study

20 pSJRGSJ1-Nde   ca. 390 bp Hind III-Nde I fragment in pRG1SJ1-RV is replaced by that from pSJ1TK19   This study

21 pSJRGSJ1-Eco   ca. 540 bp Hind III-EcoT22 I fragment in pRG1SJ1-RV is replaced by that from pSJ1TK19   This study

22 pRGSJ2Afl   ca. 440 bp Afl III–BspE I fragment in pRG2TK13B is replaced by that from pSJ2TK30   Ito et al. [22]

23 pRGSJ2ORF   the ORF in pRG2TK13 is replaced by that from pSJ2TK30   This study

24 pRGSJ2UTR-ORF   the 5′ UTR and ORF in pRG2TK13 is replaced by that from pSJ2TK30   This study

25 pRGSJ2ORF-UTR1360   the 3′ UTR terminus (75 bp) in pRGSJ2ORF was replaced by that from pSJ2TK30   This study

26

27 Viral cDNA for probe preparation

28 pSJ1BS1 [b]   ca. 330 bp Cla I–EcoR I fragment of SJNNV RNA1 cDNA in pBlueScript SK(-) [c]   Iwamoto et al. [25]

29 pSJ2BS2   ca. 360 bp BamH I–EcoR I fragment of SJNNV RNA2 cDNA in pBlueScript SK(-)   Iwamoto et al. [25]

30 pRG1BS1 [b]   ca. 330 bp Cla I–EcoR I fragment of RGNNV RNA1 cDNA in pBlueScript SK(-)   Hata et al. [17]

31 pRG2BS2   ca. 360 bp BamH I–EcoR I fragment of RGNNV RNA2 cDNA in pBlueScript SK(-)   Hata et al. [17]

32 ---

33 [a] pRG1TK5, pRG2TK13, and pRG2TK13B were described previously as pSG1TK5, pSG2TK13, and pSG2TK13B, respecvively

34 [22, 26].

35 [b] Riboprobes transcribed from these cDNA detected both RNA1 and RNA3.

36 [c] pBlueScript SK(-) was purchased from Stratagene (La Jolla, CA, USA).

37

Table 2. Viruses used in this study

| Virus | Viral cDNA clone [b] | | Reference |
	RNA1	RNA2	
Parental virus			
SJ1/SJ2	pSJ1TK19	pSJ2TK30	Iwamoto et al. [25]
RG1/RG2 [a]	pRG1TK5	pRG2TK13	Iwamoto et al. [26]
Reassortant virus			
SJ1/RG2 [a]	pSJ1TK19	pRG2TK13	Iwamoto et al. [26]
RG1/SJ2 [a]	pRG1TK5	pSJ2TK30	Iwamoto et al. [26]
Chimeric virus			
RGSJ1-Cla/RG2	pRGSJ1-Cla	pRG2TK13	This study
RGSJ1-Hpa/RG2	pRGSJ1-Hpa	pRG2TK13	This study
RGSJ1-Eco/RG2	pRGSJ1-Eco	pRG2TK13	This study
RGSJ1-Sty/RG2	pRGSJ1-Sty	pRG2TK13	This study
RGSJ1-Nhe/RG2	pRGSJ1-Nhe	pRG2TK13	This study
RGSJ1-Bst/RG2	pRGSJ1-Bst	pRG2TK13	This study
SJRGSJ1-5UTR/RG2	pSJRGSJ1-5UTR	pRG2TK13	This study
SJRGSJ1-Nde/RG2	pSJRGSJ1-Nde	pRG2TK13	This study
SJRGSJ1-Eco/RG2	pSJRGSJ1-Eco	pRG2TK13	This study
RG1/RGSJ2Afl	pRG1TK5	pRGSJ2Afl	Ito et al. [22]
RG1/RGSJ2ORF	pRG1TK5	pRGSJ2ORF	This study
RG1/RGSJ2UTR-ORF	pRG1TK5	pRGSJ2UTR-ORF	This study
RG1/RGSJ2ORF-UTR1360	pRG1TK5	pRGSJ2ORF-UTR1360	This study

[a] RG1/RG2, SJ1/RG2, and RG1/SJ2 are described previously as SG1/SG2, SJ1/SG2, and SG1/SJ2, respectively [26].

[b] In vitro transcripts from the indicated viral cDNAs were co-transfected into E-11 cells to obtain the viruses.

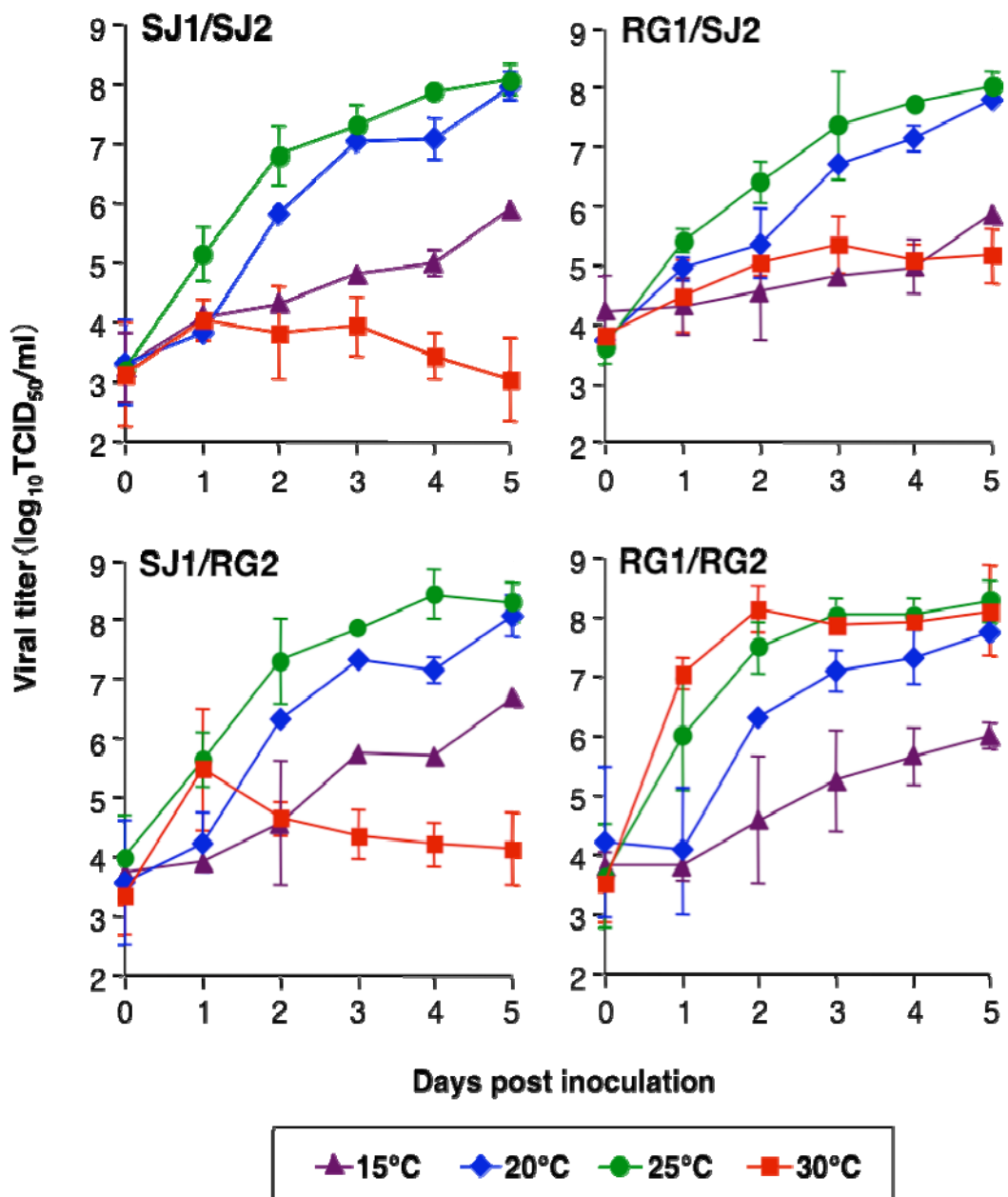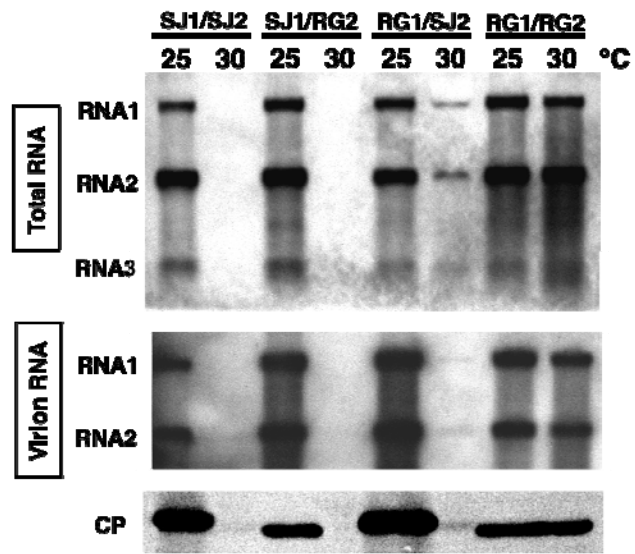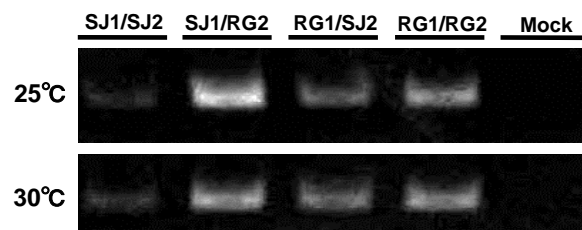1   Fig. 1

2

Days post inoculation

15°C   20°C   25°C   30°C

1   Fig. 2
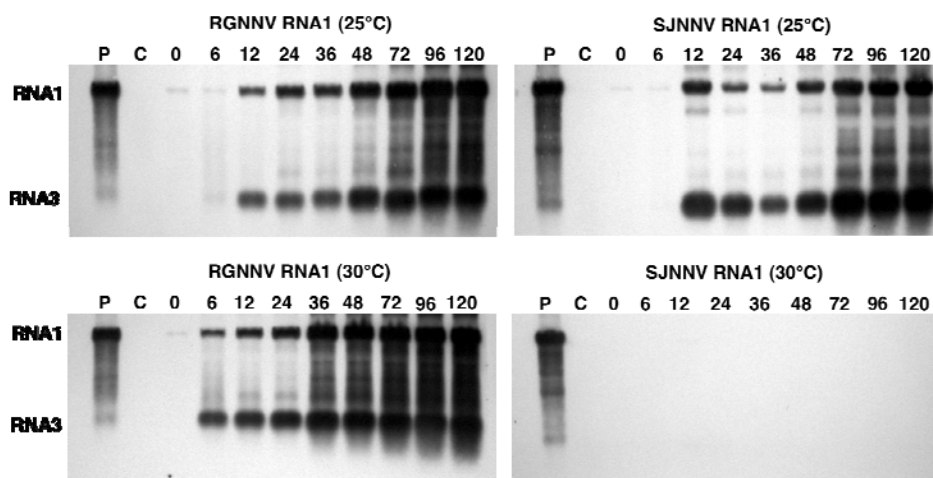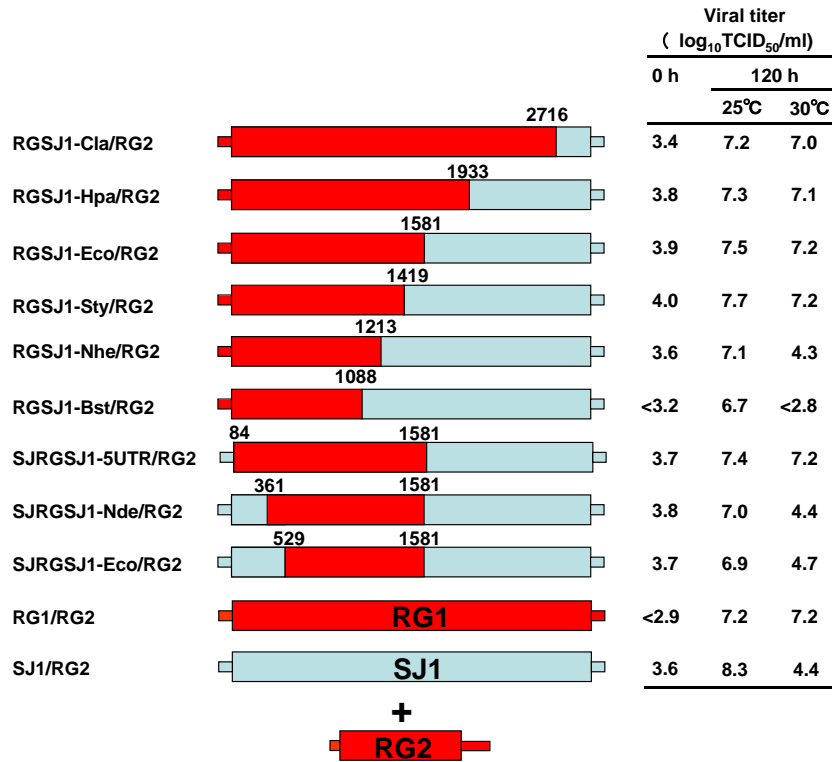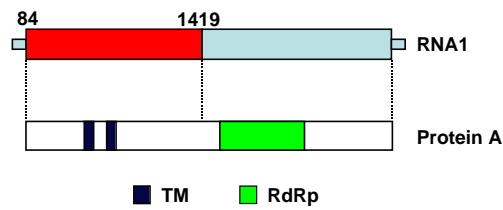
2

3

4

1

2

Fig. 3

1

2

Fig. 4

Fig. 5

1

2

A

	Viral titer ($\log_{10}$TCID$_{50}$/ml)		
	0 h	120 h	
		25℃	30℃
RGSJ1-Cla/RG2	3.4	7.2	7.0
RGSJ1-Hpa/RG2	3.8	7.3	7.1
RGSJ1-Eco/RG2	3.9	7.5	7.2
RGSJ1-Sty/RG2	4.0	7.7	7.2
RGSJ1-Nhe/RG2	3.6	7.1	4.3
RGSJ1-Bst/RG2	<3.2	6.7	<2.8
SJRGSJ1-5UTR/RG2	3.7	7.4	7.2
SJRGSJ1-Nde/RG2	3.8	7.0	4.4
SJRGSJ1-Eco/RG2	3.7	6.9	4.7
RG1/RG2	<2.9	7.2	7.2
SJ1/RG2	3.6	8.3	4.4

B