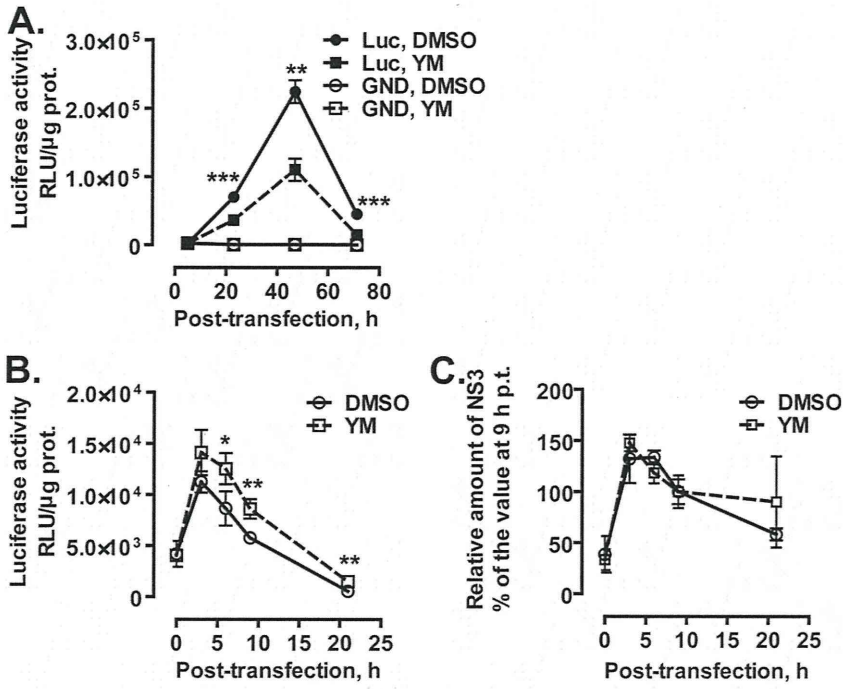

FIG 9 YM-53601 inhibits HCV RNA replication of HCV JFH-1. (A) Transient-replication assay using JFH-1 subgenomic replicons. Huh-7.5.1-8 cells were transfected with SGR-JFH1/Luc (closed symbols) or SGR-JFH1/Luc-GND (open symbols) RNAs by electroporation and then placed in serum-free medium. At 5 h posttransfection, YM-53601 (final concentration, 1.5 μM) (squares and dashed line) or DMSO (circles and solid line) was added to the medium. The cells were harvested at the indicated time points (posttransfection) and assayed for luciferase activity. (B and C) Huh-7.5.1-8 cells were pretreated with 1.5 μM YM-53601 or DMSO in serum-free medium for 42 h and then transfected with SGR-JFH1/Luc-GND RNA by lipofection. After transfection, the cells were further treated in the same medium and harvested at the indicated time points. (B) The cells were lysed and assayed for luciferase activity. (C) An equal amount of protein (10 μg/lane) in each cell lysate was subjected to immunoblotting for NS3 and GAPDH proteins, and each protein band was quantified. The relative amount of NS3 protein was calculated by dividing its intensity by that of GAPDH protein in the same lane. Data are expressed as a percentage of the relative amount at 9 h posttransfection. The value at 9 h posttransfection was not significantly different between the drug-treated and untreated cells (data not shown). Data in each graph are means ± SD for triplicate samples from one representative experiment. Similar results were obtained in at least two independent experiments. Statistical analysis was performed between drug-treated and control cells harboring the same replicon. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

reported (58, 60, 61). The foci were not detected in Huh-7.5.1-8 cells transfected with a backbone plasmid (Fig. 10A and B). Drug treatment resulted in no apparent alteration in NS4B foci (Fig. 10D) or the expression level of NS4B protein (data not shown), suggesting that the drug does not grossly alter the formation of the membranous web by NS4B protein.

RNA replication of HCV genotype 1b is not inhibited by YM-53601. To examine whether YM-53601 is able to inhibit viral RNA replication of HCV strains other than the JFH-1 strain (genotype 2a), we performed a transient-replication assay using a subgenomic replicon of the Con-1 strain (genotype 1b), FK-I_{389}Luci/NS3-3′/NK5.1 (46), and its replication-incompetent mutant, FK-I_{389}/Luci/NS3-3′/NK5.1/ΔGDD. Consistent with the previous report (46), time-dependent luciferase expression in the Con-1 replicon-transfected cells exhibited a downward-sloping pattern: luciferase activity at early time points (2.5 to 7 h posttransfection) was higher than the activity at later time points (Fig. 11A). At the early time points, the activity in the wild-type replicon-transfected cells was lower than the activity in the mutant replicon-transfected cells, indicating that RNA replication is too low to be detected at these early points. Afterwards, the activity in the wild-type replicon-transfected cells stayed higher than the activity in the mutant replicon-transfected cells, indicating that the difference between these activities was attributed to viral RNA replication. Unlike in the case of the JFH-1 replicon, treatment with YM-53601 did not lower RNA replication-dependent luciferase expression but rather enhanced it. From multiple experiments, the luciferase activity in the drug-treated cells at 46 to 50 h posttransfection was 284% ± 62% (mean ± SEM; $n = 4$) of the control activity.

To increase the impact of YM-53601, we performed a similar transient-replication assay using Huh-7.5.1-8 cells pretreated with the drug in serum-free medium for 2 days. Unexpectedly, serum-free preculture before transfection led to an overall decrease of two orders of magnitude in luciferase expression (Fig. 11B). In untreated control cells, RNA replication-dependent luciferase expression (i.e., the difference between the activity yielded by the wild-type replicon and that yielded by the mutant replicon) was not clearly found until 47 h posttransfection (compare black bars with hatched bars). However, RNA replication-dependent luciferase expression in drug-treated cells was found at and after 7 h posttransfection (compare gray bars with white bars) and was slightly higher than that in the untreated cells. Thus, the RNA replication-dependent luciferase expression does not appear to be inhibited by even a prolonged drug treatment.

Taken together, these results suggest that RNA replication of the Con-1 strain is not inhibited by YM-53601.

Entry of HCVpp of genotype 2a but not genotype 1b is blocked by YM-53601. To further investigate how YM-53601 blocks HCV production, we conducted an entry assay for HCV pseudoparticles (HCVpp), which enter cells by using HCV enve-

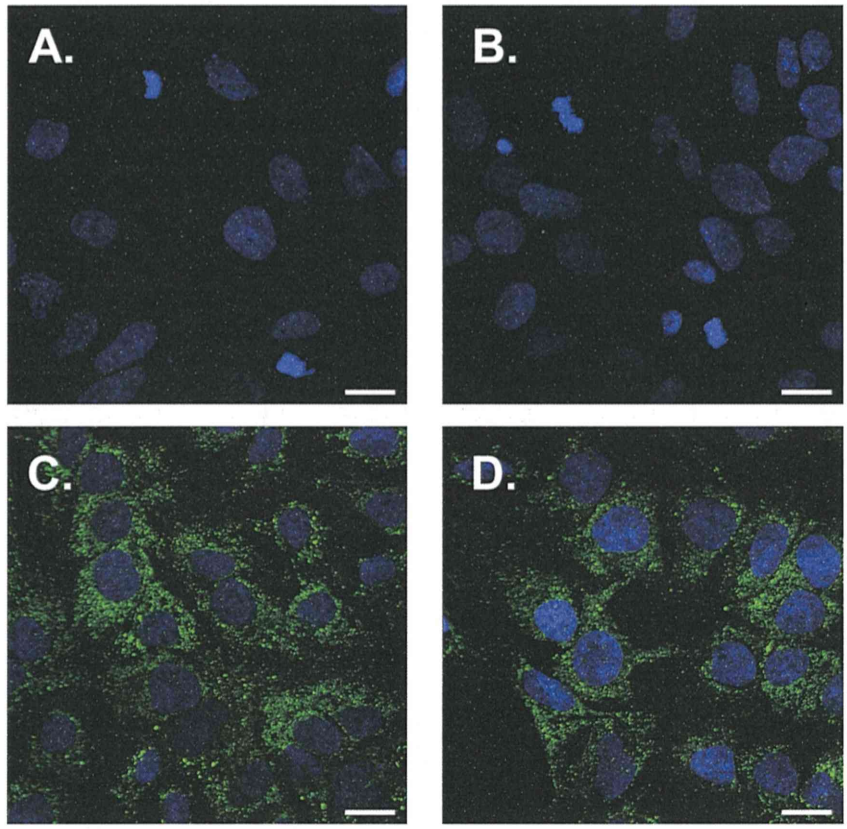

FIG 10 YM-53601 does not affect NS4B foci in Huh-7.5.1-8 cells. Huh-7.5.1-8 cells that were stably transfected with modified pCXN2 (A and B) or pCXN2/HA-TEV-NS4B (C and D) were grown on coverslips and treated with 1.5 μM YM-53601 (B and D) or DMSO (A and C) in serum-free medium for 3 days. The cells were fixed and subjected to immunofluorescence analysis using confocal microscopy. HA-tagged NS4B protein was detected with a rat anti-HA antibody followed by an Alexa Fluor 488-conjugated anti-rat antibody (green), and the nucleus was stained with DAPI (blue). Scale bars represent 20 μm.

lope protein but replicate via a retroviral system (52). Although YM-53601 was added to cells after infection (Fig. 3 and 7), a block at the step of entry of progeny virus is possible because more than one round of infection can occur under our experimental conditions. Huh-7.5.1-8 cells were preincubated with YM-53601 in serum-free medium for 2 days and then infected in the presence of the drug with HCVpp harboring envelope glycoproteins from the JFH-1 strain. The cells were thereafter incubated in the absence of the drug for 3 days, and luciferase activity, reflecting the degree of HCVpp entry into host cells, was measured. Treatment with YM-53601 reduced luciferase activity to less than 50% of the activity in untreated cells (Fig. 12, left two bars). Infection with mock HCVpp prepared without envelope glycoproteins did not yield luciferase activity (<3 relative light units [RLU]/μg protein), confirming that luciferase expression is dependent on the envelope glycoproteins (data not shown). When the drug was added only at HCVpp infection, no reduction in the luciferase expression was found (Fig. 12, third bar from left), suggesting that the drug targets cells but not HCVpp. These results are consistent with the previous report showing partial cholesterol dependency of HCV entry (14). Similarly, we tested the effect of the drug on HCVpp harboring envelope glycoproteins from genotype 1b HCV (strain TH). Drug treatment before and during infection or only during infection did not significantly alter luciferase expression (Fig. 12, right three bars). Taken together, these results suggest that YM-53601 blocks entry of HCV genotype 2a but not that of genotype 1b.

DISCUSSION

The main aim of this study was to elucidate the importance of the committed pathway of cholesterol biosynthesis in the HCV life cycle. We have shown that three types of SQS inhibitor, YM-53601 (Fig. 3), zaragozic acid A (Fig. 5), and siSQS (Fig. 6), inhibited HCV JFH-1 production in Huh-7.5.1-8 cells in a similar manner. In particular, YM-53601 exerted an antiviral effect without remarkable cell toxicity. The antiviral effect of SQS inhibition was reversed by the addition of LDL (Fig. 6 and 7), indicating that the effect is attributable to cellular cholesterol and/or cholesteryl ester deficiencies (Fig. 4 and 6). Unlike YM-53601, no antiviral effect was observed with the ACAT inhibitor Sandoz 58-035 (Fig. 8), suggesting that synthesis of cholesterol rather than that of cholesteryl esters is important for HCV production. From these findings, we conclude that the committed pathway of cholesterol biosynthesis that begins with squalene synthesis (Fig. 1) plays an important role in the HCV life cycle. This conclusion is consistent with recent studies showing that inhibition of oxidosqualene cyclase, lanosterol C_{14}-demethylase, 24-dehydrocholesterol reductase, 7-dehydrocholesterol reductase, and SQS (discussed below) leads to decreased HCV production (31–33, 62). Furthermore, we propose that SQS is a potential target for anti-HCV strategies because

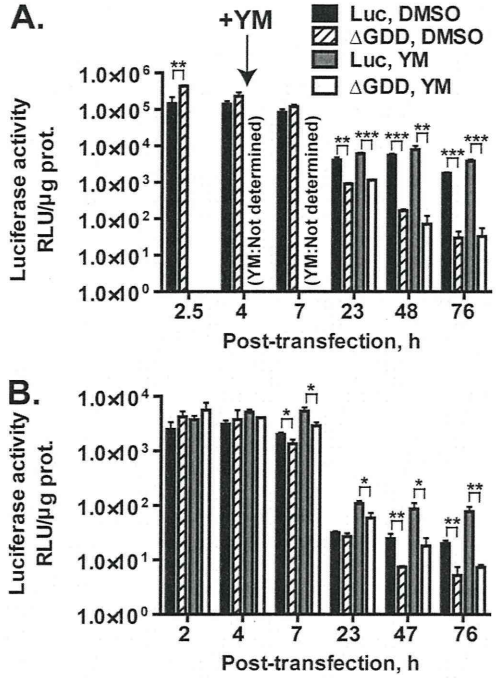

FIG 11 YM-53601 does not inhibit transient replication of Con-1 subgenomic replicons. (A) Huh-7.5.1-8 cells were transfected with FK-I_{389}Luci/NS3-3′/NK5.1 (Luc) (black and gray bars) or FK-I_{389}/Luci/NS3-3′/NK5.1/ΔGDD (ΔGDD) (hatched and white bars) RNAs by electroporation and then placed in serum-free medium. At 4 h posttransfection, YM-53601 (YM) (final concentration, 1.5 μM) (gray and white bars) or DMSO (black and hatched bars) was added to the medium. The cells were harvested at the indicated time points (posttransfection) and assayed for luciferase activity. (B) Huh-7.5.1-8 cells were pretreated with 1.5 μM YM-53601 or DMSO in serum-free medium for 47 h. The cells were transfected with FK-I_{389}Luci/NS3-3′/NK5.1 or FK-I_{389}/Luci/NS3-3′/NK5.1/ΔGDD RNAs and then further treated in the same medium. The cells were harvested at the indicated time points (posttransfection) and assayed for luciferase activity. Bars are as described for panel A. Data in each graph are means ± SD for triplicate samples from one representative experiment and are presented on a logarithmic scale because of large range of values. Some error bars are not visible due to their small sizes. Similar results were obtained in at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

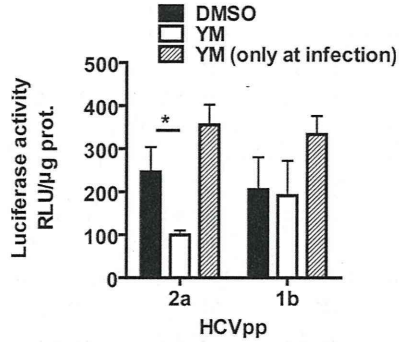

FIG 12 YM-53601 blocks entry of genotype 2a, but not genotype 1b, HCVpp. Huh-7.5.1-8 cells were grown in serum-free medium that contained 1.5 μM YM-53601 (white bars) or DMSO (black and hatched bars) for 2 days and then infected with HCVpp in the presence (white and hatched bars) or absence (black bars) of the drug. The cells were further grown in complete medium without the drug for 3 days and assayed for luciferase activity. Data are means ± SD for triplicate samples from one representative experiment. Similar results were obtained in two independent experiments. *, $P < 0.05$.

all the SQS inhibitors tested in this study exerted anti-HCV effects. It has been reported that the peak plasma concentration of YM-53601 is 0.92 μg/ml (approximately 2.5 μM) after oral administration in rats at a dose with a cholesterol-lowering effect (38, 63). This concentration is roughly close to the IC_{50} of YM-53601 for HCV production in the presence of serum. Thus, YM-53601 might exert an anti-HCV effect *in vivo*.

Using a transient-replication assay (Fig. 9A) and the HCVpp system (Fig. 12), we found that suppression of HCV RNA replication and entry is involved in the antiviral mechanism of YM-53601 against JFH-1 virus. However, the degrees of suppression of these processes were at most approximately 50% in our assays. Accordingly, these mechanisms alone may not explain the more severe inhibition of HCV production observed in the HCV cell culture system (Fig. 3). Possibly, some steps in the HCV life cycle other than RNA replication and entry might be sensitive to the drug. Alternatively, some steps which are not reproduced in the subgenomic replicon and HCVpp systems might be more sensitive to the drug.

YM-53601 inhibited transient RNA replication of the subgenomic reporter replicon from the JFH-1 strain (genotype 2a) (Fig. 9A) but somewhat enhanced that of the subgenomic replicon from the Con-1 strain (genotype 1b) (Fig. 11). Similarly, the drug inhibited entry of genotype 2a, but not genotype 1b, HCVpp (Fig. 12). These findings raise the possibility that the cholesterol requirement for HCV RNA replication and entry varies among virus genotypes. Consistent with our results, previous studies have shown that SQS inhibition by zaragozic acid A leads to an enhancement of genotype 1b RNA replication (28, 31). This effect appears to be caused by an increase in geranylgeranyl pyrophosphate, which is required for geranylgeranylation of a viral host factor, and elevated expression of HMG-CoA reductase (31). In the case of genotype 2a, the effect might be overwhelmed by antiviral effect caused by cholesterol depletion. Interestingly, genotype-specific inhibition of HCV RNA replication was also observed with inhibitors of sphingomyelin biosynthesis (19, 64, 65). Thus, major components of lipid rafts, i.e., cholesterol and sphingomyelin, appear to be similar in that they both contribute to HCV RNA replication in a genotype-dependent manner.

During preparation of this paper, Park et al. reported that siRNAs against farnesyl-diphosphate farnesyltransferase 1 (another name for SQS) and YM-53601 impair propagation of the HCV Jc1 strain (genotype 2a) in Huh-7.5 cells (62). They suggested that these agents target viral RNA replication by using a luciferase-encoding full genomic replicon of the JFH-1 strain and genotype 2a subgenomic replicon cells. These findings are consistent with our results. However, their finding that the viral RNA level in genotype 1b subgenomic replicon cells is decreased by SQS knockdown appears to argue against our results, as we could not find any antiviral effect of YM-53601 on genotype 1b RNA replication (Fig. 11). Although the reason for this discrepancy is currently unknown, differences in the culture conditions (serum-containing medium versus serum-free medium), replication assay (RT-qPCR versus reporter), methods of SQS inhibition (siRNA versus drug), and origin of the subgenomic replicon might be involved. In any case, we should evaluate the effects of SQS inhibitors on the complete life cycle of HCV genotype 1b when cell culture systems capable of supporting its growth are developed.

Our data suggest that biosynthesis of cholesterol, rather than that of cholesteryl esters, is important for HCV production (Fig. 8). Treatment with YM-53601 led to only a slight reduction in cholesterol levels (Fig. 4D) but severely impaired HCV production, implying that the drug selectively decreases relatively minor but specific pools of cellular cholesterol that are important for HCV production. Given that lipid rafts may serve as sites for viral RNA replication (15–17), assembly (19, 20), and virus entry (14, 19, 66), one scenario is that YM-53601 might selectively decrease lipid raft-associated cholesterol, thereby perturbing these processes. Consistent with this proposition, inhibition of SQS in prostate cancer cells results in a decrease of raft-associated cholesterol rather than nonraft cholesterol (67). On the other hand, a recent study has shown that purified double-membrane vesicles containing active HCV RNA replication complexes are highly enriched with cholesterol (68), although they originate from the ER, which is poor in cholesterol (69). It has also been shown that cholesterol depletion from the double-membrane vesicles decreases viral RNA levels associated with them, suggesting that cholesterol is an important structural component of HCV RNA replication complexes. Cholesterol biosynthesis (70) and HCV RNA replication (71, 72) both occur in the ER, and some cholesterol biosynthetic enzymes, including SQS, are partially copurified with components of HCV RNA replication complexes (73), implying that the cholesterol biosynthetic machinery might be closely associated with HCV RNA replication complexes in the ER. Thus, another scenario is that YM-53601 might decrease newly synthesized ER cholesterol pools, which might be preferentially used for structural components of membrane-bound viral RNA replication complexes. Preferential use of newly synthesized cholesterol in the formation of envelope membranes of human immunodeficiency virus has been found (74). Note that we could not detect any impact of YM-53601 on the morphology of NS4B-induced foci, which are considered scaffolds of viral RNA replication complexes, under fluorescence microscopy (Fig. 10). Thus, alteration in the structure of RNA replication complexes caused by YM-53601, if any, might be found at the ultrastructural level.

Our data provide evidence that the committed pathway of cholesterol biosynthesis is important for HCV production, consistent with recent studies (31–33, 62). Moreover, we found that biosynthesis of cholesterol, but not of cholesteryl esters, is important for this process. The identity of the cholesterol pools required for HCV production and the molecular mechanisms underlying the cholesterol requirement should be elucidated in future studies. Our data also provide concrete evidence that SQS is a potential anti-HCV target. Further studies are required to ascertain the anti-HCV activity of SQS inhibitors *in vivo*. SQS inhibitors are expected to exert fewer adverse effects on human cells than statins because SQS inhibitors lower cholesterol without depleting nonsterol isoprenoids (75, 76). For this reason, many compounds targeting SQS have been developed in the past by the pharmaceutical industry as potential cholesterol-lowering drugs for hypercholesterolemia. Thus, reevaluation of these compounds for potential anti-HCV activity might offer a time-saving and cost-effective approach for developing anti-HCV drugs.

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Short Communication

Production of single-round infectious chimeric flaviviruses with DNA-based Japanese encephalitis virus replicon

Ryosuke Suzuki,[1]† Tomohiro Ishikawa,[2]† Eiji Konishi,[3] Mami Matsuda,[1] Koichi Watashi,[1] Hideki Aizaki,[1] Tomohiko Takasaki[4] and Takaji Wakita[1]

Correspondence
Ryosuke Suzuki
ryosuke@nih.go.jp
Eiji Konishi
ekon@biken.osaka-u.ac.jp

[1]Department of Virology II, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

[2]Department of Microbiology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi, 321-0293, Japan

[3]BIKEN Endowed Department of Dengue Vaccine Development, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchahewi, Bangkok 10440, Thailand

[4]Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

A method for rapid production of single-round infectious particles (SRIPs) of flavivirus would be useful for viral mutagenesis studies. Here, we established a DNA-based production system for SRIPs of flavivirus. We constructed a Japanese encephalitis virus (JEV) subgenomic replicon plasmid, which lacked the C-prM-E (capsid–pre-membrane–envelope) coding region, under the control of the cytomegalovirus promoter. When the JEV replicon plasmid was transiently co-transfected with a JEV C-prM-E expression plasmid into 293T cells, SRIPs were produced, indicating successful *trans*-complementation with JEV structural proteins. Equivalent production levels were observed when C and prM–E proteins were provided separately. Furthermore, dengue types 1–4, West Nile, yellow fever or tick-borne encephalitis virus prM-E proteins could be utilized for production of chimaeric flavivirus SRIPs, although the production was less efficient for dengue and yellow fever viruses. These results indicated that our plasmid-based system is suitable for investigating the life cycles of flaviviruses, diagnostic applications and development of safer vaccine candidates.

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Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis with severe mortality in eastern and south-eastern Asia, and is estimated to be responsible for 67 900 cases annually, mostly in children (Campbell *et al.*, 2011). The virus is transmitted by *Culex* mosquito vectors between pigs and/or wild birds, and humans and horses are thought to be dead-end hosts. JEV is a member of the genus *Flavivirus* within the family *Flaviviridae*, which includes dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV). JEV is an enveloped single-stranded positive-sense RNA virus with an 11 kb genome that is translated as a single large polyprotein. The polyprotein is co-translationally cleaved by host and viral proteases into three structural proteins – capsid (C), pre-membrane (prM) and envelope (E) – and seven non-structural (NS) proteins (Sumiyoshi *et al.*, 1987).

For several flaviviruses, subgenomic replicons, which lack structural protein genes but can replicate in cells, have been constructed (Khromykh & Westaway, 1997; Pang *et al.*, 2001; Shi *et al.*, 2002). In addition, the expression of viral structural proteins in cells harbouring replicon RNA has been shown to produce single-round infectious particles (SRIPs), which are infectious, but progeny viruses cannot be spread from the infected cells, as the packaged genome lacks structural protein genes (Gehrke *et al.*, 2003; Jones *et al.*, 2005; Khromykh *et al.*, 1998; Ng *et al.*, 2007; Scholle *et al.*, 2004; Yun *et al.*, 2009). Furthermore, *trans*-packaging of replicons by the prM-E proteins from heterologous flaviviruses have been reported (Ansarah-Sobrinho *et al.*, 2008; Yoshii *et al.*, 2008).

A method for rapidly producing SRIPs of flaviviruses would be useful for viral mutagenesis studies, diagnostic applications and the production of vaccines with reduced

†These authors contributed equally to this paper.

One supplementary figure is available with the online version of this paper.

risk of infection. In this study, to establish a DNA-based production system for SRIPs, we constructed a JEV subgenomic replicon plasmid, which lacked the C-prM-E coding region, under the control of the cytomegalovirus (CMV) promoter. As DNA-based replicons can be transfected directly into eukaryotic cells without *in vitro* transcription, SRIPs can be rapidly produced by co-transfection with structural protein-expression plasmids.

In order to generate a subgenomic replicon from the JEV Nakayama strain (McAda *et al.*, 1987), viral RNA was extracted from infected Vero cells, reverse transcribed into cDNA and amplified in individual dsDNA fragments containing T7 RNA polymerase promoter and hepatitis delta virus ribozyme (HDV-RZ) as shown in Fig. 1(a). For deletion of the C-prM-E region, a synthetic antisense oligonucleotide was used to add a *Bsp*TI site at codons 17–18 of the C-coding region following the cyclization sequence, and a sense oligonucleotide was designed that added the *Bsp*TI site before the C-terminal transmembrane domain, which consists of 30 aa of the E protein coding sequence, in order to permit ligation of C to NS1. The five individual fragments required to produce a replicon-length cDNA were readily assembled into the low-copy-number plasmid pACYC177, designated pJEVrep#97. Replication of *in vitro*-transcribed RNAs derived from the plasmid was confirmed in RNA-transfected cells (data not shown). Next, to construct a DNA-based replicon plasmid, the T7 RNA polymerase promoter was replaced with the CMV promoter, and the simian virus 40 polyadenylation signal was inserted downstream of the HDV-RZ; the resulting plasmid was designated pCMV-JErep (Fig. 1a). pCMV-JErep-fs, which contains a frameshift mutation through a 4 nt insertion upstream of the GDD motif of RNA-dependent RNA polymerase in NS5, was also constructed as a negative control with no replication activity. To characterize the replication activity of the plasmid-derived replicon, 293T cells were transfected with plasmids as described previously (Suzuki *et al.*, 2013). Indirect immunofluorescence with an anti-dsRNA antibody showed positive staining in the cytoplasm of cells transfected with pCMV-JErep plasmid or infected with JEV Nakayama strain, whereas no signal was detected in the cells transfected with pCMV-JErep-fs, indicating the ability of viral RNAs transcribed intracellularly from the plasmid pCMV-JErep to replicate in cells (Fig. 1b). It should be noted that NS1 protein was detected in the cytoplasm of cells transfected with both pCMV-JErep and pCMV-JErep-fs.

We also constructed expression plasmids for JEV C-E, mature C consisting of 105 aa, and prM-E, which we designated pCAG-JECE, pCAG-JEC and pCAG-JEprME, respectively (Fig. 2a). To reduce sequence homology and intergenomic recombination potential with the truncated C and E genes in the subgenomic replicon, 21 nt mutations were incorporated into the 5′ region of the C gene and 3′ region of the E gene. These changes also include two nucleotides in the conserved 5′ cyclization sequence (CS) (Hahn *et al.*, 1987; Khromykh *et al.*, 2001), producing a sequence that was non-complementary to the 3′ CS of the replicon genome, thereby preventing replication of a recombinant genome. To produce SRIPs of JEV, 293T cells were transfected with a mixture of two (pCMV-JErep and pCAG-JECE) or three (pCMV-JErep, pCAG-JEC and pCAG-JEprME) plasmids. The infectivity of SRIPs was determined by inoculating the culture supernatant of transfected cells into Vero cells, followed by immunostaining with anti-NS1 antibody. 293T cells produced a titre of 6.9×10^5 IU ml^{-1} (Fig. 2b) or 7.9×10^5 IU ml^{-1} (Fig. 2c) 3 days after transfection with two or three plasmids, respectively. In contrast, no infectious particles were detected in the supernatant when one of the two or three plasmids was omitted or the replicon containing a frameshift mutation was introduced. The production levels of SRIPs from cells transfected with two or three plasmids were similar, as shown in Fig. 2(d).

In order to confirm that the SRIPs have only single-round infectivity potential, Vero cells were inoculated with medium harvested from 293T cells transfected with replicon and structural protein plasmids, and were examined for antigen-positive cells. SRIPs were demonstrated to be infectious in the first round (Fig. 3a). However, no antigen-positive cells were observed in a second round, in which the supernatants of the cells infected with SRIPs were transferred to naive Vero cells (Fig. 3a). As a control, supernatant from JEV-infected cells produced antigen-positive cells in second-round infection.

We then evaluated whether the SRIPs could be used in neutralization tests instead of infectious live virus by using anti-JEV sera raised in rabbits as a representative antibody. Serial fourfold dilutions of serum were mixed with aliquots of SRIPs or virus of equivalent infectivity. The virus–antibody mixture was incubated for 1 h at room temperature, followed by titration for infectivity on Vero cell monolayers in a 96-well plate. The neutralizing activity of each antibody dilution was expressed as a percentage of the infectivity obtained with the control, which was tested in the absence of any serum. Infection with SRIPs and JEV Nakayama strain were similarly neutralized by anti-JEV antibody in a dose-dependent manner, although normal serum did not affect infection with SRIPs and JEV (Fig. 3b).

Next, to examine whether SRIPs derived from other flaviviruses could be generated using our plasmid-based method, we used prM-E expression plasmids for the following viruses: DENV1, Mochizuki strain; DENV2, New Guinea C strain; DENV3, H87 strain; DENV4, H241 strain (Konishi *et al.*, 2006); WNV, NY99-6922 strain (Ishikawa *et al.*, 2007); YFV, 17D strain; and TBEV, Oshima 5-10 strain (Yoshii *et al.*, 2003). Detection of each E protein in cells transfected with prM-E expression plasmids by immunofluorescence revealed indistinguishable efficiency of transfection as shown in Fig. S1 (available in JGV Online). Efficient production of chimaeric flavivirus SRIPs by co-transfection with JEV C and JEV replicons was achieved for

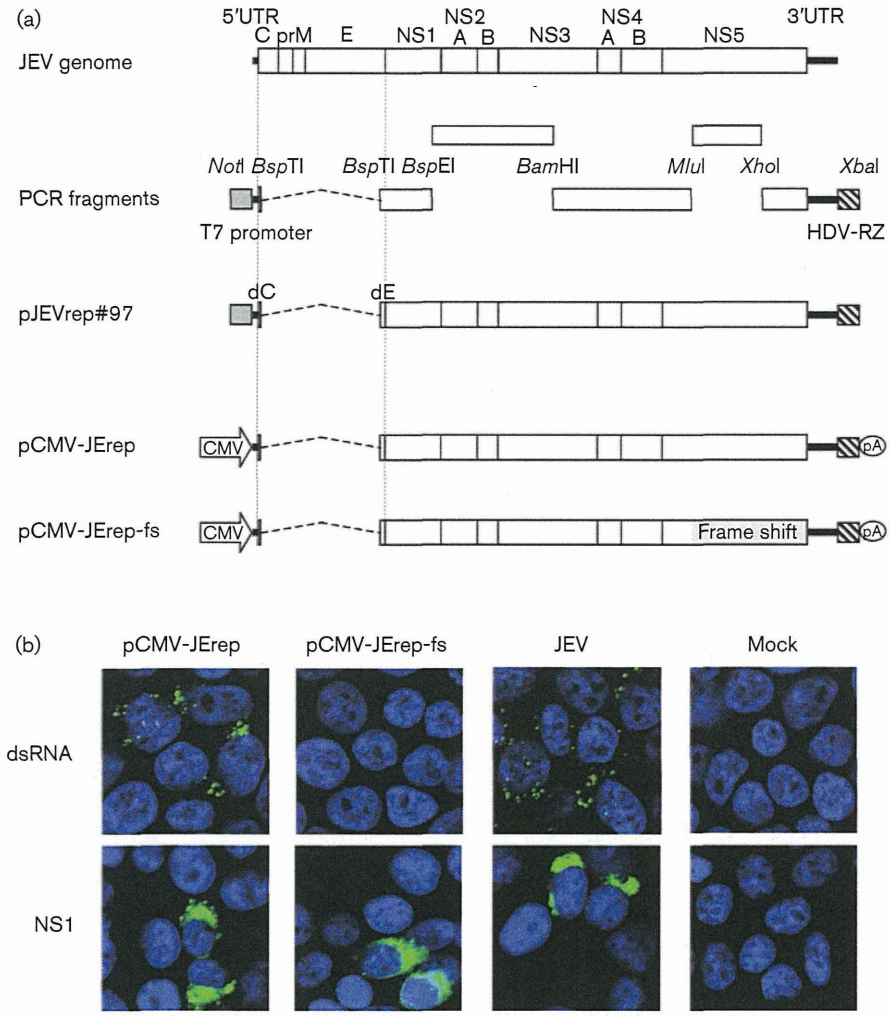

Fig. 1. (a) Schematic representation of the position of the JEV genome showing restriction enzymes sites (*Not*I, *Bsp*TI, *Bsp*EI, *Bam*HI, *Mlu*I, *Xho*I and *Xba*I), fragments used to assemble for replicon construction, position of the T7 promoter, CMV promoter (CMV), HDV-RZ and polyadenylation signal (pA). (b) 293T cells were transfected with the indicated plasmids or were infected or mock-infected with JEV. Two days post-transfection or post-infection, cells were fixed and permeabilized as described previously (Suzuki *et al.*, 2013). Samples were then incubated with anti-dsRNA antibody (J2; English & Scientific Consulting) or anti-NS1 antibody (2D5; Konishi *et al.*, 2004). Green signals were obtained with Alexa-Fluor-488-labelled goat anti-mouse IgG secondary antibody (Invitrogen). Cell nuclei were counterstained with DAPI.

WNV and TBEV, although production of SRIPs was less efficient for DENV1-4 and YFV (Fig. 3c).

It is curious that TBEV prM-E protein can be utilized efficiently for assembly of SRIPs in combination with the JEV C protein and replicon RNA producing equivalent titres to JEV and WNV, as TBEV is a tick-borne virus and is classified as a distinct serogroup from JEV. In contrast, production of DENV- and YFV-SRIPs was less efficient. The low infectious titre of SRIPs containing at least dengue prM-E may be explained by the low specific infectivity of particles encapsidated in DENV envelope protein (van der Schaar *et al.*, 2007; Winkelmann *et al.*, 2011), although we were unable to exclude the possibility that the viral assembly and/or secretion with dengue prM-E is not efficient (Chang *et al.*, 2003; Hsieh *et al.*, 2008). Adaptive mutations in structural and NS proteins could possibly enhance the production of infectious particles by improving the specific infectivity of the resulting particles (Winkelmann *et al.*, 2011). In addition, it has been reported that a chimaeric WNV genome with DENV2 prM-E genes but lacking the C gene replicates much better in DENV2-C-expressing cells than in WNV-C-expressing cells (Suzuki *et al.*, 2009), thus suggesting that the combination of homologous C protein and prM-E proteins improves the production of viral particles. Therefore, it is possible to obtain a better yield of dengue SRIPs by using DENV C protein instead of JEV C protein. Such DENV-SRIPs can be useful for studying infection-enhancing and neutralizing antibody activities.

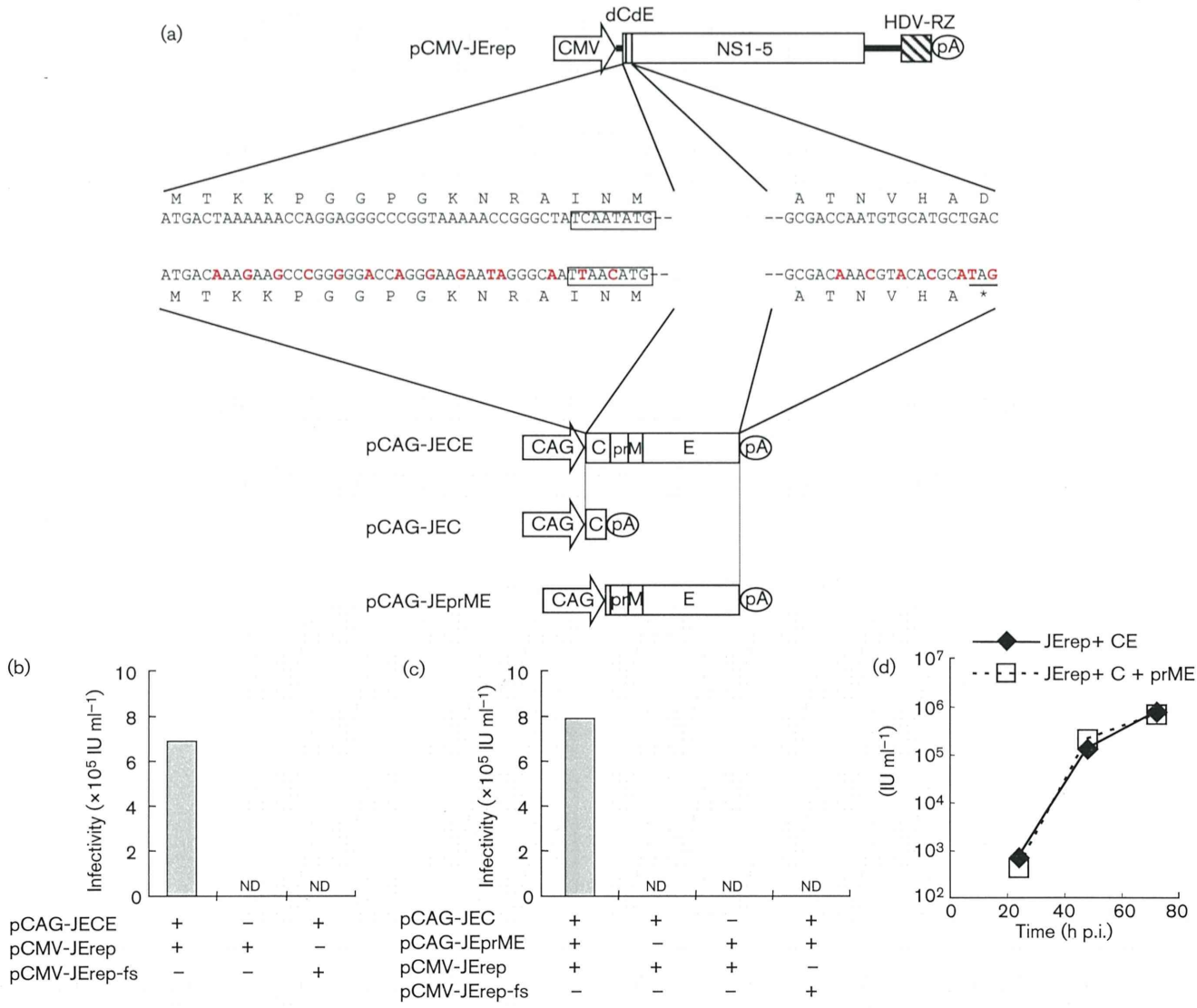

Fig. 2. Schematic representation of JEV replicon and structural protein-expression plasmids. (a) Top: JEV subgenomic replicon with deletion of structural proteins. This replicon contains a partial C and E gene. Bottom: JEV structural protein-expression plasmids showing the region of overlap with JEV replicon. Boxes indicate the 8 nt in the 5′ CS that are 100 % conserved among all mosquito-borne flaviviruses. The JEV C-E, C and prM-E coding sequences harbour silent mutations designed to prevent homologous recombinations that included two changes to the CS sequence, which must be 100 % complementary to the 3′ CS of JEV in order to permit genome replication. The termination codon is underlined. Nucleotide substitutions are shown in red. (b, c) Titres of JEV-SRIPs produced by transfection of 293T cells with replicon plasmid and structural protein-expression plasmids. Dilutions of supernatant collected at 3 days post-transfection were used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-NS1 antibody. Stained cells were then counted to determine the titres (IU ml^{-1}) produced by transfections. ND, Not detected. (d) Time course of JEV SRIP production from transfected cells. At each time point, medium was removed and frozen for subsequent titration and fresh medium was added. JEV SRIP titres were determined by assaying infectivity in Vero cells.

The plasmid-based production system offers an advantage for vaccine production in terms of stability and safety, as this method is able to reduce the chance of mutations in the structural protein region, as well as the risk of infection when compared with live virus production. In addition, our replicon plasmids have the potential for application to DNA-based vaccines, as described previously (Cao *et al.*, 2011; Chang *et al.*, 2008; Huang *et al.*, 2012).

In conclusion, we established a DNA-based production system for SRIPs of flaviviruses. This system has potential value as a basic research and diagnostic tool, and could be used to enhance the safety of neutralization assay, as well as vaccine production.