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

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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.

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...
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 BspTI site at codons 17–18 of the C-coding region following the cyclization sequence, and a sense oligonucleotide was designed that added the BspTI 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-JECE 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-JECE 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 x 10^5 IU ml^(-1) (Fig. 2b) or 7.9 x 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, NT99–6922 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...
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. 1. (a) Schematic representation of the position of the JEV genome showing restriction enzymes sites (NotI, BspTI, BspEI, BamHI, MluI, Xhol and XbaI), 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.](image-url)
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.

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**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.
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References


