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Engineering the Japanese encephalitis virus RNA genome for the expression of foreign genes of various sizes: Implications for packaging capacity and RNA replication efficiency

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Using the RNA replication machinery of Japanese encephalitis virus (JEV), the authors have established and characterized three strategies for the expression of foreign genes. Initially, ≈11 kb genomic RNA was engineered to express heterologous genes of various sizes by preferentially inserting a new cistron at the beginning of the 3′ nontranslated variable region. RNA transfection yielded recombinant viruses that initiated foreign gene expression after infecting permissive cells. JEV was capable of packaging recombinant genomes as large as ≈15 kb. However, larger genome size was inversely correlated with RNA replication efficiency and cytopathogenicity, with no significant change in infectivity. Second, a variety of self-replicating propagation-deficient viral replicons were constructed by introducing one to three in-frame deletions into the ectodomains of all the structural proteins of JEV. These replicons displayed a spectrum of RNA replication efficiency upon transfection, suggesting that remnant transmembrane domains play a suppressive role in this process. Third, the authors generated a panel of stable packaging cell lines (PCLs) providing all three JEV structural proteins *in trans*. These PCLs efficiently packaged viral replicon RNAs into single-round infectious viral replicon particles. These JEV-based virus/vector systems may provide useful tools for a variety of biological applications, including foreign gene expression, antiviral compound screening, and genetic immunization. *Journal of NeuroVirology* (2007) **13,** 522–535.

Keywords: encapsidation; foreign gene expression; Japanese encephalitis virus; packaging cell lines; *trans*-complementation; viral replicon particles

Introduction

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is closely related to dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), and Kunjin virus (KUNV). Like other flaviviruses, JEV is a small-enveloped virus with a single-stranded positive-sense RNA genome of ≈11,000 nucleotides (nt) in length, which is capped at the 5′ end and unpolyadenylated at the 3′ end (Lindenbach and Rice, 2001). The genome contains a single long open reading frame (ORF) flanked

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by *cis*-acting nontranslated regions (NTRs) for viral replication/transcription/translation at both the 5′ and 3′ ends, which are characterized by highly conserved primary sequences and secondary or tertiary structures (Markoff, 2003). The polyprotein is co- or post-translationally processed into mature proteins by cellular and viral proteases. The infectious virion is assembled by encapsidating genomic RNA into the core shell of capsid (C) proteins, which is, in turn, enveloped by two viral glycoproteins, premembrane (prM; which is further processed into the pr and M proteins) and envelope (E), embedded in the host-derived lipid membrane. The RNA genome replicates in the cytoplasm. This process is mediated by a complex of viral replicases, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Lindenbach and Rice, 2001).

Several of the flaviviruses, including JEV, display a number of characteristics that make them useful for the development of delivery vectors to express foreign genes: Several flavivirus hosts are susceptible to infection, and a wide range of cell types, including those of insect, avian, and mammalian origin, are permissive for infection and replication. Also, manipulatable genome sizes and simple procedures make possible the rapid generation of high titers of recombinant viruses. In addition, cytoplasmic RNA amplification in these viruses eliminates nuclear involvement and leads to high levels of replication and gene expression. In the past few years, heterologous genes have been expressed in the context of (i) an infectious virus genome, in the case of WNV (Pierson *et al*, 2005) and tick-borne encenphalitis virus (TBEV) (Gehrke *et al*, 2005); or (ii) a replicon lacking viral structural proteins (C, prM, and E), in the case of several flaviviruses, including DENV (Pang *et al*, 2001), YFV (Jones *et al*, 2005; Mason *et al*, 2006; Molenkamp *et al*, 2003), WNV (Fayzulin *et al*, 2006; Lo *et al*, 2003; Mason *et al*, 2006; Shi *et al*, 2002), KUNV (Khromykh and Westaway, 1997; Varnavski and Khromykh, 1999), and TBEV (Gehrke *et al*, 2005). In the case of YFV, two approaches to producing recombinant viruses using infectious cDNA technology have been utilized, depending on the antigen to be expressed. One is the generation of chimeric viruses through the exchange of structural prM/E genes (Lai and Monath, 2003). The other technique is the expression of foreign protein epitopes at the surface of recombinant viruses by engineering certain E protein–coding regions (Bonaldo *et al*, 2002; Bonaldo *et al*, 2005).

JEV is one of only two flaviviruses (the other being YFV 17D) for which an effective live attenuated vaccine (SA14-14-2 for JEV) is available for human vaccination (Hennessy *et al*, 1996; Monath, 2003; Solomon, 2003; Xin *et al*, 1988). Consequently, it is a particularly attractive candidate for the development of gene expression systems. However, in contrast to YFV, which has been extensively exploited as a vector for expressing foreign genes (Bonaldo *et al*, 2002, 2005; Bredenbeek *et al*, 2006; Jones *et al*, 2005; Lai and Monath, 2003; Molenkamp *et al*, 2003), JEV has not been used for this purpose to date. Here we report three strategies for utilizing JEV or its genetic elements as vectors to express foreign genes in a variety of cell types, taking advantage of a full-length infectious JEV cDNA (Yun *et al*, 2003) that we have previously constructed. These JEV-based expression systems can potentially serve as powerful tools in both *in vitro* and *in vivo* applications. The large packaging capacity of JEV offers a distinct advantage with regard to expressing larger genes and the treatment of associated diseases. Our findings also indicate that cross-talk may link the biogenesis of the JEV structural proteins within the ER to the level of RNA replication in the cytoplasm of infected cells.

Results

Recombinant JEV genomic RNAs of up to ≈15 kb are encapsidated into infectious virions with no significant changes in infectivity, but larger genome size is inversely correlated with RNA replication efficiency and cytopathogenicity in cell culture

To determine an appropriate site for the insertion of an additional foreign gene expression unit within the full-length genomic RNA of JEV, we initially constructed a pair of recombinant full-length JEV cDNAs by inserting an expression cassette for an enhanced *Aequorea victoria gfp* (*egfp*) gene as a reporter at two different loci within the viral genome without disrupting its single long ORF, either upstream of the initiation codon or downstream of the stop codon, in what is known as the variable region of JEV 3′ NTR (detailed description given in Figure S1, Supplemental Materials). Direct comparison of these two recombinant cDNAs revealed that the insertion of the highly structured internal ribosome entry site of the encephalomyocarditis virus (EMCV IRES) RNA sequence and the 768-bp *egfp* gene at the variable region of the JEV 3′ NTR allowed the complete replication of JEV but reduced RNA production and viral protein expression (Figures S1 and Figures 1 to 3). In contrast, insertion at the JEV 5′ NTR upstream of the initiation codon of the single long ORF had a deleterious effect on replication (Figure S1).

Next, we characterized the packaging capacity of JEV and evaluated its usefulness for expressing foreign genes, using a panel of recombinant JEV genomic RNAs of variable sizes. Based on a strategy of inserting an additional EMCV IRES-driven foreign gene expression unit at the variable region of the JEV 3′ NTR, we engineered the full-length viral genome to express three commonly used and variously sized heterologous reporter genes: *egfp* (768 bp), the luciferase gene from *Photinus pyralis* (*luc*, 1653 bp), and *lacZ* (3012 bp). The resulting three recombinant cDNA constructs were designated pJEV/FL/3*egfp*, pJEV/FL/3*luc*, and pJEV/FL/3*lacZ*, respectively (Figure 1). In parallel, we constructed

Templates used for transcription	Viral genome size (nucleotides)	Infectivity (per μg of RNA) 1st expt	Infectivity (per μg of RNA) 2nd expt	Virus titer (per ml) 48 h.p.t	Virus titer (per ml) 72 h.p.t
pJEV/FL	10,968	2.2 X 10^6 (PFU)	1.6 X 10^6 (PFU)	3.0 x 10^6 (PFU)	5.1 x 10^5 (PFU)
pJEV/FL/3*egfp*	12,367	1.1 X 10^6 (PFU or GFU)	2.0 X 10^6 (PFU or GFU)	9.0 x 10^4 (GFU)	5.2 x 10^5 (GFU)
pJEV/FL/3*luc*	13,236	0.9 X 10^6 (PFU)	0.8 X 10^6 (PFU)	3.0 x 10^4 (PFU)	3.1 x 10^5 (PFU)
pJEV/FL/3*luc*$^{REP-}$	13,153	0	0	0	0
pJEV/FL/3*lacZ*	14,753	0.6 X 10^6 (PFU or BFU)	0.8 X 10^6 (PFU or BFU)	1.1 x 10^4 (BFU)	1.3 x 10^5 (BFU)

Figure 1 Packaging of recombinant JEV genomic RNAs of up to ≈15 kb in size with no significant change in infectivity or production of recombinant viruses. A new cistron driven by EMCV IRES for the expression of three reporter genes of different sizes (*egfp*, 768 bp; *luc*, 1653 bp; *lacZ*, 3012 bp) was inserted into the variable region of JEV 3′ NTR in pJEV/FL, producing three recombinant cDNAs (pJEV/FL/3*egfp*, pJEV/FL/3*luc*, and pJEV/FL/3*lacZ*). The structures of the recombinant JEV cDNAs and parental cDNA (pJEV/FL) are shown. Viral ORFs are illustrated by thick solid lines at both termini, which denote the 5′ and 3′ NTRs of the genome. In the case of replication-competent pJEV/FL/3*luc* cDNA, replication-incompetent pJEV/FL/3*luc*$^{REP-}$ was also generated as a negative control by introducing an 83-nt internal deletion (*dotted line*) in the middle of the NS3 gene, resulting in the premature termination of viral translation at nt 5596 (*asterisk*). SP6 polymerase runoff transcription of recombinant JEV cDNA templates produced genomic RNAs ranging in size from 10,968 to 14,753 nt (viral genome size). Naïve BHK-21 cells (8×10^6) were transfected with 2 μg of synthetic RNAs transcribed from each cDNA template. The specific infectivities of the synthetic RNAs were estimated by counting the plaque-forming units (PFU) per μg of RNA, the green focus-forming units (GFU) per μg of RNA for pJEV/FL/3*egfp* under a fluorescence microscope, or the blue focus-forming units (BFU) per μg of RNA for pJEV/FL/3*lacZ* after X-gal staining, as indicated (infectivity). Virus titers in the culture supernatants were determined at 48 and 72 h post transfection by measuring the numbers of PFU per ml, GFU per ml for pJEV/FL/3*egfp,* or BFU per ml for pJEV/FL/3*lacZ*, as indicated (virus titer). h.p.t., hours post transfection.

the replication-incompetent pJEV/FL/3*luc*$^{REP-}$ (Yun *et al*, 2003) as a negative control; this construct contains an internal deletion of 83 nt in the NS3 region that prematurely terminates viral translation at nt 5596 (Figure 1). From these five recombinant cDNAs, including parental pJEV/FL, five recombinant JEV genomic RNAs of various lengths (10,968 to 14,753 nt) were synthesized by *in vitro* run-off transcription using SP6 RNA polymerase (Figure 1, viral genome size).

Using these newly generated RNAs, we initially sought to identify the mechanism by which the genome size of JEV might influence the specific infectivity and replication of the viral genomic RNA, expression of viral proteins, and production of infectious viral particles. We found that the synthetic RNAs derived from pJEV/FL/3*egfp*, pJEV/FL/3*luc*, and pJEV/FL/3*lacZ,* when introduced into susceptible BHK-21 cells, displayed specific infectivities of 1.1–2.0 $\times 10^6$, 0.8–0.9 $\times 10^6$, and 0.6–0.8 $\times 10^6$ plague forming units (PFU)/μg, respectively, analagous to that of synthetic RNA derived from parental pJEV/FL (1.6–2.2 $\times 10^6$ PFU/μg) (Figure 1, infectivity). We also saw no evidence of differences in specific infectivity by confocal microscopy for pJEV/FL/3*egfp*-derived or pJEV/FL/3*lacZ*-derived RNA-transfected cells. However, we observed a delay in the accumulation of infectious viral particles in supernatant fractions from recombinant RNA-transfected BHK-21 cells, and this delay was directly correlated with the lengths of the heterologous reporter genes inserted (Figure 1, virus titer). This correlation was reflected by the formation of smaller plaques (Figure 2a), delayed accumulation of genomic RNAs as measured by real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) (Figure 2b), and delayed expression of viral proteins as visualized by immunoblotting with a JEV-specific hyperimmune antiserum (Figure 2c) in recombinant RNA-transfected BHK-21 cells. Thus, JEV was capable of encapsidating recombinant genomes as large as ≈15 kb, but the packaging of these larger genomes was negatively correlated with RNA replication efficiency and cytopathogenicity in cell culture and not with infectivity.

We then examined the functional integrity of the recombinant RNAs by analyzing the expression of various lengths of heterologous reporter genes inserted into the viral genome (Figure 3).

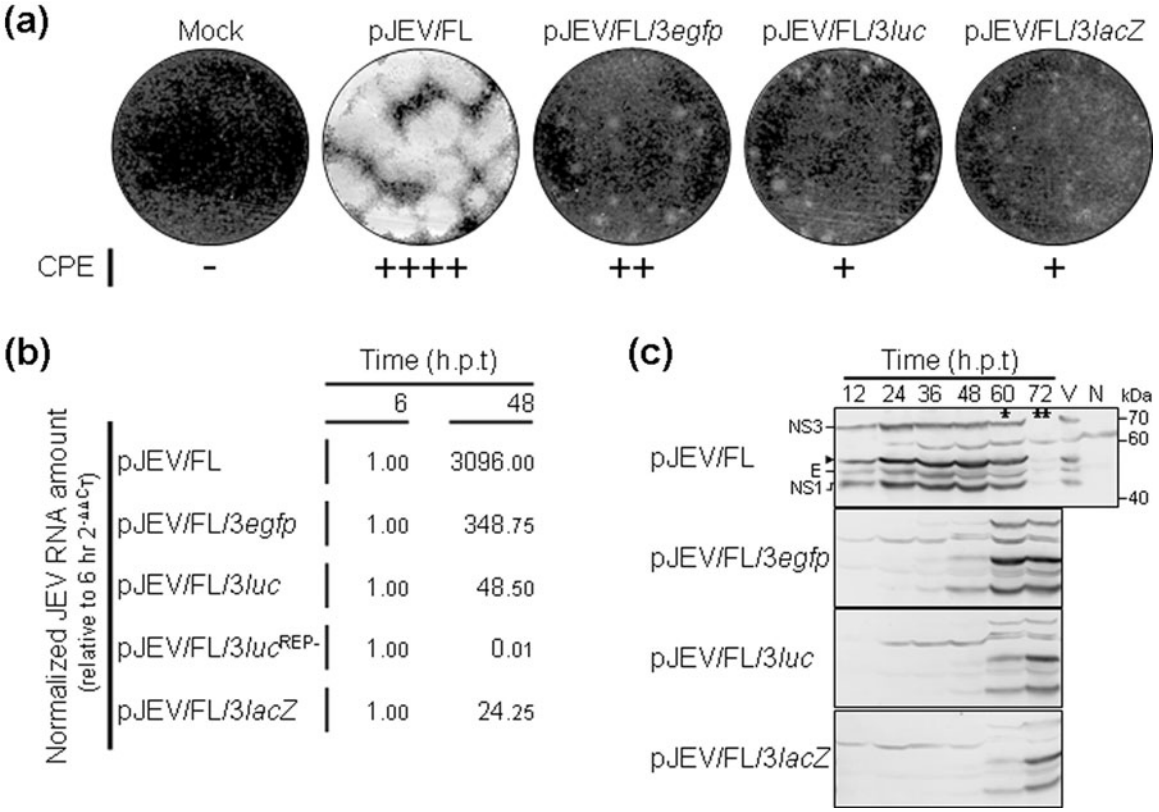

Figure 2 Negative correlation of the JEV genomic RNA size with the level of RNA replication and extent of cytopathogenicity. Naïve BHK-21 cells (8×10^6) were mock-transfected or transfected with 2 μg of the parent or recombinant JEV RNAs transcribed from the relevant JEV cDNA, as indicated. (**a**) Representative plaque morphology. Transfected cells were overlaid with agarose and stained 5 days later with crystal violet. (**b**) Level of JEV RNA production. Total cellular RNA was isolated from transfected cells at the indicated time points. Real-time quantitative RT–PCR was used for the quantitation of JEV-specific RNA and β-actin RNA for normalization of total RNA levels. The $2^{-\Delta\Delta CT}$ method was used to analyze the changes in JEV RNA levels, relative to those at 6 h post transfection, obtained from real-time quantitative PCR experiments. Data are shown from one of two independent experiments, which yielded similar results. (**c**) Levels of JEV protein accumulation. Transfected cells were lysed with 1× sample loading buffer at the indicated time points, and the protein extracts were resolved on 10% SDS-polyacrylamide gels. Viral proteins were visualized by immunoblotting with a JEV-specific hyperimmune antiserum. The positions of the viral proteins (E, NS1, and NS3) and a cleavage-related intermediate (*arrowhead*) are indicated on the left. Molecular mass markers in kDa are specified on the right. * and ** indicate that >50% and >99% of the pJEV/FL-derived RNA-transfected cells died and detached from the bottom of the culture dishes, respectively. V, JEV CNU/LP2-infected BHK-21 cells as a reference; N, naïve BHK-21 cells. h.p.t., hours post transfection.

In pJEV/FL/3*egfp*-derived RNA-transfected BHK-21 cells, confocal microscopy disclosed green fluorescence in both the nucleus and cytoplasm of cells expressing EGFP (Figure 3a), because enhanced green fluorescent protein (EGFP) is small (≈30 kDa) enough to diffuse between the nucleus and cytoplasm. As expected, fluorescence was not observed in mock-transfected cells (Figure 3a). Flow cytometry analyses demonstrated that green fluorescent cells constituted 99.8% of the transfected cells, when compared to mock-transfected cells (Figure 3b). In pJEV/FL/3*lacZ*-derived RNA-transfected BHK-21 cells, the X-gal staining pattern of *lacZ*-expressing cells, as revealed by light microscopy, indicated that 99.6% of the transfected cells expressed *lacZ* (Figure 3c). We also monitored the luciferase activity of BHK-21 cells over time after transfection with either the replication-competent pJEV/FL/3*luc*-derived RNA or with replication-incompetent pJEV/FL/3*luc*$^{REP-}$-derived RNA as a negative control (Figure 3d). We found that the level of luciferase activity varied over time, depending on the presence or absence of viral replication.

Self-replicating, self-limiting JEV replicons constructed by deleting one, two, or all viral structural genes display a spectrum of RNA replication and protein expression

To independently express foreign genes using the JEV RNA replication machinery, we used pJEV/FL/3*luc* to generate a panel of self-replicating, self-limiting viral replicons that meet stringent safety concerns. We employed the *luc* reporter as the heterologous gene because it allows sensitive and quantitative monitoring of viral replication. In all, we constructed three sets of nine viral replicon vectors by introducing one to three in-frame deletions into the ectodomains of all the JEV structural proteins in

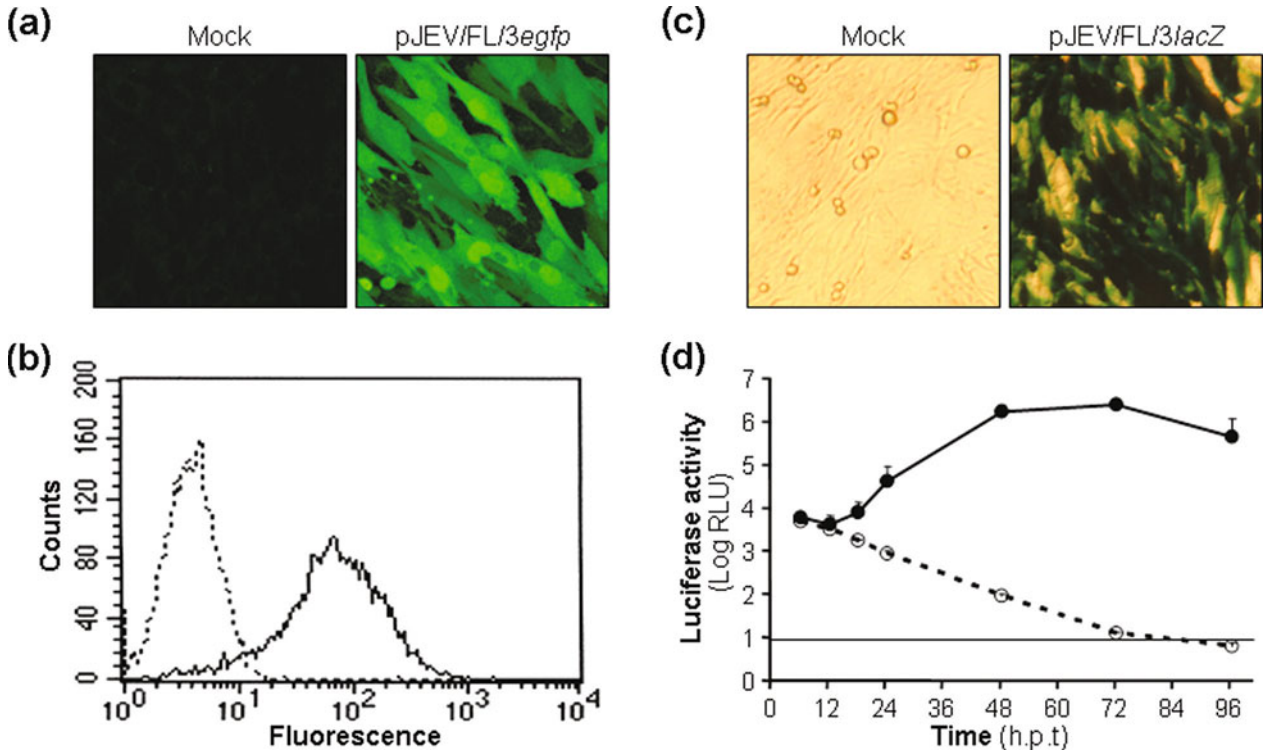

Figure 3 Functional assays for the expression of three reporter genes encoded in each of the three recombinant JEV genomic RNAs of variable sizes. Naive BHK-21 cells (8×10^6) were mock-transfected or transfected with 2 μg of the recombinant JEV RNAs transcribed from each cDNA, as indicated: pJEV/FL/3*egfp* (**a, b**), pJEV/FL/3*lacZ* (**c**), pJEV/FL/3*luc* or pJEV/FL/3*luc*$^{REP-}$ (**d**). (**a, b**) Expression of *egfp*. Transfected cells were prepared for confocal microscopy (**a**) and flow cytometric analysis (**b**). dotted line, mock-transfected cells; solid line, cells transfected with pJEV/FL/3*egfp*-derived RNA. (**c**) Expression of *lacZ*. Transfected cells were processed for X-gal staining. (**d**) Expression of *luc*. Transfected cells were seeded on 6-well plates at a density of 6×10^5 cells per well. At the indicated time points, cell lysates were assayed for luciferase activity. Experiments were performed in triplicate. Data are presented as mean values, with the standard deviations indicated by error bars solid circles, pJEV/FL/3*luc*-derived RNA-transfected cells; open circles, pJEV/FL/3*luc*$^{REP-}$-derived RNA-transfected cells; —, the level of background luminescence of naïve BHK-21 cells. h.p.t., hours post transfection.

order to allow us to compare the influence of each deletion on the competence and level of RNA replication: (i) a first set of four (pJEV/Rep/3*luc*/ΔCC, pJEV/Rep/3*luc*/ΔC, pJEV/Rep/3*luc*/ΔprM, and pJEV/Rep/3*luc*/ΔE) bearing a single deletion in each of the viral structural protein-coding regions; (ii) a second set of three (pJEV/Rep/3*luc*/ΔC+ΔprM, pJEV/Rep/3*luc*/ΔC+ΔE, and pJEV/Rep/3*luc*/ΔprM+ΔE) containing a double deletion in the coding regions of particular structural proteins; and (iii) a third set of two (pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE and pJEV/Rep/3*luc*/ΔCtoE) lacking all the structural proteins (Figure 4). In all cases, the region of deletion was carefully determined with respect to the membrane topology of each structural protein so that the transmembrane domains located in the C-termini remained intact. pJEV/Rep/3*luc*/ΔCC was identical to pJEV/Rep/3*luc*/ΔC, except that an additional 5′ deletion (nt 132 to 201) extended to include the proposed cyclization sequence motif in the 5′ region of the C gene, which is required for replication in other mosquito-borne flaviviruses, such as DENV, YFV, KUNV, and WNV (Alvarez *et al*, 2005; Bredenbeek *et al*, 2003; Corver *et al*, 2003; Hahn *et al*, 1987; Khromykh *et al*, 2001; Lo *et al*, 2003; You *et al*, 2001).

We then asked whether the viral replicon RNAs derived from nine cDNA templates were competent in replication by analyzing viral protein expression over time after transfection into BHK-21 cells. Two RNA samples derived from replication-competent pJEV/FL/3*luc* and replication-incompetent pJEV/FL/3*luc*$^{REP-}$ were transfected in parallel to serve as positive and negative controls, respectively. Immunoblotting using an anti-JEV NS1 antiserum (Figure 5a) and a JEV-specific hyperimmune antiserum (data not shown) showed that with the exception of pJEV/Rep/3*luc*/ΔCC-derived RNA, all viral replicon RNAs harboring a single, double, or triple in-frame deletion in JEV structural genes produced a detectable amount of viral proteins during the first 24 h post transfection. Protein levels increased until 48 h post transfection and were maintained until 72 h post transfection because of a lack of cell-to-cell spread. In contrast, we did not detect any viral proteins in BHK-21 cells transfected with pJEV/Rep/3*luc*/ΔCC-derived RNA or with replication-incompetent pJEV/FL/3*luc*$^{REP-}$-derived RNA (Figure 5a and data not shown). As expected, in BHK-21 cells transfected with replication-competent pJEV/FL/3*luc*-derived RNA,

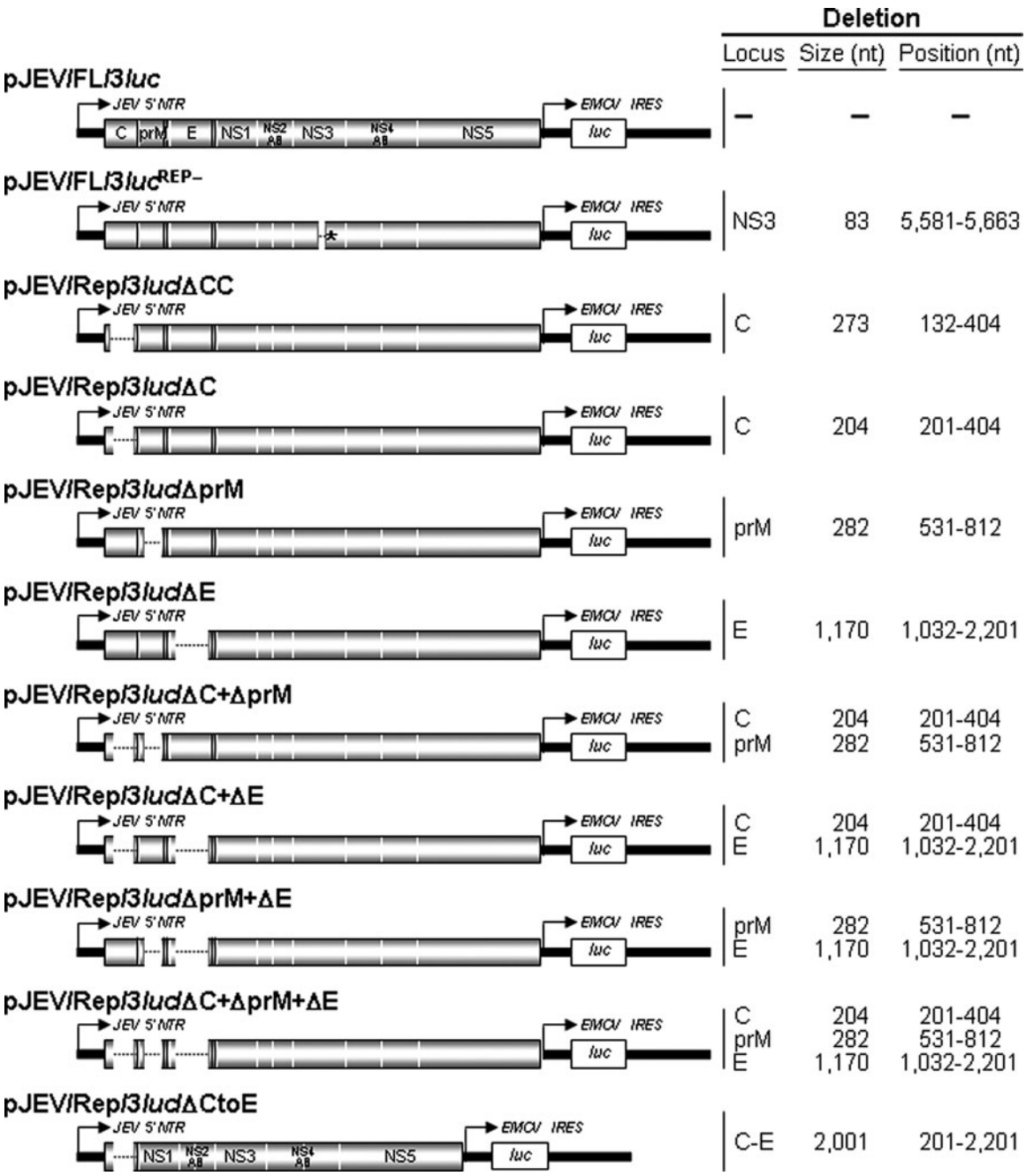

Figure 4 Schematic presentation of JEV replicons. All JEV replicons designed to express the *luc* gene were constructed on the basis of pJEV/FL/3*luc* by introducing one, two, or three internal in-frame deletions (*dotted lines*) in the ectodomain-coding regions of all three viral structural proteins (C, prM, and E). The first set of four replicons (pJEV/Rep/3*luc*/ΔCC, pJEV/Rep/3*luc*/ΔC, pJEV/Rep/3*luc*/ΔprM, and pJEV/Rep/3*luc*/ΔE) contained a single internal in-frame deletion in each structural gene of JEV. pJEV/Rep/3*luc*/ΔCC was identical to pJEV/Rep/3*luc*/ΔC, except for an additional 5′ deletion (nt 132-201) that extended to the proposed cyclization sequence motif in the 5′ region of the C gene. The second set of three replicons (pJEV/Rep/3*luc*/ΔC+ΔprM, pJEV/Rep/3*luc*/ΔC+ΔE, and pJEV/Rep/3*luc*/ΔprM+ΔE) contained two internal in-frame deletions in the structural genes. The third set of two replicons (pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE, and pJEV/Rep/3*luc*/ΔCtoE) lacked all three JEV structural proteins. pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE contained three internal in-frame deletions introduced into each of the three structural genes, whereas pJEV/Rep/3*luc*/ΔCtoE contained the 35 N-terminal and 24 C-terminal amino acids of the C protein, immediately followed by the N-terminus of the NS1 protein and the rest of the viral genome. One or two transmembrane domains located at the C-terminus of each structural protein (one for C and two for prM and E) are represented by black vertical bars. The locus, size, and position of the internal in-frame deletions introduced in the ectodomain-coding regions of all three viral structural proteins are shown on the right.

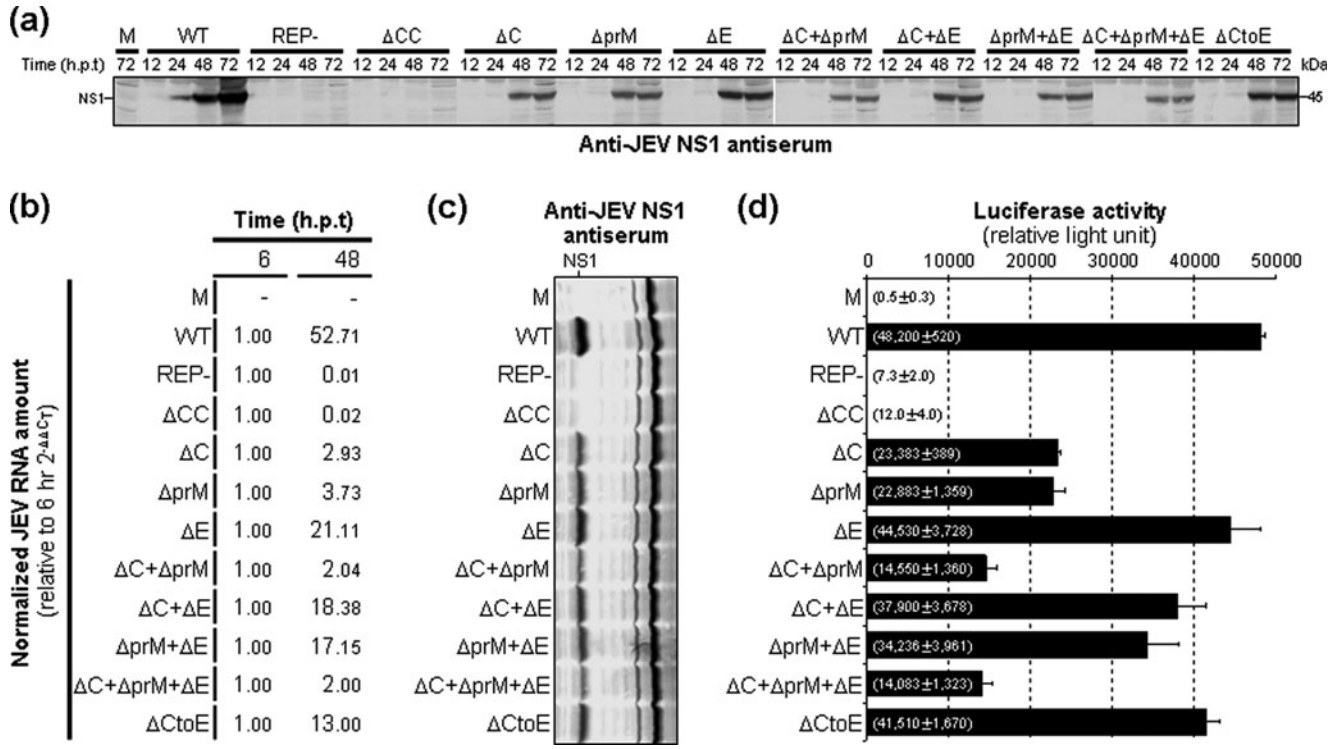

Figure 5 The spectrum of replication efficiencies exhibited by a panel of JEV replicon RNAs. Naïve BHK-21 cells (8×10^6) were mock-transfected or transfected with 2 μg of the parent or JEV replicon RNAs transcribed from each cDNA, as indicated. M, mock; WT, pJEV/FL/3*luc*; REP-, pJEV/FL/3*luc*$^{REP-}$; ΔCC, pJEV/Rep/3*luc*/ΔCC; ΔC, pJEV/Rep/3*luc*/ΔC; ΔprM, pJEV/Rep/3*luc*/ΔprM; ΔE, pJEV/Rep/3*luc*/ΔE; ΔC+ΔprM, pJEV/Rep/3*luc*/ΔC+ΔprM; ΔC+ΔE, pJEV/Rep/3*luc*/ΔC+ΔE; ΔprM+ΔE, pJEV/Rep/3*luc*/ΔprM+ΔE; ΔC+ΔprM+ΔE, pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE; ΔCtoE, pJEV/Rep/3*luc*/ΔCtoE. h.p.t, hours post-transfection. (**a**) Levels of JEV NS1 protein accumulation over time post-transfection. Transfected cells were lysed with 1× sample loading buffer at the indicated time points, and the protein extracts were resolved on 10% SDS-polyacrylamide gels. Viral proteins were visualized by immunoblotting with an anti-JEV NS1 rabbit antiserum. (**b–d**) Levels of JEV RNA production (**b**), JEV NS1 protein accumulation (**c**), and luciferase activity (**d**) at 48 h post transfection. (**b**) Real-time quantitative RT-PCR experiments. Transfected cells were used for the isolation of total cellular RNA at the indicated time points. Real-time quantitative RT-PCR was employed for the quantitation of JEV-specific RNA and β-actin RNA for normalization of total RNA levels. We utilized the $2^{-\Delta\Delta CT}$ method to analyze the changes in JEV RNA levels, relative to those at 6 h post transfection from real-time quantitative PCR experiments. Data are shown from one of two independent experiments, which yielded similar results. (**c**) Western blot analyses. Equal amounts of protein extracts from transfected cells at 48 h post transfection were separated on 10% SDS-polyacrylamide gels. The JEV NS1 protein was visualized by immunoblotting with an anti-JEV NS1 rabbit antiserum. (**d**) Luciferase assays. Equal amounts of cell lysates from transfected cells at 48 h post transfection were used to determine luciferase activity. Experiments were performed in triplicate, and data are presented as mean values with the standard deviations, indicated by error bars.

JEV NS1 (Figure 5a) and other JEV-specific viral proteins (data not shown) were detectable at 24 h post transfection and gradually accumulated until 72 h post transfection. Thus, with the exception of the pJEV/Rep/3*luc*/ΔCC-derived RNA, all the viral replicon RNAs harboring a single, double, or triple in-frame deletion in JEV structural genes were replication-competent.

However, differences became evident between 48 and 72 h post transfection (Figure 5a). In repeated experiments, direct comparison of the levels of viral replicon RNAs by real-time quantitative RT–PCR showed that production of the pJEV/Rep/3*luc*/ΔE-derived RNA was consistently higher than that by all other viral replicon RNAs harboring a single, double, or triple in-frame deletion in JEV structural genes (Figure 5b). At 48 h post transfection, production of the two viral replicon RNAs derived from either pJEV/Rep/3*luc*/ΔC or pJEV/Rep/3*luc*/ΔprM was ≈5.7- to 7.2-fold lower than that of pJEV/Rep/3*luc*/ΔE-derived RNA. Also, production of the two viral replicon RNAs derived from either pJEV/Rep/3*luc*/ΔC+ΔE or pJEV/Rep/3*luc*/ΔprM+ΔE was ≈1.2-fold lower, and that of pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE-derived RNA was ≈10.6-fold lower than that of pJEV/Rep/3*luc*/ΔE-derived RNA. In addition, production of pJEV/Rep/3*luc*/ΔC+ΔprM-derived RNA was ≈1.4- to 1.8-fold lower than that of the two viral replicon RNAs derived from either pJEV/Rep/3*luc*/ΔC or pJEV/Rep/3*luc*/ΔprM at 48 h post-transfection. In all cases, the spectrum of RNA production clearly correlated with the accumulation levels of the JEV NS1 protein (Figure 5c) and the expression profiles of the LUC protein (Figure 5d).

Thus, two in-frame deletions introduced in the ectodomains of the C and prM proteins, individually or in combination, in the context of either

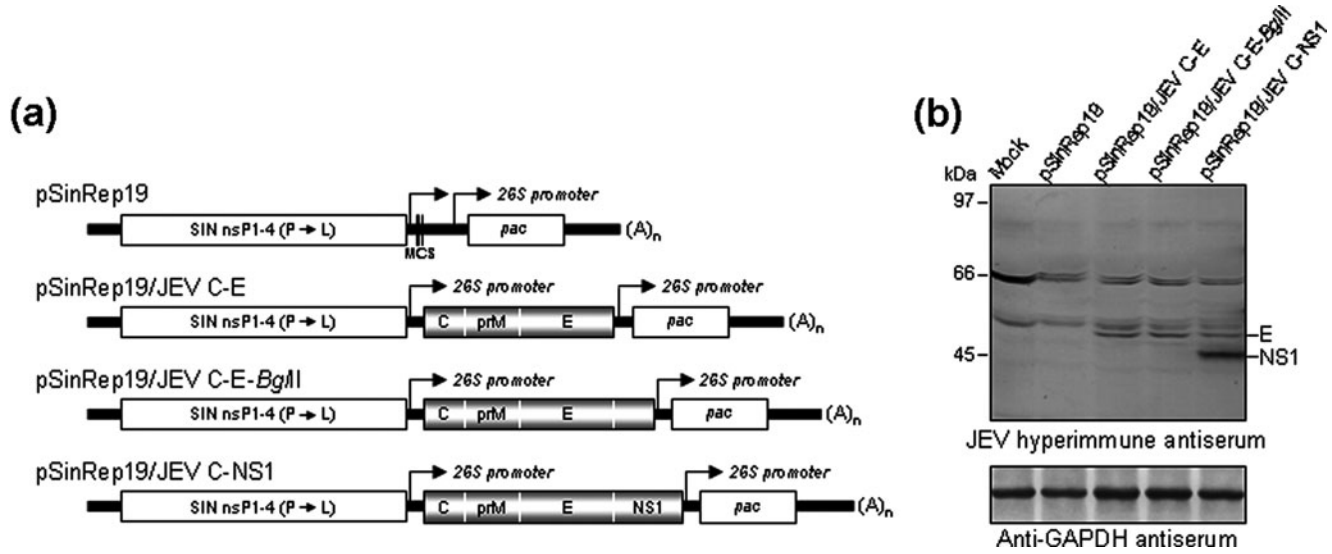

Figure 6 Construction and characterization of the packaging system for JEV replicon RNAs. (**a**) Schematic presentation of three JEV structural protein expression vectors based on the Sindbis virus–derived expression vector, pSinRep19. A foreign gene and the *pac* gene were expressed using separate subgenomic promoters (26S promoter), indicated by arrows. The pSinRep19/JEV C-E vector contains the entire coding sequence of JEV C through E. The pSinRep19/JEV C-E-*Bgl*II vector includes the complete coding sequence of JEV C through E, followed by the N-terminal 58 residues of NS1. The pSinRep19/JEV C-NS1 encompasses the complete coding sequence of JEV C through NS1. MCS, multiple cloning sites. (**b**) Expression of JEV structural proteins. Naïve BHK-21 cells were mock-transfected or transfected with RNA transcripts derived from each JEV structural protein expression vector, as indicated. Cell lysates were harvested 48 h later. Equal amounts of cell lysates were resolved by SDS-PAGE and probed with a JEV-specific hyperimmune antiserum (*top panel*). In parallel, GAPDH protein was detected as a loading and transfer control with an anti-GAPDH rabbit antiserum (*bottom panel*). The positions of viral proteins E and NS1 are specified on the right, and the molecular mass markers in kDa on the left.

pJEV/Rep/3*luc*/ΔE or parental pJEV/FL/3*luc,* acted synergistically in suppressing RNA production. This finding suggested that expression of only the transmembrane domains of JEV structural proteins, in the absence of functional proteins, might suppress genomic RNA replication. This possibility was supported by a direct comparison of pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE to pJEV/Rep/3*luc*/ΔCtoE, which contains the 35 N-terminal and 24 C-terminal amino acids of the C protein, immediately followed by the N-terminus of the NS1 protein and the rest of the viral genome (Figure 4). RNA production by pJEV/Rep/3*luc*/ΔCtoE was ≈6.5-fold higher than that by pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE at 48 h post-transfection (Figure 5b), indicating that the removal of two pairs of transmembrane domains (one at the end of the prM and the other at the end of the E) in pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE increased the RNA replication efficiency. Consistently, RNA levels were reflected in the accumulation of the JEV NS1 protein (Figure 5c) and the expression profiles of LUC protein (Figure 5d).

Generation of stable packaging cell lines capable of encapsidating JEV-derived replicon vector RNAs into single-round infectious viral replicon particles

The utility of JEV replicon-based expression vectors was expanded by developing a *trans*-complementation system that expressed all three JEV structural proteins *in trans* and allowed the encapsidation of JEV replicon RNAs into single-round infectious, propagation-deficient viral replicon particles (VRPs). For the ectopic expression of JEV structural proteins, we utilized a Sindbis virus–based heterologous gene expression vector, pSinRep19 (Agapov *et al*, 1998), that contains the puromycin *N*-acetyltransferase (*pac*) gene as a dominant selectable marker. We constructed a set of three JEV structural protein expression vectors containing the entire coding sequence of JEV C through E (pSinRep19/JEV C-E), JEV C through E plus the 58 N-terminal residues of NS1 (pSinRep19/JEV C-E-*Bgl*II), and JEV C through NS1 (pSinRep19/JEV C-NS1) (Figure 6a). Protein expression was evaluated in BHK-21 cells after transfection with synthetic RNAs transcribed *in vitro* from the corresponding vector. Immunoblot analysis of lysates from transfected cells with a JEV-specific hyperimmune antiserum revealed that equal amounts of the JEV E protein were expressed in cells transfected with one of the three vector RNAs, whereas JEV NS1 was identified only in BHK-21 cells transfected with the vector RNA transcribed from pSinRep19/JEV C-NS1 (Figure 6b). GAPDH was employed as a loading control (Figure 6b).

We took two approaches to producing JEV VRPs (Figure 7a). The first involved the transient co-transfection of JEV replicon RNA with one of the three JEV structural protein expression vector RNAs. Titering and monitoring of VRP production

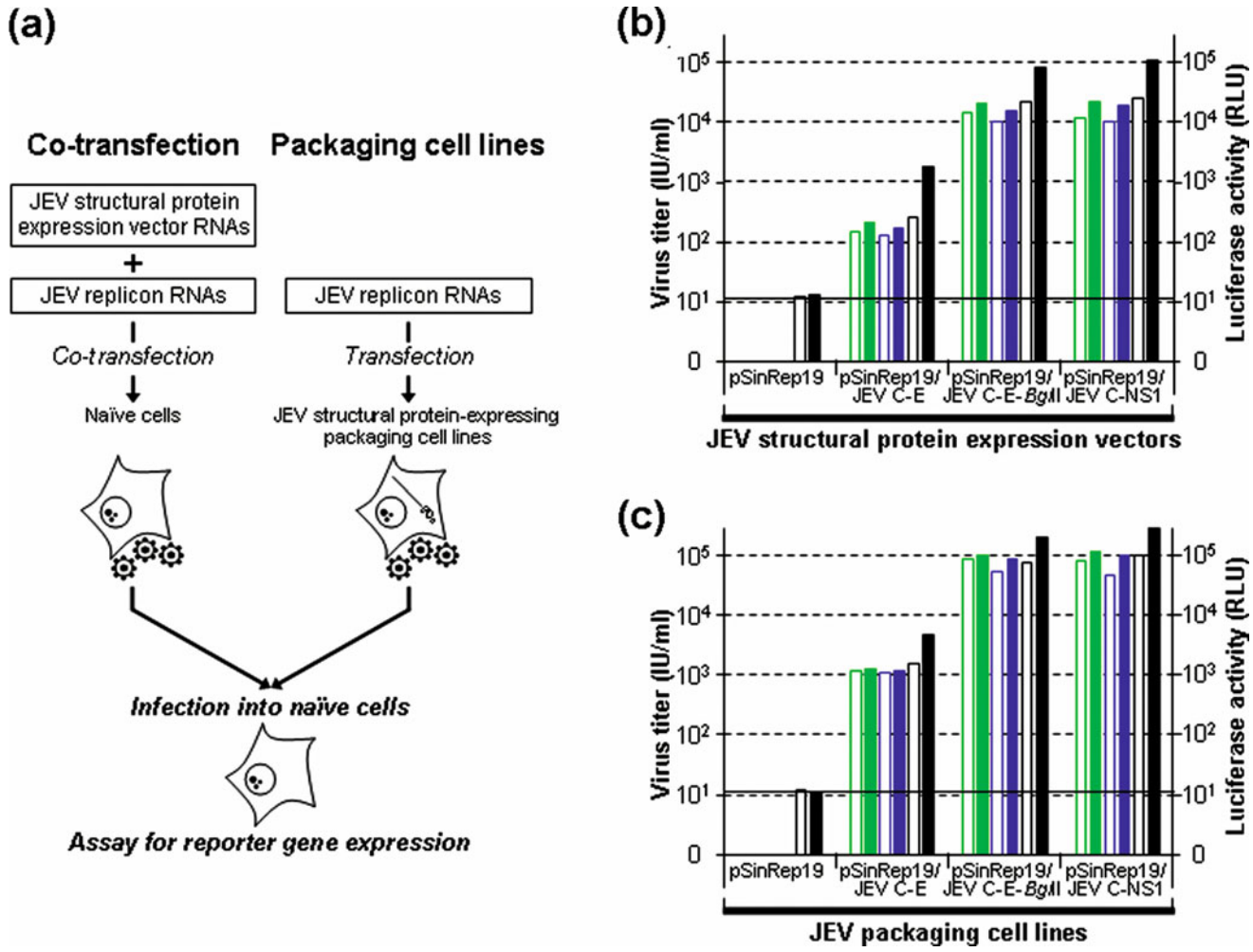

Figure 7 Production of single-round infectious propagation-deficient JEV VRPs. (**a**) Schematic illustrating the generation of JEV VRPs by (i) cotransfection of JEV structural protein expression vector RNAs with JEV replicon RNAs or (ii) transfection of JEV structural protein-expressing PCLs with JEV replicon RNAs. (**b, c**) Production of JEV VRPs. Two approaches were utilized: One involved the transient cotransfection of naïve BHK-21 cells with two vector RNAs, including a JEV structural protein expression vector RNA along with a JEV replicon RNA, as indicated (**b**). The other procedure involved the transfection of JEV PCLs with one of the JEV replicon RNAs (**c**). The JEV replicon RNAs used were as follows: □ green, pJEV/Rep/3*egfp*/ΔC+ΔprM+ΔE; ■ green, pJEV/Rep/3*egfp*/ΔCtoE; □ blue, pJEV/Rep/3*lacZ*/ΔC+ΔprM+ΔE; ■ blue, pJEV/Rep/3*lacZ*/ΔCtoE; □ black, pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE; ■ black, pJEV/Rep/3*luc*/ΔCtoE. The supernatant fractions were collected at 48 h post transfection and used to infect naïve BHK-21 cells for the titration of VRPs, followed by the examination of the respective reporter gene expression. −, the level of background luminescence of naïve BHK-21 cells for luciferase activity.

was performed by infecting naïve BHK-21 cells and assaying for reporter gene expression from the JEV replicon RNA packaged into VRPs. Cotransfection of pSinRep19/JEV C-NS1-derived vector RNA with *egfp*-expressing JEV replicon RNA derived from either pJEV/Rep/3*egfp*/ΔC+ΔprM+ΔE or pJEV/Rep/3*egfp*/ΔCtoE in several experiments produced 1.1–4.3 × 10^4 infectious units/ml (IU/ml) of VRPs (Figure 7b). Similar results were obtained using *lacZ*-expressing JEV replicon RNA derived from either pJEV/Rep/3*lacZ*/ΔC+ΔprM+ΔE or pJEV/Rep/3*lacZ*/ΔCtoE (Figure 7b). No apparent differences were evident when pSinRep19/JEV C-E-*Bgl*II–derived vector RNA was used for the expression of JEV structural proteins instead of that from pSinRep19/JEV C-NS1 (Figure 7b). In contrast, cotransfection of the pSinRep19/JEV C-E-derived vector RNA with one of four JEV replicon RNAs expressing either *egfp* or *lacZ* consistently produced ≈100-fold fewer VRPs (Figure 7b). These observations were confirmed by cotransfecting one of three pSinRep19-based JEV structural protein expression vector RNAs with one of two *luc*-expressing JEV replicon RNAs derived from either pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE or pJEV/Rep/3*luc*/ΔCtoE (Figure 7b).

The second approach to producing JEV VRPs was based on using stable packaging cell lines (PCLs), established by initially transfecting naïve BHK-21 cells with one of three pSinRep19-based vector RNAs expressing JEV structural proteins and subsequently

selecting with puromycin. The selected cells stably expressed JEV structural proteins with no deleterious effects on the host cells (data not shown). These PCLs were more efficient in producing JEV VRPs than were the parental BHK-21 cells. In all cases, approximately 5- to 10-fold higher VRP titers were obtained upon transfection of these PCLs, with one of six JEV replicon RNAs expressing the *egfp*, *luc*, or *lacZ* gene based on either pJEV/Rep/ΔC+ΔprM+ΔE or pJEV/Rep/ΔCtoE, when compared to the cotransfection of the parental BHK-21 cells with two vector RNAs (Figure 7c). In order to verify that the virus was truly propagation deficient, we attempted to amplify any very low amounts of propagation-competent virus particles that might have been present. We did not detect any propagation-competent virus particles in our packaging system after three passages of the undiluted supernatant containing 3×10^5 IU VRPs on naïve BHK-21 cells (data not shown). Thus, we have successfully demonstrated that *trans*-complementation-based packaging systems for JEV replicon vectors can produce single-round infectious, propagation-deficient JEV VRPs.

Discussion

Here we report the development of a JEV-based vector system for foreign gene expression that utilizes a full-length infectious JEV cDNA. We have established and fully characterized three strategies for the expression of foreign genes using the RNA replication machinery of JEV. The first strategy made use of recombinant infectious vector RNAs/viruses encoding foreign genes of various sizes, preferentially at the variable region of JEV 3′ NTR, and showed an inverse correlation between genome length and gene expression. The second approach involved a variety of replication-competent, propagation-deficient viral replicon vector RNAs showing a spectrum of RNA replication efficiencies. The third procedure made use of stable packaging cell lines for the production of single-round infectious, propagation-deficient JEV VRPs. These data strongly suggest that our JEV-based vector systems represent attractive potential candidates for foreign gene expression and antiviral compound screening in a wide variety of cells *in vitro*, and possibly *in vivo* for immunization applications.

When we determined the maximum extra sequence length that JEV could accommodate, we found to our surprise that JEV was capable of encapsidating recombinant JEV genomic RNAs encoding heterologous reporter genes as large as ≈15 kb into infectious viral particles. Because JEV replicon RNA derived from pJEV/Rep/ΔCtoE is ≈9 kb in size, we estimate that a foreign gene of at least 6 kb can be packaged into JEV VRPs. This result is of particular significance for the expression of lengthy genes, such as the cystic fibrosis transmembrane conductance regulator, whose coding sequence is ≈4.5 kb (Flotte *et al*, 1993). A large packaging capacity of JEV is also useful in case two or more expression units need to be added (Agapov *et al*, 1998). On the other hand, we have found that the replication kinetics and final virus titers in cell culture proportionally decreased as the inserted gene length increased. Our data indicate that size is a nonspecific factor affecting the ability of the RNA to serve as a template for replication, with smaller RNAs generally being better templates. The simplest explanation for this effect is that as the length of the RNA template increases, the number of minus- and plus-strand elongation cycles also increases; however, we cannot rule out the possibility that larger RNAs replicate less efficiently because of differences in GC content or in their secondary and/or tertiary structures.

We have also found that two recombinant JEVs harvested from BHK-21 cells transfected with pJEV/FL/3*egfp*- or pJEV/FL/3*lacZ*-derived RNA are capable of transducing a wide variety of cell types, including SH-SY5Y (human neuroblastoma), HeLa (human cervix adenocarcinoma), MOLT-4 (human lymphoblastic leukemia), Vero (monkey kidney), Neuro-2a (mouse neuroblastoma), MDCK (dog kidney), CRFK (cat kidney), BHK-21 (hamster kidney), C6/36 (mosquito larva), and primary rat neurons (data not shown). It is important to note, however, that foreign genes inserted at the beginning of the JEV 3′ NTR appeared to be unstable, in that the longer foreign genes more rapidly lost their activity during serial passages. When we infected BHK-21 cells at an multiplicity of infection (MOI) of 1 with two recombinant JEVs expressing the *egfp* or *lacZ* gene, we found that the percentage of infected cells expressing both JEV proteins and the corresponding reporter gene decreased with each passage, with ≈50% (pJEV/FL/3*egfp*) and ≈30% (pJEV/FL/3*lacZ*) of the cells expressing both proteins at passages 3 and 2, respectively (data not shown). In light of reports concerning other flavivirus vector systems (Fayzulin *et al*, 2006; Gehrke *et al*, 2005; Pierson *et al*, 2005), this finding was not unexpected, because the increase in genome size was associated with a decreased rate of viral replication and vice versa, providing the opportunity for deletion of all or a portion of the inserted foreign gene that is unnecessary for viral replication. Thus, although the application of such JEV-based vector systems appears to be limited in part by the long-term instability of the viral genome when it contains a foreign gene of a larger size, the genome should still be stable enough to produce recombinant JEVs carrying such a foreign gene during the first 3 to 4 days of RNA transfection.

Vectors based on self-replicating replicons of several flaviviruses have recently been constructed and characterized for potential application to gene expression in mammalian cells and the development of novel antiviral therapeutics and vaccines (Gehrke *et al*, 2005; Jones *et al*, 2005; Khromykh and Westaway, 1997; Molenkamp *et al*, 2003; Pang *et al*, 2001; Shi *et al*, 2002; Varnavski and Khromykh,

1999). A relatively small genome size and simple procedure allow for rapid generation of recombinants, and continuous synthesis of dsRNA intermediates during replication yields an enhanced immune response. In the present study, we have systematically manipulated the genome of JEV for the first time to generate a panel of nine viral replicons by introducing one to three internal in-frame deletions in the ectodomains of structural proteins. Of these, all except pJEV/Rep/3*luc*/ΔCC were replication-competent. It is important to note that pJEV/Rep/3*luc*/ΔCC is identical to pJEV/Rep/3*luc*/ΔC except for an additional 5′ deletion (nt 132 to 201) in the coding region of the C protein; therefore, this region is apparently essential for RNA replication. This finding is consistent with the proposed cyclization sequence motif in the 5′ region of the C gene, which is known to be required for replication in other mosquito-borne flaviviruses, such as DENV, YFV, KUNV, and WNV (Alvarez *et al*, 2005; Bredenbeek *et al*, 2003; Corver *et al*, 2003; Hahn *et al*, 1987; Khromykh *et al*, 2001; Lo *et al*, 2003; You *et al*, 2001). Furthermore, we have also engineered a variety of JEV replicon vector RNAs that are packaged when the structural proteins are supplied *in trans*, using a Sindbis virus-based expression system, as previously reported for other flavivirus replicon-derived vectors (Fayzulin *et al*, 2006; Gehrke *et al*, 2003; Harvey *et al*, 2004; Khromykh *et al*, 1998; Mason *et al*, 2006; Scholle *et al*, 2004).

The eight self-replicating *luc*-expressing JEV replicons constructed here displayed a wide spectrum of RNA replication and protein expression and various levels of cytopathogenicity. Specifically, the levels of RNA production and viral protein accumulation were synergistically reduced by an internal in-frame deletion introduced, either individually or in combination, into the ectodomains of the C and prM proteins of either pJEV/Rep/3*luc*/ΔE or parental pJEV/FL/3*luc*. According to our current understanding, the endoplasmic reticulum (ER) is the principal site at which JEV protein synthesis, virus assembly, and final maturation take place (Lindenbach and Rice, 2001). JEV infection causes extensive proliferation of the secretory apparatus, including rough ER and Golgi complexes, in BHK-21 cells (Wang *et al*, 1997). The ER is extremely sensitive to changes in homeostasis, and in response to a variety of stimuli, certain signals are transduced from the ER to both the cytoplasm and nucleus, resulting in adaptation for survival or induction of apoptosis (Kaufman, 1999; Pahl, 1999). Recent studies have shown that JEV infection induces ER stress, triggering the unfolded protein response, which in turn initiates a unique signaling cascade from the ER to the nucleus (Su *et al*, 2002). Accordingly, we hypothesize that the transmembrane domains of JEV structural proteins expressed from the C-terminus in the ER suppress the replication of each viral replicon RNA. This hypothesis was supported by direct comparison of RNA production by pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE with that of pJEV/Rep/3*luc*/ΔCtoE, which lacks two pairs of transmembrane domains in the C-terminal portions of the prM and E proteins remaining in pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE. RNA production by pJEV/Rep/3*luc*/ΔCtoE was ≈6.5-fold higher than that of pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE at 48 h post-transfection. Alternatively, it is conceivable that the unnatural repeating of transmembrane domains leads to incorrect or inefficient translocation of the NS1 protein to the rough ER, thereby potentially interfering with the biogenesis of the remainder portion of the polyprotein and resulting in the inefficient RNA replication.

In summary, the JEV-based vector system described in this article represents a promising system for delivering foreign genes to cells, not only *in vitro* for the expression of foreign genes and screening of antiviral compounds but potentially also *in vivo* for the development of genetic vaccines. The large packaging capacity of JEV should facilitate the design of vectors for expressing larger genes and treatment of associated diseases. Also, the unexpected influence of the transmembrane domains within the viral structural proteins on RNA replication may provide novel information regarding cross-talk that connects the biogenesis of functional structural proteins of flaviviruses within the ER and the levels of RNA production and gene expression in the cytoplasm of infected cells.

Materials and methods

Cells

BHK-21 cells were maintained in alpha minimal essential medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, vitamins, and antibiotics (Yun *et al*, 2003). The stable JEV replicon packaging cell lines derived from BHK-21 cells were maintained in the presence of 1 μg/ml puromycin.

Construction of plasmids

All plasmids were constructed using standard molecular biology protocols, and the regions amplified by PCR were verified by sequencing. The recombinant JEV-derived vectors used in this study were constructed based on pBAC^{SP6}/JVFLx/*Xba*I (Yun *et al*, 2003), designated "pJEV/FL" in this report. The nucleotide position refers to the complete nucleotide sequence of JEV CNU/LP2 (GenBank accession number AY585243). Only the salient features of these plasmids are described here. Details of the cloning strategies are provided in Supplemental Materials, and computer-readable sequence files are also available upon request.

Recombinant full-length JEV vectors: pJEV/FL/3-*egfp* was constructed by inserting the EMCV IRES-driven EGFP expression cassette immediately downstream of the first 41 nucleotides of JEV 3′ NTR,

followed by the entire JEV 3′ NTR. pJEV/FL/3*luc* contained the *luc* gene in lieu of the *egfp* gene of pJEV/FL/3*egfp*. pJEV/FL/3*luc*$^{REP-}$ was identical to pJEV/FL/3*luc*, except that an 83-nucleotide deletion (nt 5581 to 5663) was introduced into the middle of the NS3 gene, resulting in premature termination of viral translation at nt 5596 (Yun *et al*, 2003). pJEV/FL/3*lacZ* contained the *lac*Z gene encoding the bacterial β-galactosidase enzyme in exchange for the coding sequence of *egfp* of pJEV/FL/3*egfp*.

Propagation-deficient JEV replicon vectors: A panel of nine JEV replicon vectors expressing the *luc* gene was constructed to facilitate the monitoring of replication. These vectors, based on pJEV/FL/3*luc*, were produced by introducing one to three internal in-frame deletions in the coding sequences of particular JEV structural proteins. All deletions were identified by a novel *Xho*I site that resulted in the insertion of Leu and Glu. The first set of four viral replicon vectors (pJEV/Rep/3*luc*/ΔCC, pJEV/Rep/3*luc*/ΔC, pJEV/Rep/3*luc*/ΔprM, and pJEV/Rep/3*luc*/ΔE) contained a 273-nucleotide deletion (nt 132 to 404) in the C gene, a 204-nucleotide deletion (nt 201 to 404) in the C gene, a 282-nucleotide deletion (nt 531 to 812) in the prM gene, and a 1170-nucleotide deletion (nt 1032 to 2201) in the E gene, respectively. The second set of three viral replicon vectors (pJEV/Rep/3*luc*/ΔC+ΔprM, pJEV/Rep/3*luc*/ΔC+ΔE, and pJEV/Rep/3*luc*/ΔprM+ΔE) contained a pair of internal in-frame deletions in the coding regions of two JEV structural proteins and are designated accordingly. The third set of two viral replicon vectors (pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE and pJEV/Rep/3*luc*/ΔCtoE) lacked all three JEV structural proteins. pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE contained all three internal in-frame deletions, namely a 204-nucleotide deletion (nt 201 to 404) in the C gene, a 282-nucleotide deletion (nt 531 to 812) in the prM gene, and a 1170-nucleotide deletion (nt 1032 to 2201) in the E gene. pJEV/Rep/3*luc*/ΔCtoE contained a 2,001-nucleotide deletion (nt 201 to 2201) in the viral genome, excluding the 35 N-terminal and 24 C-terminal amino acids of the C protein and followed immediately by the N-terminus of the NS1 protein and the rest of the viral genome. We also generated a separate set of four viral replicon vectors (pJEV/Rep/3*egfp*/ΔC+ΔprM+ΔE, pJEV/Rep/3*egfp*/ΔCtoE, pJEV/Rep/3*lacZ*/ΔC+ΔprM+ΔE, and pJEV/Rep/3*lacZ*/ΔCtoE) designed to express either *egfp* or *lacZ,* by replacing the *luc* gene of pJEV/Rep/3*luc*/ΔC+ΔprM+ΔE or pJEV/Rep/3*luc*/ΔCtoE with the corresponding reporter gene.

JEV structural protein expression vectors: pSinRep19 (Agapov *et al*, 1998), a Sindbis virus–based heterologous gene expression vector, was engineered to express three JEV structural proteins: pSinRep19/JEV C-E contained the entire coding sequence of JEV C through E under the control of its own subgenomic RNA promoter. pSinRep19/JEV C-E-*Bgl*II contained the coding sequence of JEV C through E plus the 58 N-terminal residues of NS1, whereas pSinRep19/JEV C-NS1 included the complete coding sequence of JEV C through NS1.

RNA transcription and transfection
Purified pJEV/FL and its derivatives were linearized by digestion with *Xba*I, followed by treatment with mung bean nuclease. Purified pSinRep19 and its derivatives were linearized by digestion with *Xho*I. Linearized plasmids were used for *in vitro* transcription reactions employing SP6 RNA polymerase, as described previously (Yun *et al*, 2003). After transcription, reaction mixtures were further incubated with 10 U DNase I for 30 min and extracted with phenol-chloroform-isoamylalcohol. RNA yield was quantified on the basis of [^{3}H]UTP incorporation, as measured by RNA absorption to DE-81 filter paper (Whatman, Maidstone, UK). RNA integrity was assessed by agarose gel electrophoresis. RNA (2 μg) was transfected into cells by electroporation, as described previously (Yun *et al*, 2003).

Real-time quantitative RT–PCR
Total RNA was extracted from duplicate wells with TRIzol reagent (Invitrogen, Carlsbad, CA). Specifically, 50 ng of total cellular RNA was used for reverse transcription using Superscript II RT (Gibco-BRL Life Technologies, Gaithersburg, MD) with primers specific for the JEV NS3 region, as well as BHK-21 β-actin RNA to normalize total RNA levels. JEV and BHK-21 β-actin cDNAs were generated by reverse transcription at 45°C for 30 min, followed by inactivation of reverse transcriptase at 95°C for 10 min. JEV-specific and BHK-21 β-actin–specific cDNAs were amplified with the iQ Supermix Quantitative PCR System (Bio-Rad Laboratories, Hercules, CA) and detected with the iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories). A fraction (1/10th) of the reverse transcription reaction was used for 50 cycles of amplification at 95°C for 15 s and 60°C for 1 min. JEV forward and reverse primers were 5′-ATCCAACTCAACCGCAAGTC and 5′-TCTAAGATGGTGGGTTTCACG, respectively. The probe sequence (nt 5837 to 5856) was 5′-6FAMCATCTCTGAAATGGGGGCTA-BHQ1 (Integrated DNA Technologies, Coralville, IA). The forward and reverse primers for BHK-21 β-actin were 5′-ACTGGCATTGTGATGGACTC and 5′-CATGAGGTAGTCTGTCAGGTC, respectively. The probe sequence was 5′-HEX-CCAGCCAGGTCCAGACGCAGG-BHQ2 (Integrated DNA Technologies Inc.). The $2^{-\Delta\Delta C_T}$ method was used to analyze relative changes in JEV RNA levels from real-time quantitative RT-PCR experiments (Winer *et al*, 1999).

Immunoblotting
The experimental procedures used are described in detail elsewhere (Yun *et al*, 2003). One-twentieth

(4×10^5 cells) of the electroporated cells described above was plated on a 6-well plate. At the indicated time points after transfection, cells were lysed with 200 μl of 1× sample loading buffer (80 mM Tri-HCl [pH 6.8], 2.0% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1 M dithiothreitol [DTT], 0.2% bromophenol blue). One-tenth of the lysate was resolved by electrophoresis on SDS–12% polyacrylamide gels and electroblotted onto methanol-activated polyvinylidene difluoride membranes. JEV proteins were probed with a mouse hyperimmune antiserum (1:1000 dilution) specific for JEV, obtained from the American Type Culture Collection (VR-1259AF). JEV NS1 proteins were stained with a rabbit antiserum (1:1000 dilution) raised against the glutathione *S*-transferase-fused N-terminal 166 amino acids of JEV NS1 protein (nt 2478 to 2975). GAPDH proteins were detected with a rabbit antiserum (1:5000 dilution) from the LabFrontier, Seoul, Korea. The immunoreactive proteins were visualized by the binding of alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgGs (1:1000 dilution; Jackson ImmunoResearch Labs, West Grove, PA) and subsequent incubation with the substrates 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

Expression of heterologous reporter genes of various sizes

Immunofluorescence microscopy: For *egfp* expression, cells were seeded in a 4-well chamber slide for 36 to 48 h. After incubation, they were fixed in phosphate-buffered saline (PBS) containing 0.37% (*v*/*v*) formaldehyde and mounted in 0.2 ml of 80% glycerol. Images were generated on a Zeiss Axioskop confocal microscope equipped with a fluorescein filter using Bio-Rad MRC 1024 and LaserSharp software.

Flow cytometry analysis: Cells were trypsinized, washed once with PBS, and resuspended in 0.37% (*v*/*v*) formaldehyde in PBS, followed by analysis of *egfp* expression using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Dead cells were excluded by appropriate gating for forward and side light-scattering, and 1×10^4 viable cells were counted per sample.

Luciferase assay: Luciferase activity, with luciferin as substrate, was measured in cell lysates by using a commercial assay according to the manufacturer's recommendations (Promega, Madison, WI). Each experiment was performed in triplicate, and the mean values are presented.

β-Galactosidase assay: Cells were washed once with PBS, fixed with 0.05% (*v*/*v*) glutaraldehyde in PBS for 15 min at room temperature, and carefully washed three times with PBS. They were assessed for β-galactosidase activity by incubation in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS) and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Sigma-Aldrich, St. Louis, MO) at 37°C.

Generation of stable packaging cell lines for JEV replicon RNAs

Naïve BHK-21 cells were transfected with pSinRep19-based JEV structural protein expression vector RNAs, as specified in the text. After transfection, cells were seeded for ≈24 h, and the medium was replaced with fresh complete medium containing 10 μg/ml puromycin. Thereafter, cells were maintained in the presence of 1 μg/ml puromycin and either passaged or frozen, as were the parental BHK-21 cells.

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