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Author(s)	Sakai, Mizuki; Muto, Memi; Hirano, Minato et al.
Citation	Virus research, 203, 36-40 https://doi.org/10.1016/j.virusres.2015.03.006
Issue Date	2015-05-04
Doc URL	https://hdl.handle.net/2115/61469
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Virus research v.203 p.36-40.pdf

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Title

Virulence of tick-borne encephalitis virus is associated with intact conformational viral RNA structures in the variable region of the 3′-UTR

Running title: The variable region of 3'-UTR as a virulence factor in TBEV

Mizuki Sakai, Memi Muto, Minato Hirano, Hiroaki Kariwa, Kentaro Yoshii

Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

Corresponding author: Kentaro YOSHII, PhD., D.V.M.,
Postal address: Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University, kita-18 nishi-9, kita-ku, Sapporo, Hokkaido 060-0818, Japan
Tel/fax: +81-11-7-6-5213
Email: kyoshii@vetmed.hokudai.ac.jp

Word count for abstract: 156

Word count for text: 1660

Abstract

Tick-borne encephalitis virus (TBEV) is maintained between ticks and mammals in nature and causes severe neurological disease in human. However, the mechanism of viral pathogenicity is unknown. Previously, we showed that the deletion in the variable region of the 3′-untranslated region (UTR) is involved in the pathogenicity of the strains from the Far-Eastern subtype of TBEV. To investigate the detailed function of the variable region, we constructed recombinant TBEV with partial deletions in the region. In a mouse model, the partial deletions drastically increased the virulence of the virus, with no effect on virus multiplication in mouse brain. Furthermore, the mutations did not affect the production of subgenomic flavivirus RNA from the 3′-UTR, and the induction of interferon (IFN) and IFN-stimulated genes. These data suggested that the conformational structure of the variable region is associated with the pathogenicity of the Far-Eastern subtype of TBEV. These findings provide a foundation for further research to identify the pathogenic mechanisms of TBEV.

Key words

Tick-borne encephalitis virus, 3'-untranslated region, virulence factor, pathogenicity

Text

Tick-borne encephalitis (TBE) virus (TBEV), a member of the genus *Flavivirus* in the family *Flaviviridae*, causes potentially fatal encephalitis in humans. It is a major arbovirus that causes thousands of cases of severe neurological illness annually (Abbott et al., 2005; Kaiser et al., 2007). TBE is a significant public health problem in endemic

areas of European and Asian countries (Lindquist and Vapalahti, 2008; Mansfield et al., 2009). Mortality rates vary from about 0.5 to 30%, and neurological sequelae can occur in 30 to 60% of survivors (Grard et al., 2007; Gritsun et al., 2003; Heinz and Kunz, 2004).

TBEV is a positive-stranded RNA virus with a genome of ~11 kb that encodes a long polyprotein in a single open reading frame (ORF), flanked by 5′- and 3′-untranslated regions (UTRs). The 5′- and 3′-UTRs are believed to be associated with viral genome replication (Khromykh et al., 2001; Kofler et al., 2006).

The 3′-UTR of TBEV is divided into two domains: the 5′-terminal variable region and 3′-terminal core element. The core element is highly conserved among TBEV strains and contains a sequence that is essential for viral genome replication (Kofler et al., 2006). The variable region is considered to be essential for the natural transmission cycle of TBEV, but the sequence and length vary among TBEV strains. Notably, several strains contain sequence deletions or a polyA insertion in the variable region (Mandl et al., 1991; Ruzek et al., 2008); however, strains isolated from ticks and wild rodents contain no deletions or polyA insertions in the variable region (Bredenbeek et al., 2003). In contrast, deletions or a polyA sequence insertion in the variable region were found in strains passaged in mammalian cell culture (Mandl et al., 1991). Deletions were also observed in TBEV strains isolated from human patients (Formanova et al., 2015; Leonova et al., 2013; Mandl et al., 1998). These reports suggest that the deletions or polyA insertions are caused by viral adaptation to mammalian cell and are related to viral virulence in severe cases in human. It is also suggested that direct repeats in the region is associated with RNA replication, providing the strategy for flavivirus survival in a variety of natural hosts (Gritsun and Gould, 2007). However, the role of this region

71 in the virulence remains unclear.

72 The variable region was reported to play no role in viral replication and
73 virulence in laboratory mice (Mandl et al., 1998). However, in our previous study
74 regarding virulence factors of the Far-Eastern subtype of TBEV, we demonstrated that
75 a deletion in the variable region is associated with differential virulence observed
76 between a highly virulent strain, Sofjin-HO (accession no. AB062064) (Chumakov and
77 Levkovich et al., 1985) and a low virulent strain, Oshima 5-10 (accession no. AB062064)
78 (Chiba et al., 1999). In this study, we investigated in more detail how partial deletions
79 in the variable region affect the viral pathogenicity during TBEV infection.

80 MFOLD (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Markham
81 and Zuker, 2005; Zuker, 2003) was used to predict the RNA secondary structures in the
82 variable regions of the Sofjin and Oshima strains as descrived previously (Gritsun et al.,
83 2014) (Fig. 1). The variable region of Oshima includes seven stem loop (SL) structures
84 (SL1 to SL7), whereas the variable region in Sofjin lacks SL3, SL4 and SL5. To confirm
85 whether the specific RNA sequence or secondary structure of the region is associated
86 with the difference in virulence between Sofjin and Oshima, we employed reverse
87 genetics using an infectious cDNA clone of the Oshima 5-10 strain (Oshima-IC-pt)
88 (Hayasaka et al., 2004) (Fig. 2A), as described previously (Sakai et al., 2014).
89 Oshima-based mutant viruses were constructed with the following deletions: SL3, SL4
90 and SL5, 10443–10649 nt (Oshima-dSL3/4/5); SL3 and SL4, 10443–10567 nt
91 (Oshima-dSL3/4); and SL5, 10568–10649 nt (Oshima-dSL5). In addition, we constructed
92 a recombinant virus containing a 35-base polyA insertion between 10493 and 10494 nt
93 (Oshima_polyA) to investigate the effect of the insertion found in other strains with
94 high passage history in cultured cells (Fig. 2A).

95 The virulence of the recombinant viruses was compared with those of the parental
96 infectious cDNA clones, Oshima-IC-pt and Sofjin-IC-pt (Takano et al., 2011).
97 The growth of the mutant viruses was examined in mouse neuroblastoma NA
98 cells at a MOI of 1. Virus titers were determined by plaque assay using baby hamster
99 kidney (BHK-21) cells as described previously (Sakai et al., 2014). Plaque assays in NA
100 cells showed that the mutant viruses had growth similar to that of Oshima-IC-pt, but
101 significantly lower than that of Sofjin-IC-pt (Fig. 2B). This result suggested that the
102 partial deletions or the poly A insertion in the mutant viruses had no effect on viral
103 multiplication in cultured cells.
104 Mutant and parental viruses (1,000 pfu) were infected subcutaneously into
105 C57BL/6 mice to investigate their virulence *in vivo*. All mutant viruses with partial
106 deletions or polyA insertion exhibited virulence similar to Sofjin, as shown by survival
107 curves, days of onset and mortality (Fig. 2C, Table 1). The partial deletion mutants
108 lacking the SL3 to SL5 structures showed no difference in the increased virulence of
109 Oshima_dSL3/4 and Oshima_dSL5, indicating that both of the partial deletions of the
110 SL3/4 and SL5 region increased the virulence in mice and that the intact
111 conformational structure containing SL3 to SL5 was important in the virulence. In the
112 RNA structures of the 3'-UTR predicted by MFOLD (Fig. S1), the deletion of SL3/4 did
113 not affect the other stem loop structure, whereas the deletion of SL5 changed the
114 structure of SL3 and SL4. Insertion of polyA sequence also affected the loop structure of
115 SL3. This suggested that the perturbation of the predicted structure of SL3/4 might be
116 related to the increase of the virulence.
117 To examine the correlation between disease development and viral replication
118 in organs, the viral loads in the blood, spleen and brain were compared in mice

119 inoculated with the Sofjin-IC-pt, Oshima_dSL3/4/5, Oshima_dSL3/4, Oshima_dSL5,
120 Oshima_polyA or Oshima-IC-pt viruses (Fig. 3). Organs were homogenized, and
121 prepared as 10% suspensions (w/v) in PBS with 10% FCS. The suspensions were
122 clarified by centrifugation (4,000 rpm for 5 min, 4°C). Transient viremia was observed
123 in the mice infected with each virus, which almost disappeared by 5-7 dpi. Increases in
124 viral replication were observed in the spleen after viremia (from 3 dpi). Compared with
125 Oshima-IC-pt-infected mice, the viral titer in blood was lower in the mice infected with
126 deletion or insertion mutant viruses. The viruses were detected in the brain by 7 dpi in
127 the mice inoculated with Sofjin-IC-pt, with titers reaching 1.1×10^8 pfu/ml and 3.4×10^9
128 pfu/ml at 7 and 9 dpi, respectively; these titers were significantly higher than those in
129 mice infected with the Oshima-based mutant viruses or Oshima-IC-pt ($p < 0.05$). No
130 significant difference was observed between titers in the brains infected with each
131 mutant virus and Oshima-IC-pt.
132 Taken together, these data suggested that the deletion in the variable region of
133 the 3′-UTR decreased virus multiplication in periphery, but enhanced the virulence in
134 the mouse brain. One explanation for this heightened virulence could be that the intact
135 conformational structure containing SL3 to SL5 interacted with host factors involved in
136 controlling the pathogenicity. It has been reported that several host proteins, such as La,
137 p100, FBP1 and Mov34, bind to the 3′-UTRs of flaviviruses (Chien et al., 2011; De
138 Nova-Ocampo et al., 2002; Lei et al., 2011; Ta and Vrati, 2000; Vashist et al., 2009;
139 Vashist et al., 2011). The La protein was shown to bind to the 5′- and 3′-UTR of
140 Japanese encephalitis virus and to affect the viral multiplication process
141 (Garcia-Montalvo et al., 2004; Vashist et al., 2011; Yocupicio-Monroy et al., 2007).
142 Interaction of the intact conformational structure with host factors might alter host

response in the brain. Also, several studies showed that immune response in peripheral organ affected the viral pathogenicity in brain (Ruzek et al., 2011). There is a possibility that the difference in the viremia might affect the host peripheral immune response associated with the pathogenicity in brain.

Subgenomic flavivirus RNA (sfRNA) is the product of degradation of viral genomic RNA in flavivirus infection (Chang et al., 2013; Pijlman et al., 2008). sfRNA is thought to be involved in virus replication in cultured cells and virulence in mice by interfering with host protective responses, such as RNA interference machinery and type I interferon (IFN) reaction (Chang et al., 2013). We performed Northern blot analysis to confirm whether the production of sfRNA is affected by the deletions or polyA insertion. Total RNA was extracted from virus-infected BHK cells, and RNA (4 μg) was subjected to denaturing gel electrophoresis in a 2% agarose and 2% formaldehyde gel followed by transfer onto membranes. Hybridization was carried out in digoxigenin (DIG) hybridization solution at 68°C using 0.5 ng/ml of positive- or negative-sense DIG-labeled RNA for 3′-UTR (10277–11100 nt of Oshima-IC-pt). After hybridization, the bound probes were reacted with alkaline phosphatase conjugated anti-DIG Fab fragments, and the bands were visualized (Roche Diagnostics, Basel, Switzerland). As shown in Fig. 4, two different sizes of sfRNAs (~300 or 500 nt) were produced in the infected BHK-21 cells (Fig. 4A), as reported previously (Pijlman et al., 2008). However, among the viruses, there was no difference in the size of sfRNA and ratio of amount of sfRNA to genomic RNA (data not shown).

We next performed reporter assays to investigate the effect of the deletions or polyA insertion in the variable region on the induction of IFN-β and interferon-stimulated genes (ISGs) (Fig. 4B and C). NA cells and human

neuroblastoma SYM-1 cells were transfected with 1 μg of pISRE-Luc cis-reporter plasmid or 4×IRF-3-Luc reporter plasmid (kindly provided by Dr. Rongtuan Lin, Jewish General Hospital-McGill University, Montreal, Canada), respectively. At 24 hours post-infection (hpi), the cells were inoculated with Oshima-IC-pt, Oshima_dSL3/4/5 or Sofjin-IC-pt at a MOI of 1, respectively. To activate the ISRE promoter, NA cells were treated with 500 U/ml mouse IFN-α (PBL Assay Science, Piscataway, NJ) at 24 hpi, and the cells were collected 3 h after the treatment. The SYM-1 cells were collected at 48 hpi. The cells were lysed and the luciferase activities were determined using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. The promoter activity of IFN-ß was significantly higher in cells infected with Sofjin-IC-pt than Oshima-IC-pt or Oshima-dSL3/4/5 (Fig. 4B). The IFN-stimulated regulatory element (ISRE) promoter activity decreased in the cells infected with Oshima-IC-pt and Sofjin-IC-pt (Fig. 4C). However, there was no significant difference between Oshima and Oshima_dSL3/4/5 infection. These results indicated that the deletion in the variable region affected neither the sfRNA production nor the induction of the type I IFN and ISGs.

In summary, we found that partial deletions and polyA insertion in the variable region increased virulence in the mouse model. These data suggested that the variable region might have a role for the alteration of host responses in the brain. Identification of such host factors may clarify the mechanism of pathogenicity in TBEV infections. These findings provide an important foundation for identifying the pathogenicity of TBEV and developing prevention and therapeutic strategies for TBE.

Acknowledgement

191 This work was supported by Grants-in-Aid for Scientific Research (2478093, 26660220,
192 22780268, 21405035 and 25・1563) from Ministry of Education, Culture, Sports, Science
193 and Technology of Japan, and Health Sciences Grants for Research on Emerging and
194 Re-emerging Infectious Disease from the Ministry of Health, Labor and Welfare of
195 Japan.
196

Figure legends

Fig. 1 The RNA secondary structures of the variable region.

The RNA secondary structures of the variable region (10377–10757 nt of Oshima and 10377–10550 nt of Sofjin) were predicted by Mfold (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). Seven stem loop (SL) structures (SL1–7) were observed in the variable region of Oshima-IC-pt, and four SLs (SL1, SL2, SL6 and SL7) were observed in Sofjin-IC-pt.

Fig. 2 Effect of deletions and polyA insertion in the variable region.

(A) Schematic representation of the recombinant virus genomes. Oshima_dSL3/4/5, Oshima_dSL3/4 or Oshima_dSL5 lacked SL3-5, SL3-4 or SL5, respectively. Oshima_polyA contained a 35-base polyA insertion in the position of the deletion in the variable region of Sofjin. **(B)** Growth curve analysis in NA cells. NA cells were infected with each virus at a MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data are the means ± SD of three independent experiments. *At 24 and 48 hpi, significant differences were observed between Sofjin-IC-pt and the other viruses ($p<0.05$). No significant difference between Oshima-IC-pt and each mutant virus was observed. **(C)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the mutant viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed square), Oshima_dSL3/4/5 (closed triangle), Oshima_dSL3/4 (open triangle), Oshima_dSL5 (closed diamond), Oshima_polyA (open diamond) and Oshima-IC-pt (closed circle).

Fig. 3 Effects of the deletions or polyA insertion in variable region on viral multiplication in organs.

Mice were infected subcutaneously with 1,000 pfu of Sofjin-IC-pt, Oshima_dSL3/4/5, Oshima_dSL3/4, Oshima_dSL5, Oshima_polyA and Oshima-IC-pt. Virus titers in the blood **(A)**, spleen **(B)**, and brain **(C)** at the indicated days after infection were determined by plaque assays. The horizontal dashed lines indicate the limits of detection for the assay (100 pfu/ml). Error bars represent the SD (n=3). At 9 days p.i., there were two surviving mice infected with Sofjin-IC-pt or Oshima_polyA. An asterisk (*) or dagger (†) denotes a significant difference compared with Sofjin-pt-IC and Oshima-IC-pt, respectively (p <0.05).

Fig. 4 Deletion in the variable region had no effect on the production of sfRNA and the IFN-mediated antiviral response.

(A) BHK-21 cells were infected with each virus and harvested at 48 hpi. The cells were lysed and RNA was extracted. Total RNA (4 μg) were loaded into each well and genomic RNA (gRNA) and sfRNA were detected by Northern blotting, using a DIG-labeled probe to detect the 3′-UTR. **(B, C)** SYM-1 and NA cells were transfected with 1 μg of pISRE-Luc cis-reporter plasmid or 4×IRF-3-Luc reporter plasmid, respectively. At 24 hpi, the cells were inoculated with each virus (NC: negative control). To activate the ISRE promoter, NA cells were treated with 500 U/ml mouse IFN-α at 24 hpi (PC: positive control), and NA cells were harvested 3 h after the treatment. SYM-1 cells were collected at 48 hpi. The cells were lysed and the luciferase activities were measured. The data are presented as means (± SD) of three independent replicates. Asterisks (*) and (**) denote a significant difference (p <0.05 or 0.01).

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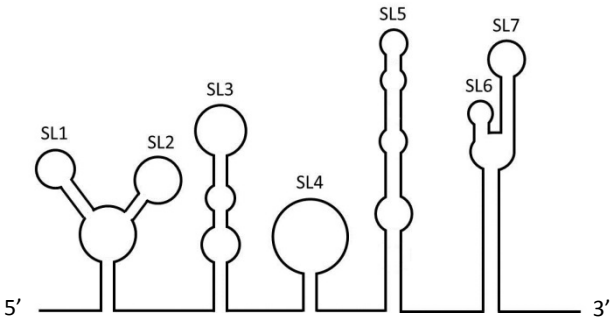
Table 1. Physical differences among mice infected with Sofjin-IC-pt, Oshima-IC-pt or the recombinant viruses[a].

Virus	Onset of disease (days)	Survival time (days)	Morbidity (%)	Mortality (%)
Sofjin-IC-pt	7.7 ± 0.8**	9.0 ± 1.5**	100	100
Oshima-IC-pt	11.9 ± 1.7	18.4 ± 5.3	90	50
Oshima_dSL3/4/5	8.6 ± 0.5**	10.4 ± 1.6*	100	100
Oshima_dSL3/4	8.6 ± 0.8**	9.7 ± 0.7**	100	100
Oshima_dSL5	8.2 ± 0.4**	9.7 ± 0.4**	100	100
Oshima_polyA	9.1 ± 0.7**	10.0 ± 1.1**	100	100

[a] Ten mice were infected with each viruses. One asterisk (*) or double astarisk (* *) denotes a significant difference between Oshima-IC and the other viruses ($p<0.05$ or 0.01) , respectively.

Fig.1

Oshima-IC-pt

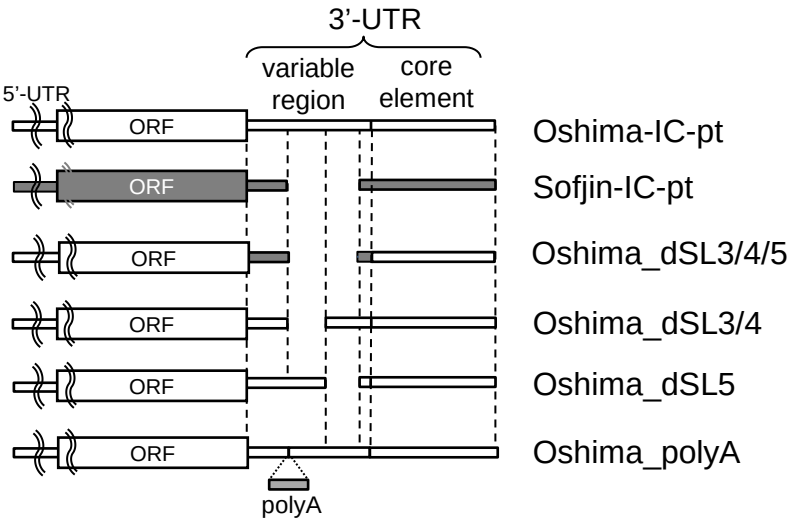


Sofjin-IC-pt

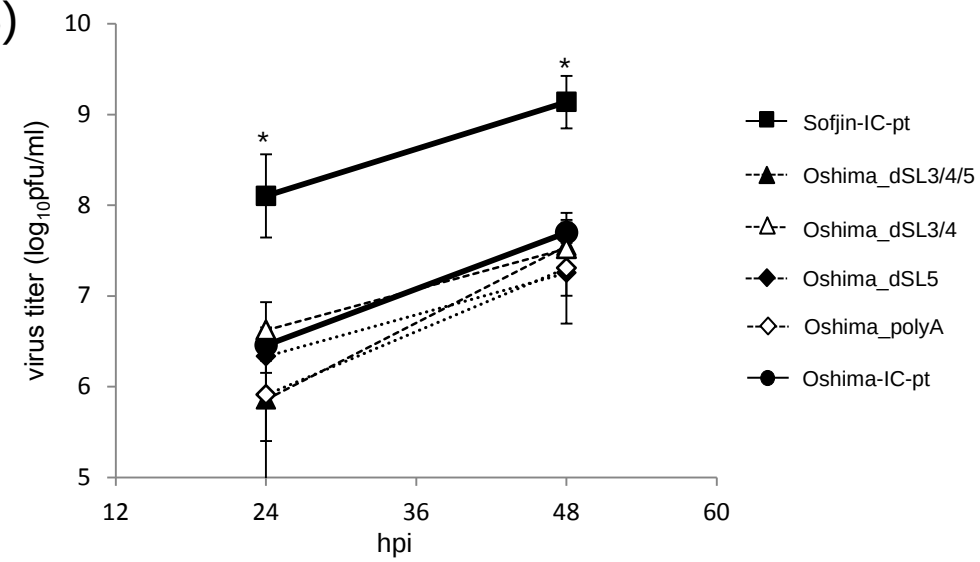


Fig.2

(A)


3'-UTR
variable region
core element
5'-UTR
ORF
polyA
Oshima-IC-pt
Sofjin-IC-pt
Oshima_dSL3/4/5
Oshima_dSL3/4
Oshima_dSL5
Oshima_polyA

(B)


virus titer (log_{10}pfu/ml)
hpi
Sofjin-IC-pt
Oshima_dSL3/4/5
Oshima_dSL3/4
Oshima_dSL5
Oshima_polyA
Oshima-IC-pt

(C)

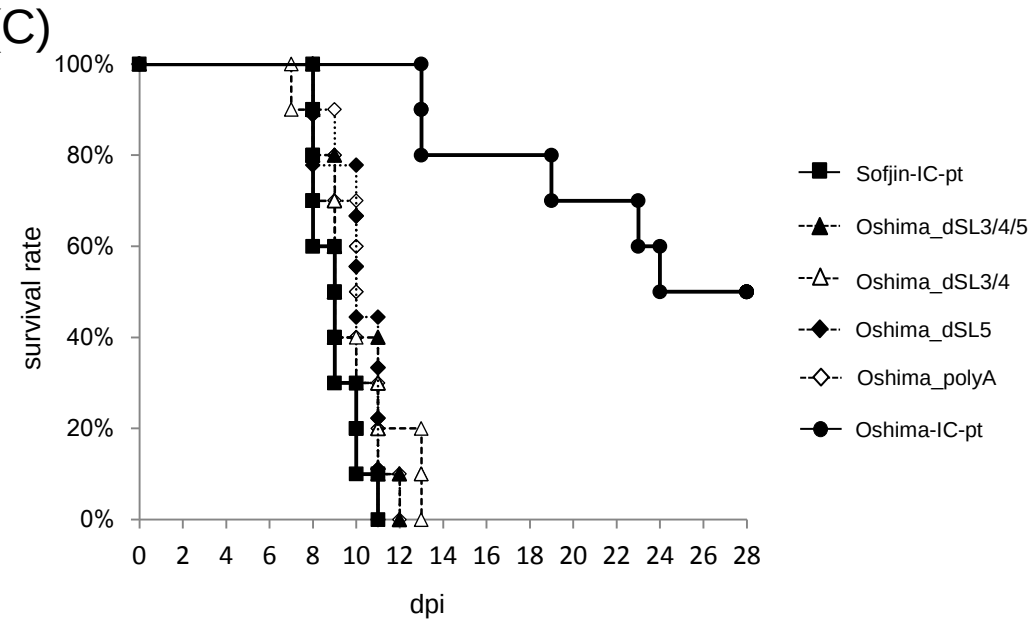

survival rate
dpi
Sofjin-IC-pt
Oshima_dSL3/4/5
Oshima_dSL3/4
Oshima_dSL5
Oshima_polyA
Oshima-IC-pt

Fig.3

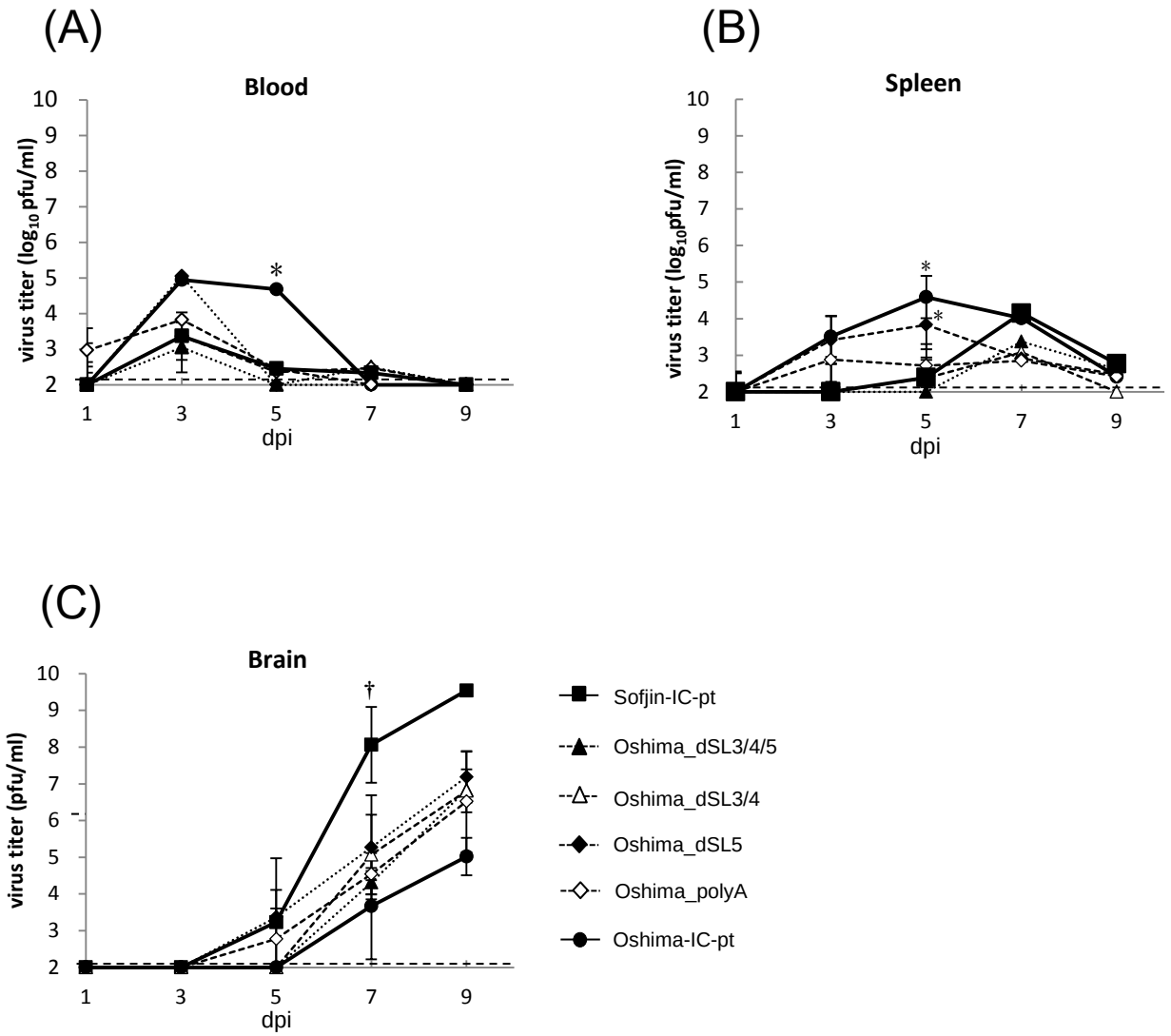

(A)
Blood
virus titer (log_{10} pfu/ml)
dpi
(B)
Spleen
virus titer (log_{10}pfu/ml)
dpi
(C)
Brain
virus titer (pfu/ml)
dpi
Sofjin-IC-pt
Oshima_dSL3/4/5
Oshima_dSL3/4
Oshima_dSL5
Oshima_polyA
Oshima-IC-pt

Fig.4

(A)

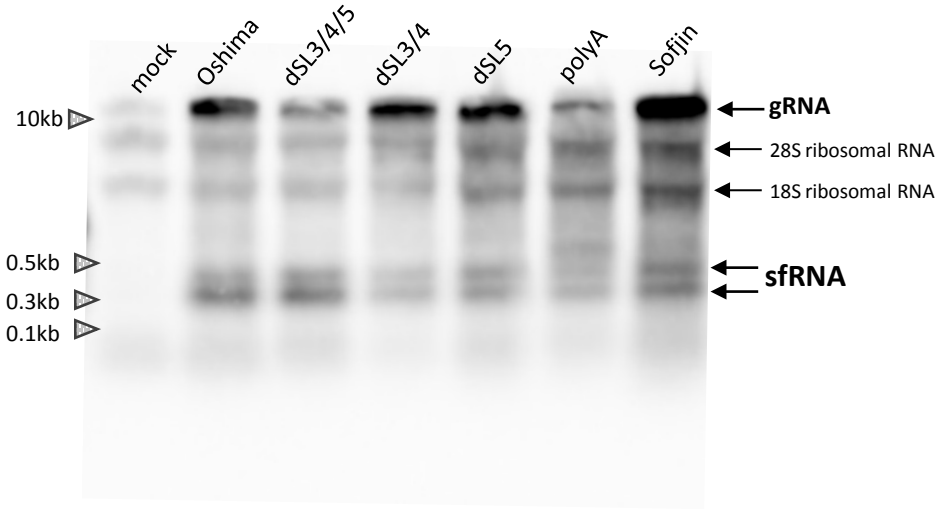
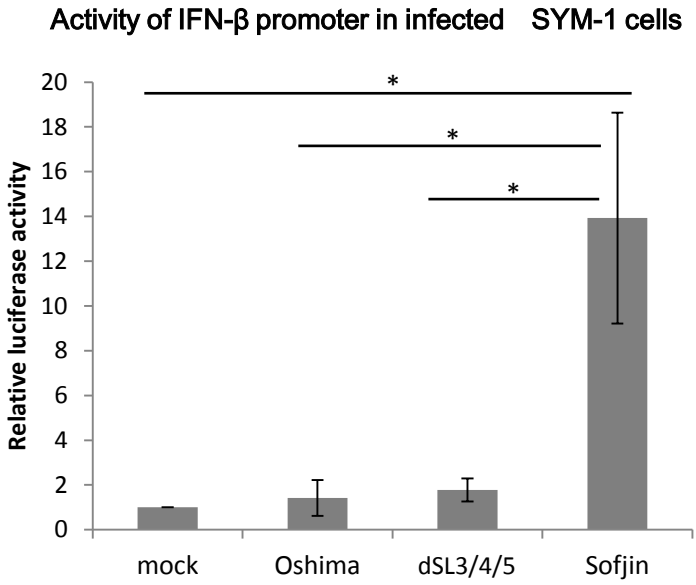
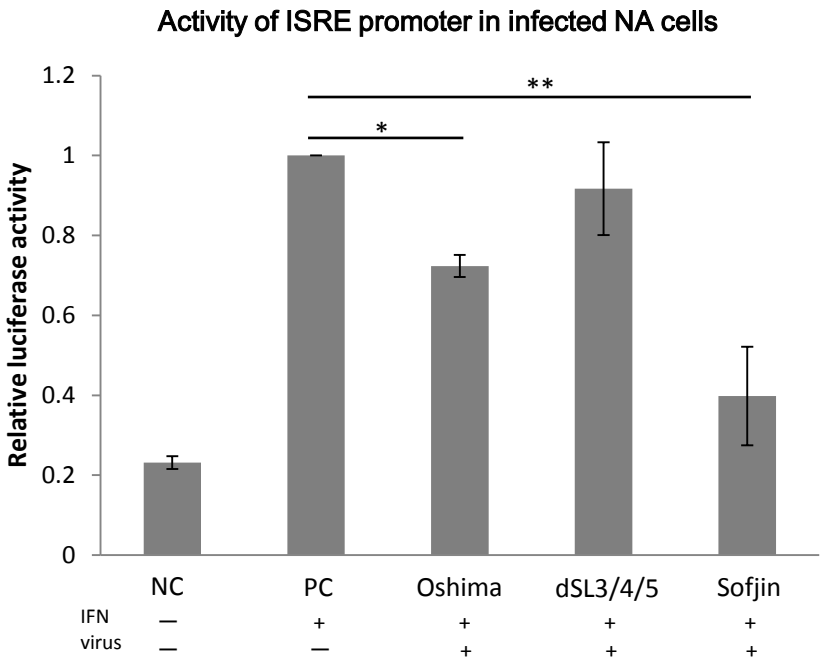

mock
Oshima
dSL3/4/5
dSL3/4
dSL5
polyA
Sofjin
10kb
0.5kb
0.3kb
0.1kb
gRNA
28S ribosomal RNA
18S ribosomal RNA
sfRNA

Fig.4

(B)


Activity of IFN-β promoter in infected SYM-1 cells
Relative luciferase activity
20
18
16
14
12
10
8
6
4
2
0
*
*
*
mock
Oshima
dSL3/4/5
Sofjin

(C)


Activity of ISRE promoter in infected NA cells
Relative luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0
**
*
NC
PC
Oshima
dSL3/4/5
Sofjin
IFN
virus
—
+
+
+
+
—
—
+
+
+