In Vivo Dynamics of Reporter *Flaviviridae* Viruses

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ABSTRACT Recombinant viruses possessing reporter proteins have been generated for virus research. In the case of the family *Flaviviridae*, we recently generated recombinant viruses, including the hepatitis C virus of the genus *Hepacivirus*, Japanese encephalitis virus (JEV) of the genus *Flavivirus*, and bovine viral diarrhea virus of the genus *Pestivirus*; all three viruses possess an 11-amino-acid subunit derived from NanoLuc luciferase (HiBiT). Here, we further developed the recombinant viruses and investigated their utility *in vivo*. Recombinant viruses harboring HiBiT in the E, NS1, or NS3 protein constructed based on the predicted secondary structure, solvent-accessible surface area, and root mean square fluctuation of the proteins exhibited comparable replication to that of the wild-type virus *in vitro*. The recombinant JEV carrying HiBiT in the NS1 protein exhibited propagation in mice comparable to that of the parental virus, and propagation of the recombinant was monitored by the luciferase activity. In addition, the recombinants of classical swine fever virus (CSFV) possessing HiBiT in the E^{ns} or E2 protein also showed propagation comparable to that of the wild-type virus. The recombinant CSFV carrying HiBiT in E^{ns} exhibited similar replication to the parental CSFV in pigs, and detection of viral propagation of this recombinant by luciferase activity was higher than that by quantitative PCR (qPCR). Taken together, these results demonstrated that the reporter *Flaviviridae* viruses generated herein are powerful tools for elucidating the viral life cycle and pathogenesis and provide a robust platform for the development of novel antivirals.

IMPORTANCE *In vivo* applications of reporter viruses are necessary to understand viral pathogenesis and provide a robust platform for antiviral development. In developing such applications, determination of an ideal locus to accommodate foreign genes is important, because insertion of foreign genes into irrelevant loci can disrupt the protein functions required for viral replication. Here, we investigated the criteria to determine ideal insertion sites of foreign genes from the protein structure of viral proteins. The recombinant viruses generated by our criteria exhibited propagation comparable to that of parental viruses *in vivo*. Our proteomic approach based on the flexibility profile of viral proteins may provide a useful tool for constructing reporter viruses, including *Flaviviridae* viruses.

KEYWORDS *Flaviviridae*, bioinformatics, *in vivo* dynamics, reporter virus

The first description of a reporter assay was a chloramphenicol acetyltransferase assay reported in 1980 (1). Since that time, reporter assays have been widely developed using an array of naturally occurring fluorescent proteins and genetically engineered derivatives for the monitoring and visualization of various intracellular events, including gene expression, protein localization, trafficking, interaction, and signaling pathways (2). In virus research, both fluorescent and bioluminescent proteins

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have been used to examine viral life cycles, tropism, and transmission. In particular, the bioimaging of viral infection has been achieved by using recombinant viruses possessing a reporter protein, thereby allowing imaging to identify the specific sites of viral replication; this approach has already been applied for several viruses (3). However, there are still limited applications of the recombinant viruses—especially small RNA viruses such as *Flaviviridae* viruses—because the accommodation of foreign genes into the viral genome is often difficult to achieve.

The family *Flaviviridae* viruses possess single-stranded positive-sense RNA and consist of four genera: *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus* (4, 5). Flaviviruses cause widespread morbidity and mortality throughout the world (6). For example, the Zika virus (ZIKV), which causes severe neurological complications in adults and congenital Zika syndrome in infants during pregnancy, was initially an endemic virus in Africa and Asia but is now considered a global public health concern (7). This is because flaviviruses are found in arthropods, and some of them, including dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV), and ZIKV are occasionally transmitted to humans by mosquito bites (8). These flaviviruses are believed to replicate in the resident dendritic cells or epidermal keratinocytes of the skin (9). Subsequently, viruses disseminate to the local lymph nodes either in association with infected migratory dendritic cells or as free viruses that are transduced directly into the lymphatic fluid, leading to viremia (6). After that, viruses spread to the target cells and display tissue-specific virulence; however, the molecular mechanisms associated with the genetic determinants and spreading toward the designated tissues are still poorly understood. In regard to the other members of the family *Flaviviridae*, the pestiviruses comprise bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus of sheep (10). They infect even-toed ungulate animals, leading to losses in the livestock industry; CSFV is a particularly contagious agent in swine (11). The establishment of prophylactic and therapeutic measures is crucial, because the “stamping-out” policy and antigen detection are gold standards for controlling CSFV infection (12). In our recent study, NanoLuc binary technology (NanoBiT) (13) was employed for the construction of recombinant reporter *Flaviviridae* viruses (14). Using NanoBiT, we have successfully generated recombinant *Flaviviridae* viruses, including DENV, JEV, hepatitis C virus (HCV), and BVDV, through the insertion of a smaller HiBiT NanoLuc subunit (15) and used them for drug screening. Although the recombinant HCV was able to propagate *in vivo*, the usefulness of other recombinant viruses for monitoring spatiotemporal dynamics *in vivo* remains to be investigated.

In this study, we further investigated the criteria for the insertion of foreign genes into viral proteins based on protein structure and succeeded in the construction of recombinant flaviviruses carrying HiBiT that were fully applicable for the study of *in vivo* dynamics. The luciferase assay exhibited sensitivity and specificity comparable to those of the conventional quantitative PCR (qPCR) assay in pigs and mice upon infection with the recombinant CSFV and JEV, suggesting that the reporter viruses generated in this study could be a powerful tool for the investigation of viral pathogenesis and development of novel antivirals.

RESULTS

Impairment of viral propagation in mice by the insertion of a reporter gene in the N terminus of the JEV NS1 gene. In our previous work, we constructed a recombinant JEV that incorporated a gene cassette encoding HiBiT luciferase and a linker sequence in frame in the N terminus of the NS1 gene (JEV NS1_N-HiBiT) and showed that it was capable of replicating *in vitro* (14). In the present study, to determine the pathogenicity of the recombinant JEV *in vivo*, we intraperitoneally inoculated mice with the wild-type or recombinant JEV ($n = 9$ mice per group), and the survival rate was monitored for 3 weeks. The mice inoculated with wild-type virus showed the typical clinical manifestations, and 6 of them were dead within 2 weeks. In contrast, mice infected with the recombinant JEV showed fewer clinical signs, and only

FIG 1 Survival rate of the mice infected with recombinant JEV carrying the HiBiT tag in the N terminus of NS1. Percentages of surviving mice ($n = 9$) after intraperitoneal inoculation with 5×10^4 FFU of either the wild-type or recombinant viruses are shown. Significant difference was determined using the log rank test. *, $P < 0.05$.

one mouse died at 10 days postinoculation (Fig. 1), suggesting that the *in vitro* replication level of the recombinant JEV was similar to that of the wild-type JEV, but the former was slightly attenuated by insertion of the HiBiT gene.

Determination of suitable loci for insertion of the HiBiT gene into a JEV genome to achieve robust propagation *in vitro*. To further investigate an ideal locus for the insertion of the HiBiT into JEV, we determined the candidate loci based on the protein structures already published (16–18). In addition to NS1, we selected the E protein and nonstructural protein NS3 for the screening (Fig. 2A). NS3 possesses multiple functions in viral replication, including protease and helicase functions (19). To avoid dysfunction of the enzymatic activities, we chose the N-terminal domain of NS3 (1 to 21 amino acids [aa]) for insertion. Based on their secondary structures, the coil and turn sites, but not the β -sheet and α -helix sites, were considered suitable for the insertion. We calculated the solvent-accessible surface area of each amino acid residue of the proteins and selected the residues exposed to the surface area (Fig. 2B). The amino acid sequences of 25 species of flaviviruses (Table 1) were aligned and compared using MAFFT software (20), and the amino acid conservation profiles were calculated as entropy profiles (Fig. 2C). We chose the loci with less conservation as suitable sites. By these criteria based on the secondary structure of the proteins, we selected 27, 30, and 8 sites in the E, NS1, and NS3 proteins, respectively. Then, we evaluated the molecular dynamics of amino acid residues of viral proteins as the root mean square fluctuation (RMSF) values, and the candidate loci were further selected based on the resulting RMSF values (Fig. 2D) and the three-dimensional (3D) structures. In total, 32 loci were finally selected as key residues of each protein (12, 13, and 7 sites in E, NS1, and NS3, respectively) according to these criteria (Fig. 2E to G).

Fragments of cDNA covering the entire region of the recombinant JEV were generated by the circular polymerase extension reaction (CPER) method (14, 21), and infectious titers in the culture supernatants and intracellular luciferase activities in Huh7 cells expressing the NanoLuc protein (LgBiT) were determined at 8 days posttransfection with the CPER products. Among the 32 candidates we examined, two viruses carrying the HiBiT in the central domain of E (residues 275 and 276), one virus carrying the HiBiT in the C-terminal domain of NS1 (residue 349), and two viruses carrying the HiBiT in the N-terminal domain of NS3 (residues 19 and 20) were recovered with significant luciferase expression (Fig. 2H). All 5 insertion sites were located on the surfaces of the respective proteins.

Huh7 cells expressing LgBiT were inoculated with the parental and recombinant JEVs at a multiplicity of infection (MOI) of 0.1, and then the infectious titer in the culture supernatants, intracellular luciferase activities, and viral RNA were determined (Fig. 3A). The recombinant virus carrying HiBiT in position 349 in NS1 (JEV NS1₃₄₉-HiBiT) exhibited propagation similar to that of wild-type JEV. The remaining recombinants exhibited a slight reduction in viral replication. The luciferase activity in cells infected with the recombinants, but not with wild-type JEV, was increased in accord with the intracellular RNA.

TABLE 1 Twenty-five flaviviruses used for the amino acid alignment

Virus no.	Species	Virus name	Strain	Virus abbreviation	GenBank accession no.
1	<i>Bagaza virus</i>	Bagaza virus	ARD138018_DIAWARA_SEN_2000	BAGV	MF380426
2	<i>Dengue virus</i>	Dengue virus-1	Mochizuki	DENV	AB074760
3	<i>Dengue virus</i>	Dengue virus-2	New Guinea-C	DENV	M29095
4	<i>Dengue virus</i>	Dengue virus-3	H87	DENV	KU050695
5	<i>Dengue virus</i>	Dengue virus-4	H241	DENV	AY947539
6	<i>Ilheus virus</i>	Ilhéus virus	Original	ILHV	AY632539
7	<i>Japanese encephalitis virus</i>	Japanese encephalitis virus	AT31	JEV	AB196923
8	<i>Kedougou virus</i>	Kédougou virus	DakAr D1470	KEDV	AY632540
9	<i>Kokobera virus</i>	Kokobera virus	AusMRM 32	KOKV	AY632541
10	<i>Kyasanur Forest disease virus</i>	Alkhurma hemorrhagic fever virus	1176	KFDV	AF331718
11	<i>Langat virus</i>	Langat virus	TP21	LGTV	EU790644
12	<i>Louping ill virus</i>	Louping ill virus	369T2	LIV	Y07863
13	<i>Murray Valley encephalitis virus</i>	Murray Valley encephalitis virus	MRM3929	MVEV	AY898809
14	<i>Ntaya virus</i>	Ntaya virus	IPDIA	NTAV	JX236040
15	<i>Omsk hemorrhagic fever virus</i>	Omsk hemorrhagic fever virus	Bogoluvovska	OHFV	AY193805
16	<i>Powassan virus</i>	Powassan virus	Nadezdinsk-1991	POWV	EU670438
17	<i>Royal Farm virus</i>	Royal Farm virus	Karashi (LEIV 2247)	RFV	AF013381
18	<i>Saint Louis encephalitis virus</i>	St. Louis encephalitis virus	Argentine 66	SLEV	AY632544
19	<i>Tembusu virus</i>	Tembusu virus	JS804	TMUV	JF895923
20	<i>Tick-borne encephalitis virus</i>	Tick-borne encephalitis virus	Sofjin-HO	TBEV	AB062064
21	<i>Usutu virus</i>	Usutu virus	SAAR-1776	USUV	AY453412
22	<i>West Nile virus</i>	West Nile virus	NY99-6922	WNV	AB185915
23	<i>Yellow fever virus</i>	Yellow fever virus	17D	YFV	JX949181
24	<i>Wesselsbron virus</i>	Wesselsbron virus	AV259	WESSV	JN226796
25	<i>Zika virus</i>	Zika virus	MR766	ZIKV	NC_012532

To assess the utility of the HiBiT recombinant viruses for immunoblotting, HiBiT luciferase expression in Huh7 cells infected with the recombinant viruses was assessed by chemiluminescent staining by using the counterpart protein LgBiT (Fig. 3B, top). The results indicated that expression of the HiBiT-tagged viral proteins was detectable by LgBiT despite the preparation of specific antibodies against the viral proteins. It was also shown that NS1 was efficiently secreted into the extracellular space (22), and large amounts of the recombinant NS1 and small amounts of E but not NS3 protein were detected in the culture supernatants upon infection with the recombinant viruses (Fig. 3B, bottom).

Next, to examine the genetic stability of the recombinant viruses, we passaged the viruses in Huh7 cells five times and then determined the infectious titers and luciferase activities (Fig. 3C). Upon infection with the JEV NS1₃₄₉-HiBiT, the infectious titers and luciferase activities were almost stable during the passages. In contrast, the infectious titers were elevated on the second passage and remained at the same levels thereafter in cells infected with the other recombinants, but no significant change in the luciferase activity was observed during the passages (Fig. 3C). Furthermore, we passaged the viruses for more five rounds (in total 10 times) and analyzed the whole-genome sequence. Although we observed the amino acid changes after the 10 serial passages of the respective viruses (Table 2), the HiBiT gene was maintained after the serial passages, suggesting that recombinant JEVs possessing the HiBiT gene in the loci

FIG 2 Legend (Continued)

secondary structure of each protein is denoted using red to indicate the structures of the coil and turn components and cyan to indicate the β -sheet and α -helix components. (B) The solvent-accessible surface area (ASA) of the whole chain (red) and side chain (green) was calculated from the 3D structures of the JEV E (Protein Data Bank accession number 5WSN), NS1 (modeling based on Protein Data Bank accession number 4O6D), and NS3 (modeling based on Protein Data Bank accession number 2WV9) and shown in line graphs. (C) The amino acid conservation was calculated as an entropy profile and shown in bar graphs. (D) The molecular dynamics of amino acid residues of viral proteins is shown as the value of the root mean square fluctuation (RMSF). The candidate positions of insertion are plotted in the respective viral protein molecules, E (E), NS1 (F), and NS3 (G). (H) Thirty-two recombinant viruses (12 viruses of E, 13 of NS1, and 7 of NS3) were generated by the CPER method. At 8 days posttransfection, the virus titers in the supernatants and luciferase activities of the transfected cells were determined by TCID₅₀ and a luciferase assay, respectively.

FIG 3 *In vitro* characteristics of the six HiBiT recombinants of JEV. (A) Huh7 cells were infected with the parental JEV and the five recombinant JEVs. The intracellular JEV RNA and luciferase activity and infectious titers in culture supernatants were determined at the indicated time points by reverse

(Continued on next page)

TABLE 2 Nucleotide and amino acid substitutions after the 10 serial cell passages of the HiBiT recombinant viruses

Recombinant virus	Substitution	
	Nucleotide ^a	Amino acid ^b
JEV E ₂₇₅ -HiBiT	T2311C G6530A	E I430L — ^c
JEV E ₂₇₆ -HiBiT	C7914(C/A)	NS5 L65I
JEV NS1 ₃₄₉ -HiBiT	T1943(A/T) C8351A	— —
JEV NS3 ₁₉ -HiBiT	G312(G/A)	C A73K
JEV NS3 ₂₀ -HiBiT	A1023(A/T)	E S16C
CSFV E ^{ns} -HiBiT	ND ^d	ND
CSFV E2-HiBiT	ND	ND

^aNumbers indicate the position from 5' end of the virus genome.^bNumbers indicate the position from N terminus of each virus protein.^c—, synonymous substitution.^dNot detected.

chosen based on protein structure were genetically stable after multiple passages *in vitro*.

Characterization of *in vivo* propagation of the recombinant JEV. To determine the *in vivo* propagation of JEV NS1₃₄₉-HiBiT, mice were inoculated with wild-type and recombinant JEV ($n = 9$ mice per group) and the survival rate was determined. Mice showed typical clinical signs of encephalitis upon inoculation with wild-type or recombinant JEV, and 4 mice died during the observation period (Fig. 4A), indicating that the virulence of NS1₃₄₉-HiBiT in mice was comparable to that of wild-type JEV. Next, we injected the recombinant virus into 13 mice and monitored the virus replication by luciferase activity. The luciferase signals were detected in all of the inoculated mice by 4 days postinfection and recuperated (Fig. 4B). The significant luciferase activity was detected from the mice showing severe clinical manifestations. The sera obtained from these mice exhibited high luciferase activity again 1 week postinfection, and high virus titers and luciferase activity were observed in the brain (Fig. 4C), suggesting that these mice died by encephalitis. In addition, these results showed that the *in vivo* viral dynamics could be successfully evaluated by luciferase activity.

Characterization of recombinant CSFV carrying the HiBiT gene in the viral envelope proteins *in vitro*. In pestivirus, several groups have reported the construction of recombinant BVDVs carrying tags in the N termini of the envelope proteins E^{ns} and E2 (23–25) and we also generated recombinant BVDV carrying HiBiT in the envelope E2 (14). Because we have previously established experimental infection of CSFV in pigs (26), *in vivo* evaluation was conducted by using CSFV. We generated two recombinant CSFVs carrying the HiBiT in either the E^{ns} or E2 envelope protein (CSFV E^{ns}-HiBiT and CSFV E2-HiBiT) (Fig. 5A). Infectious titers in the culture supernatants were determined in porcine SK6 cells upon infection with parental and recombinant CSFVs at an MOI of 0.1. Both recombinant viruses exhibited propagation comparable to that of the wild-type CSFV (Fig. 5B). To determine the effect of insertion of HiBiT in the structural proteins of CSFV on the physical properties of viral particles, parental and recombinant CSFVs were analyzed by buoyant density ultracentrifugation, and infec-

FIG 3 Legend (Continued)

transcription-quantitative PCR (qRT-PCR), a luciferase assay, and TCID₅₀, respectively. The experiments were conducted independently two times, and the data were analyzed statistically by an ANOVA. *, $P < 0.05$. (B) BHK-21 cells were inoculated at an MOI of 1 per cell with the five recombinant viruses as indicated. At 40 hpi, culture supernatants and cells were collected and subjected to detection of cellular β -actin and HiBiT-tagged proteins by Western blotting using the anti- β -actin antibody or the recombinant LgBiT protein. (C) The five recombinant JEVs were passaged on Huh7 cells for five rounds from the transfected cells, and 100- μ l aliquots of the culture supernatants were used to infect naive cells. At 48 hpi, the culture supernatants were collected and their virus titers were determined before subjecting them to the next passage. The luciferase activities in cells infected with the recombinant viruses were measured during the passages.

FIG 4 Investigation of the *in vivo* utility of the improved recombinant JEV. Nine mice per group were injected intraperitoneally with 1×10^6 FFU of the wild-type or the improved HiBiT recombinant JEV. (A) The percentage of surviving mice was monitored. The data were analyzed statistically by the log rank test. (B) Thirteen mice were injected intraperitoneally with 1×10^6 FFU of the improved HiBiT recombinant JEV. Viremia was evaluated by luciferase activity. ‡, mice euthanized for reasons of animal welfare on the day indicated. (C) To examine viral growth in the brain, the cerebra of the dead mice were subjected to measurements of viral RNA copy and luciferase activity. The data were analyzed statistically by Student's *t* test. *, $P < 0.05$.

tious titers and viral RNA in each fraction were determined (Fig. 5C). Peaks of infectious titer and viral RNA of wild-type and recombinant viruses were detected at densities of 1.05 to 1.06 g/ml, suggesting that the insertion of HiBiT into either E^{ns} or E2 had no effect on the physical properties of CSFV particles.

Next, we examined the luciferase expression in cells infected with the recombinant CSFV (Fig. 5D). Luciferase activities in cells infected with the E^{ns} recombinant were significantly higher than those in cells infected with the E2 recombinant, indicating that the E^{ns} protein is a more suitable HiBiT insertion site than the E2 protein for purposes of monitoring viral propagation, both because the E^{ns} protein is secreted into the culture supernatants and because E^{ns} is an envelope glycoprotein (27). To further evaluate the genetic stability of the recombinant viruses, we passaged the viruses in SK6 cells five times

A Classical swine fever virus (CSFV)

FIG 5 Construction and characterization of the recombinant CSFVs carrying the HiBIT gene in the envelope proteins. (A) A schematic representation of CSFV. Arrows indicate the insertion sites of the HiBIT luciferase gene. (B) Porcine SK6 cells were inoculated with the parental and two recombinant CSFVs at an MOI of 0.1. The virus titers were determined at the indicated time points by TCID₅₀. The experiments were conducted independently three times and the data were analyzed statistically by an ANOVA. (C) The culture supernatants of cells upon infection with the parental and recombinant CSFVs at an MOI of 1 were subjected to density gradient fractionation, and the viral RNA copies (top) and infectious titers (bottom) for each fraction were determined. (D) Intracellular luciferase activities at 72 hpi were determined by a luciferase assay. The experiments were conducted independently three times and the data were analyzed statistically by an ANOVA. *, $P < 0.05$. (E) The recombinant CSFVs were passaged on SK6 cells for five rounds, and 100- μ l aliquots of the culture supernatants were used to infect naive cells. At 72 hpi, the culture supernatants were collected and their virus titers were determined before subjecting them to the next passage.

FIG 6 Evaluation of the *in vivo* utility of the recombinant CSFV carrying HiBiT in *E^{ms}*. Two groups of 3-week-old SPF pigs ($n = 4$ pigs each) were inoculated intramuscularly with $10^{7.0}$ TCID₅₀ of the wild-type and the recombinant CSFV carrying the HiBiT in *E^{ms}*. (A) Leukocyte counts were determined on days 0 and 5 pi, and are shown as the means $\pm$ SDs. (B) The infectious titers in the tonsil homogenates were determined by TCID₅₀ and are shown as the means $\pm$ SDs. (C) Serum samples were collected at the indicated time points and subjected to measurements of viral RNA copies (from 100 μ l of the sample), luciferase activity (10 μ l), and virus titers (1 ml). All of the data were analyzed statistically by Student's *t* test. *, $P < 0.05$.

and then determined the infectious titers and sequence of the inserted gene. Infectious titers were maintained at around 10^6 50% tissue culture infective dose (TCID₅₀)/ml during the passages (Fig. 5E), and there were no mutations or deletions at the insertion site of the luciferase gene after the 10 passages (Table 2), indicating that recombinant CSFVs carrying the HiBiT gene are genetically stable *in vitro*.

Characterization of *in vivo* propagation of the recombinant CSFV. Because the *E^{ms}* protein is detected in the blood of infected animals (28), we examined the characteristics of propagation of the recombinant CSFV in pigs. Specific-pathogen-free (SPF) pigs were inoculated intramuscularly with parental and *E^{ms}*-HiBiT CSFVs ($n = 4$ per group). Because CSFV primarily infects the tonsils and causes leukopenia and viremia (11), we determined the leukocyte counts and infectious titers in the tonsils and the viral RNA, infectious titer, and luciferase activity levels in the sera. All pigs showed mild leukocytopenia and no significant difference in viral replication in tonsils between the infections with parental and recombinant CSFV (Fig. 6A and B), suggesting that insertion of the HiBiT tag in *E^{ms}* has no effect on the pathogenicity of CSFV in pigs. Although viral RNA in the serum samples from infected pigs was below the detectable limit by qPCR, luciferase activities were detected and increased in correlation with virus titers (Fig. 6C), indicating that luciferase activity was a more sensitive index of viral growth than qPCR.

DISCUSSION

In this study, we determined the criteria for insertion of foreign peptides to generate recombinant flaviviruses for *in vivo* investigation. The construction of recombinant viruses was achieved using a combination of existing systems to which several improvements were applied in order to achieve robust viral replication, virulence, and stable expression of the reporter. The recombinant flaviviruses exhibited higher sensitivity for the monitoring of virus replication than the conventional methods and permitted the examination of spatiotemporal dynamics *in vivo*.

The previous version of the recombinant flaviviruses possessing HiBiT reporters (14) was proved to be useful for drug screening *in vitro*, but its replication was attenuated in mice (Fig. 1). The impairment of virus replication *in vivo* is problematic for the investigation of virulence, efficacy of drugs, and spatiotemporal dynamics. The change in virus replication might have been due to the size of the foreign gene incorporated into the viral genome and/or the site for insertion dampening the function of viral proteins. To solve these problems, we investigated the criteria to determine the reporter gene insertion site in viral proteins and evaluated the propagation and reporter gene expression upon infection with the recombinant viruses. The secondary structures of the β -sheet and α -helix are common structural elements of numerous proteins and generally possess functional roles or serve as the protein backbone (29). In contrast, the "turn" is generally observed at the junction of the other elements of the secondary structure. The other structural element, i.e., the coil, is not characterized by any regular hydrogen bonding pattern and is found in the N and C termini of the proteins and/or between regular secondary structure elements. Therefore, we selected the latter two types of structures for insertion of foreign peptides (Fig. 2A). Because the amino acid residues near the luciferase element must be exposed on the surfaces of proteins in order to access the counterpart protein and to avoid disruption due to protein folding, we calculated the solvent exposure as the accessible surface area of each residue (Fig. 2B). In addition, we chose the less-conserved site of the amino acid sequence of viral proteins (Fig. 2C). Based on the criteria related to the secondary structure, we were able to reduce the number of the candidate loci, but we also needed to account for the 3D structure of each protein, since the conformational dynamics of the protein is generally a critical element for the protein function (30). Thus, the molecular dynamics of viral proteins was simulated (Fig. 2D). Finally, we obtained five recombinant viruses that exhibited levels of replication comparable to that of the wild-type virus, and all five of the sites we identified for the insertion of foreign peptides were located on the protein surface (Fig. 2E to G). Therefore, by combining these results with *in silico* analyses, we were able to determine suitable sites for the insertion of foreign peptides into viral proteins.

The five recombinant JEVs carrying the reporter genes within structural or nonstructural proteins exhibited comparable replication *in vitro*, and the recombinants carrying the tag in the nonstructural protein NS1 showed pathogenicity in mice. Recently, three different groups reported using whole-genome transposon mutagenesis screening of flaviviruses to identify tolerant loci for the insertion of foreign genes (31–33). Although the suitable loci identified in our present study overlapped those identified in these previous studies, our method has the additional advantage of narrowing down the number of viruses for screening. In the case of CSFV and BVDV, we found that the N termini of E^{ns} and E2 met the criteria for insertion (34, 35). Collectively, these data suggest that these strategies were applicable for the construction of recombinants for other RNA viruses.

Rapid and accurate diagnosis is an important countermeasure to control infectious diseases induced by infection with viruses, including *Flaviviridae*. Flavivirus NS1 and pestivirus E^{ns} are essential components of virus replication (36–40); both are secreted into the blood of infected patients/animals during the acute phase of infection (28, 41), and both have been used as antigenic markers for the detection of viral infection (42, 43). In this study, we showed that recombinant viruses carrying HiBiT-tagged NS1 or

E^{ns} were effective for examining the pathogenicity of the viruses in animal experiments. We used the live attenuated CSF vaccine virus GPE⁻, which has been proved to be safe and to confer robust immunity (44) after induction of mild leukopenia and slight replication in pigs (45, 46). All pigs infected with the recombinant CSFV carrying the HiBiT luciferase gene in E^{ns} showed slight viremia, and the detection rate of viral RNA in sera was higher by luciferase assay than by conventional qPCR. In addition, we were able to measure the luciferase activities in mice upon infection with the recombinant JEV carrying HiBiT in NS1 (Fig. 4B), suggesting that the NanoBiT luciferase assay is useful to detect viral replication and would be applicable for diagnosis in a clinical setting.

In conclusion, recombinant flaviviruses possessing the small luciferase in combination with protein-based criteria to determine the suitable site for accommodation of the reporter are powerful and versatile tools to elucidate tissue tropism and virulence of *Flaviviridae* viruses both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Ethical statement. The animal experiments performed using mice were approved by the Committee for Animal Experiments of Osaka University, and the experiments performed using pigs were approved by the Committee for Animal Experiments of Hokkaido University. All experiments complied with the national and international animal experimentation guidelines.

Plasmids. The lentiviral vectors pCSII-EF-LgBiT and pCSII-EF-GFP, into which the gene cassettes of LgBiT luciferase and AcGFP, respectively, were inserted, were generated previously (14). CSFV was derived from the full-length cDNA of the CSFV strain GPE⁻ passaged in swine kidney cells from the vaccine seed (GenBank accession number [D49533](#)). The cDNA clones encoding the viral sequence for transfection were flanked by a modified T7 promoter sequence at the 5' end and a SrfI restriction site at the 3' end (46). The recombinant cDNA clone encoding the HiBiT luciferase gene was constructed by using a KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan) and the respective oligonucleotide primers. The plasmids used in this study were confirmed by sequencing with an ABI 3130 genetic analyzer (Thermo Fisher Scientific, Waltham, MA).

Cell lines. All of the mammalian cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂. The human hepatocellular carcinoma-derived Huh7 cells, human embryonic kidney-derived 293T cells, and baby hamster kidney fibroblast-derived BHK-21 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank (JCRB0403, JCRB9068, and JCRB9020, respectively). Huh7 and 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM; Nakalai Tesque, Kytoto, Japan) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). BHK-21 cells were maintained in minimal essential medium (MEM; MilliporeSigma, Burlington, MA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% FBS. The porcine kidney-derived SK6 cells (47) were propagated in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 5% BVDV antibody-free FBS (Japan Bio Serum), and 5% horse serum (Thermo Fisher Scientific). The *Aedes albopictus* mosquito-derived cell line C6/36 was obtained from the JCRB cell bank (IFO50010) and grown in Leibovitz's L-15 medium (Thermo Fisher Scientific) with 10% tryptose phosphate broth and FBS at 28°C.

Antibodies. Mouse monoclonal antibodies to β-actin, double-stranded RNA (dsRNA), and pestivirus NS3 were purchased from Sigma-Aldrich, English & Scientific Consulting Kft, and TropBio, respectively. Alexa Fluor (AF) 488-conjugated anti-rabbit and mouse IgG antibodies were purchased from Thermo Fisher Scientific. Rat anti-hemagglutinin (HA) antibody was purchased from Roche Diagnostics.

Immunoblotting. SDS-PAGE and Western blotting were performed as described previously (14). The concentration of the SDS polyacrylamide gels was 5% to 20%. The proteins were transferred to polyvinylidene difluoride membranes (MilliporeSigma) and incubated with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Thermo Fisher Scientific). The NanoLuc complex (HiBiT and LgBiT) was stained with a Nano-Glo HiBiT blotting system (Promega, Madison, WI). All signals were detected by use of an Amersham Imager 600 image analyzer system (GE Healthcare, Chicago, IL).

Preparation of viruses. All of the cDNA-derived CSFVs and the recombinants thereof were recovered as described previously (48). In brief, plasmid constructs were linearized at the SrfI site located at the end of the viral genomic cDNA sequence, and RNA was obtained by runoff transcription using a MEGascript T7 kit (Life Technologies). The *in vitro* transcribed RNA (5 µg) was electroporated into SK6 cells using a Gene Pulser Xcell electroporation system (Bio-Rad) and then plated onto fresh medium. The supernatants were collected from the electroporated cells, and the infectious titers were determined by the 50% tissue culture infective dose (TCID₅₀) per milliliter. The HiBiT recombinant JEVs were generated by circular polymerase extension reaction (CPER) as described previously (14, 21). The seven fragments of cDNA covering the entire genome of JEV strains AT31 (GenBank accession number [AB196926](#)) and their untranscribed region (UTR) linkers were cloned into pCR-Blunt II-TOPO vectors (Thermo Fisher Scientific). The plasmids were completely sequenced as described above. Next, eight PCR fragments were generated and subjected to CPER. The CPER products were transfected into Huh7 cells with Trans IT LT-1 transfection reagent (Mirus Bio, Madison, WI). The culture supernatants were harvested on day 8 posttransfection. The infectious titers were determined and are expressed as focus-forming units (FFU).

per ml. The viruses were propagated in C6/36 cells in order to obtain sufficient virus yields for the animal experiments.

Luciferase assay. Luciferase activity was measured by mixing 100 μ l of substrate from a Nano-Glo HiBIT Lytic assay system (Promega) with 10 μ l of samples from culture supernatants or serum. To measure the luciferase activity of the tissues or cells, 100 μ l of substrate was directly added. The signals of luciferase were measured using AB-2270 Luminescencer Octa (ATTO, Japan) according to the protocols provided by the manufacturer.

Virus replication kinetics. *In vitro* growth kinetics of the parental and recombinant viruses was evaluated in the susceptible cell lines. Huh7 cells were inoculated with JEV at an MOI of 0.1. The supernatants of Huh7 cells were collected at 12, 24, 48, and 72 h postinoculation (hpi). The replication kinetics of CSFV was determined in SK6 cells by inoculation at an MOI of 0.1, with the collection of cell culture supernatants at 12, 24, 48, and 72 hpi. The virus titers were determined in duplicates using the respective cell lines.

Quantitative RT-PCR. For the quantification of viral RNA copies, total RNA was extracted from cells by using a PureLink RNA Mini kit (Thermo Fisher Scientific), and then first-strand cDNA synthesis and a quantitative RT-PCR were performed by using a TaqMan RNA-to-C_T 1-Step kit and a ViiA7 real-time PCR system (Thermo Fisher Scientific), respectively, according to the manufacturers' protocols, and the previously reported primer sets (49, 50). Fluorescent signals were determined by the ViiA7 system.

Buoyant density fractionation. The density-dependent fraction assay was conducted as described previously (14). The culture supernatants of cells infected with the parental and recombinant CSFVs were concentrated with the use of Spin-X UF centrifugal concentrators (Corning, Corning, NY). A linear gradient of 10 to 40% OptiPrep (Axis-Shield, Dundee, UK) was used for the separation, and the supernatants were spun at 32,000 rpm for 16 h at 4°C by using an SW41 rotor (Beckman Coulter, Brea, CA). Aliquots of 12 consecutive fractions were collected from the top to bottom, and the density, infectious titer, and viral RNA were determined for each fraction.

Animal experiments. For JEV infection, female 3-week-old mice of the ICR or C57BL/6NCrSlc strains were purchased from CLEA Japan and kept in specific-pathogen-free environments under a 12-h light/dark cycle (lights on at 8:00 am) at room temperature (23 $\pm$ 2°C). Groups of mice (n = 9) were intraperitoneally inoculated with 5×10^4 FFU (125 μ l) of the viruses for ICR mice and 1×10^6 FFU (100 μ l) for C57BL/6NCrSlc mice. The mice were observed for 3 weeks after inoculation to determine survival rates. Thirteen C57BL/6NCrSlc mice were inoculated with the improved HiBIT virus, and blood was collected in tubes at designated time points. The serum samples were subjected to measurements of luciferase activity.

To assess the pathogenicity of the cDNA-derived CSFVs, groups of four 3-week-old crossbred Landrace $\times$ Duroc $\times$ Yorkshire SPF pigs (Yamanaka Chikusan, Hokkaido, Japan) were injected intramuscularly with 10^7 TCID₅₀ of virus from the cell culture supernatant (1 ml) and kept for 5 days according to the previous study (26). All pigs were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) in a biosafety level 3 (BSL3) facility of the Faculty of Veterinary Medicine, Hokkaido University. Blood was collected in tubes containing or without EDTA (TERUMO, Tokyo, Japan) on 0, 3, and 5 days postinoculation (dpi). Total leukocytes in the EDTA-treated blood were counted using a pocH-100iV Diff apparatus (Sysmex, Hyogo, Japan). The serum samples obtained from the whole blood were subjected to measurement of virus titers, qPCR, and measurement of luciferase activity. All pigs were euthanized at 5 dpi, and tissues from tonsils were collected aseptically. The collected samples were homogenized in DMEM to obtain a 10% suspension for virus isolation and titrated according to the previous method (26).

Sequence variability analysis. Twenty-five amino acid sequences of flaviviruses were downloaded from NCBI GenBank, from which the NS1, NS3, and E protein sequences were extracted. Then the sequences were aligned using MAFFT version 7.311 (51). The virus names and accession numbers used in this study are listed in Table 1. Shannon entropy was used as a measure of the amino acid variability (52). The Shannon entropy at the n -th position was calculated from the following formula:

$$H_n = - \sum_{i=1}^{20} p_i \log_2 p_i$$

where p_i is the frequency of the amino acid residue.

Protein structure analysis. The 3D structures of the JEV NS1 and NS3 were constructed by homology modeling based on the crystal structures of WNV NS1 (PDB: 4O6D) and Murray Valley encephalitis virus NS3 (PDB: 2WV9), respectively. Of the 100 models for each protein generated with MODELLER 9v6 (53), the model with the lowest score for probability density function was selected. The model was evaluated using Profiles-3D (54). The coordinates of the JEV E structure were taken from the electron microscopy structure (PDB: 5WSN). The secondary structure and solvent accessibility in each structure were calculated using Molecular Operating Environment (MOE) software (version 2018; Chemical Computing Group, Canada).

Molecular dynamics simulations. Molecular dynamics (MD) simulations were performed for the NS1 monomer, NS3 monomer, and E dimer of JEV. Protonation states of the ionizable residues were assigned at pH 7.0 using the PDB2PQR web server (55). All missing hydrogen atoms were added using the LEaP module in AMBER 16 (Conflex USA, San Diego, CA) (56). The ff14SB force field was used for the proteins (57). The total charges of the proteins were neutralized by the addition of chloride counterions. Then, the systems were solvated in a truncated octahedral box of TIP3P water molecules with a distance of at least 10 Å around the protein. All energy minimization and MD simulations were performed using the pmemd.cuda program in the AMBER 16, with a cutoff radius of 10 Å for the nonbonded interactions. First, the locations of hydrogen atoms, water molecules, and counterions were optimized to remove bad contacts. Then, each system was minimized by applying harmonic restraints with a force constant of 10

kcal/mol-Å² to backbone atoms of a protein and finally allowing all atoms to move freely. Each energy minimization was performed using the steepest descent method for 2,000 steps followed by the conjugate gradient method for 2,000 steps. After minimization of energy, the system was gradually heated from 0 K to 310 K over 300 ps with harmonic restraints (with a force constant of 1.0 kcal/mol-Å²). An additional 2 rounds of MD (50 ps each at 310 K) were performed under restraint weights of 0.5 and 0.1 kcal/mol-Å², respectively. Then, an unrestrained production run of 100 ns was performed, and the production trajectories were collected every 10 ps. All MD simulations were performed using the NPT ensemble and the Langevin dynamics to control the temperature (58). The time step was 2 fs, and the SHAKE algorithm was used to constrain all bond lengths involving hydrogen atoms (59). Long-range electrostatic interactions were treated using the particle mesh Ewald method (60). Analysis of the trajectories was performed using the CPPTRAJ module of AmberTools17 (56).

Statistical analysis. Results are expressed as the means ± standard deviations (SDs) or standard errors. The significances of differences were determined by log rank test, Student's *t* test, or analysis of variance (ANOVA) model.

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We declare no competing financial interests.

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