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# Construction and applications of yellow fever virus replicons

Christopher T. Jones, Chinmay G. Patkar, Richard J. Kuhn*

*Department of Biological Sciences, Purdue University, 915 W. State Street, West Lafayette, IN 47907-2054, United States*

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**Abstract**

Subgenomic replicons of yellow fever virus (YFV) were constructed to allow expression of heterologous reporter genes in a replication-dependent manner. Expression of the antibiotic resistance gene neomycin phosphotransferase II (Neo) from one of these YFV replicons allowed selection of a stable population of cells (BHK-REP cells) in which the YFV replicon persistently replicated. BHK-REP cells were successfully used to *trans*-complement replication-defective YFV replicons harboring large internal deletions within either the NS1 or NS3 proteins. Although replicons with large deletions in either NS1 or NS3 were *trans*-complemented in BHK-REP, replicons that contained deletions of NS3 were *trans*-complemented at lower levels. In addition, replicons that retained the N-terminal protease domain of NS3 in *cis* were *trans*-complemented with higher efficiency than replicons in which both the protease and helicase domains of NS3 were deleted. To study packaging of YFV replicons, Sindbis replicons were constructed that expressed the YFV structural proteins in *trans*. Using these Sindbis replicons, both replication-competent and *trans*-complemented, replication-defective YFV replicons could be packaged into pseudo-infectious particles (PIPs). Although these results eliminate a potential role of either NS1 or full-length NS3 in *cis* for packaging and assembly of the flavivirus virion, they do not preclude the possibility that these proteins may act in *trans* during these processes.


*Keywords:* Flavivirus; Replicon; *Trans*-complementation; Genome packaging

## Introduction

The flavivirus genus of enveloped positive-sense RNA viruses constitutes over 70 members many of which cause significant disease in humans. The ~11 kb flavivirus genome encodes a single large polyprotein that is processed co- and posttranslationally by viral and host cell proteases to yield three structural proteins (C, prM, and E) from the N-terminus, followed by seven nonstructural (NS) proteins in the order NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Lindenbach and Rice, 2001). The structural proteins comprise the virion along with a host-derived membrane bilayer and a single copy of the viral RNA genome. The NS proteins, for which several enzymatic activities have been reported, are responsible for replication of the viral RNA genome. In addition, one or more of the NS proteins may also be involved in virus assembly. Recent studies of Kunjin virus (KUN) and yellow fever virus (YFV), the prototype flavivirus, have suggested that the processes of genome replication and virus assembly are coupled (Khromykh et al., 2001; Kummerer and Rice, 2002). Understanding the mechanism by which flaviviruses replicate their genomes and the apparent coupling of this process to virus assembly holds, at the very least, the promise of novel antiviral therapeutics. Insight into the replication of flaviviruses has been accelerated in recent years with the successful construction of full-length cDNA clones for several members of the flavivirus genus including YFV (Bredenbeek et al., 2003), dengue virus (DEN) (Kinney et al., 1997), West Nile virus (WNV) (Yamshchikov et al., 2001), KUN (Khromykh and Westaway, 1994), and tick-borne encephalitis virus (TBE) (Mandl et al., 1997), among others. Since infectious RNA can be generated in vitro from such full-length cDNA clones, a reverse genetics approach can be used to understand the effects of a wide range of genetic perturbations that can be introduced in the flavivirus genome.

* Corresponding author. Fax: +1 765 496 1189.
*E-mail address:* kuhnr@purdue.edu (R.J. Kuhn).

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Despite these molecular genetic advances, routine laboratory manipulations of many of these viruses remain difficult due to their highly pathogenic properties. An alternative approach to studying infectious, potentially dangerous, flaviviruses has been described for several flaviviruses, including WNV, DEN, and TBE, and involves the construction of noninfectious subgenomic replicons derived from the corresponding full-length parental cDNA clone (Gehrke et al., 2003; Pang et al., 2001; Shi et al., 2002). Replicons possess the complement of genetic elements necessary for autonomous amplification of their genomes in cells, but lack the structural genes necessary for the production of progeny virus particles. As a consequence, replicons are particularly well suited for studying aspects of viral genome replication independently of the process of virus assembly. In addition, amplification of replicon RNA in the cytoplasm of cells makes them excellent vectors for transient and even stable high level expression of heterologous genes. Replicons of KUN have been described for this purpose (Varnavski and Khromykh, 1999) and as the basis for potentially safe and effective vaccine vectors (Anraku et al., 2002; Harvey et al., 2003). Similarly, replicons of other flaviviruses, including DEN and YFV, have been described and are being pursued as potentially useful tools for the development of novel vaccines (Molenkamp et al., 2003; Pang et al., 2001). In contrast, the development of flavivirus replicons as a tool for understanding the processes of genome replication and virus assembly has been investigated only for KUN (Westaway et al., 2003). However, members of the flavivirus genus are quite diverse, as evidenced by the causation of a wide range of disease symptoms (from asymptomatic to hemorrhagic disease and death) and in differences of vector transmission (tick versus mosquito). Therefore, the development of replicons for studying other flavivirus members, preferably those that demonstrate significant phylogenetic divergence, is vital for discerning properties that are common to the genus flavivirus from those specific to a particular member.

We report the construction of subgenomic replicons derived from a full-length cDNA clone of the 17D strain of YFV. Using multiple strategies, various reporter genes including green fluorescence protein (GFP), luciferase, and neomycin phosphotransferase II (Neo) were expressed from these replicons in a RNA replication-dependent manner. Using a Neo-expressing YFV replicon, a stable population of cells (BHK-REP) was selected and used to *trans*-complement replication-defective YFV replicons harboring large in-frame deletions in either the NS1 or NS3 proteins (ΔNS1 and ΔNS3, respectively). Sindbis replicons that expressed the YFV structural proteins were also constructed and could be used to package YFV replicons into pseudo-infectious virus-like particles (PIPs). Furthermore, using these Sindbis replicons, we demonstrate packaging of both *trans*-complemented ΔNS1 and ΔNS3 YFV replicons in BHK-REP cells. The ability to package *trans*-complemented ΔNS3 replicons in our system was unexpected since recent findings suggested that full-length NS3 was required in *cis* for virus assembly of the distantly related flavivirus KUN (Liu et al., 2002). The replicons described in this report should facilitate further investigation of various aspects of the YFV lifecycle, including genome replication and virus assembly.

## Results

### *Construction and characterization of YFV replicons*

Replicons have been described for several flaviviruses including KUN, DEN, WNV, and YFV (Khromykh and Westaway, 1997; Molenkamp et al., 2003; Pang et al., 2001; Shi et al., 2002). The common feature of these replicons is a large internal in-frame deletion that corresponds to the removal of most of the structural protein coding region, which renders the resulting replicons noninfectious. In all cases, retention of a small portion of the structural protein coding sequence has been shown to be essential for autonomous replication. This sequence, which has been previously recognized as an RNA cyclization sequence (Hahn et al., 1987), is located within the coding sequence of the first 20 codons of the capsid protein. The importance of this region for genome replication was recently mapped by deletion analysis in YFV (Corver et al., 2003). It was determined that retention of the first 20 codons of the capsid protein, specifically nucleotides 147–166 (YFV numbering), conferred nearly wild-type levels of replication. This observation was consistent with earlier studies of KUN replicons, which indicated that retention of at least the first 20 codons of the capsid protein was necessary for the construction of a functional replicon (Khromykh and Westaway, 1997). Thus, for construction of a YFV replicon, an in-frame deletion of nearly the entire structural protein coding sequence was made, which retained the first 21 residues of the YFV capsid protein coding sequence. In addition, the C-terminal 24 residues of the E protein, which function as a signal sequence for the NS1 protein, were preserved. Replication of the resulting YFV replicon (YFRP in Fig. 1) was first evaluated by RT-PCR. Cytoplasmic RNA from BHK15 cells electroporated with YFV or YFRP in vitro-transcribed RNA was harvested 24 h post-electroporation and subjected to RT-PCR analysis (Fig. 2A). Both plus and minus strand-specific RT-PCR products were detected in YFV- and YFRP-transfected cells, which confirmed the ability of YFRP to autonomously replicate in cells.

Varnavski and Khromykh (1999) previously described a series of KUN-based replicons that were modified to allow expression of reporter genes in cells transfected with replicon RNA. Importantly, the level of reporter gene expression was found to correlate to levels of genome replication, suggesting that such replicons could serve as

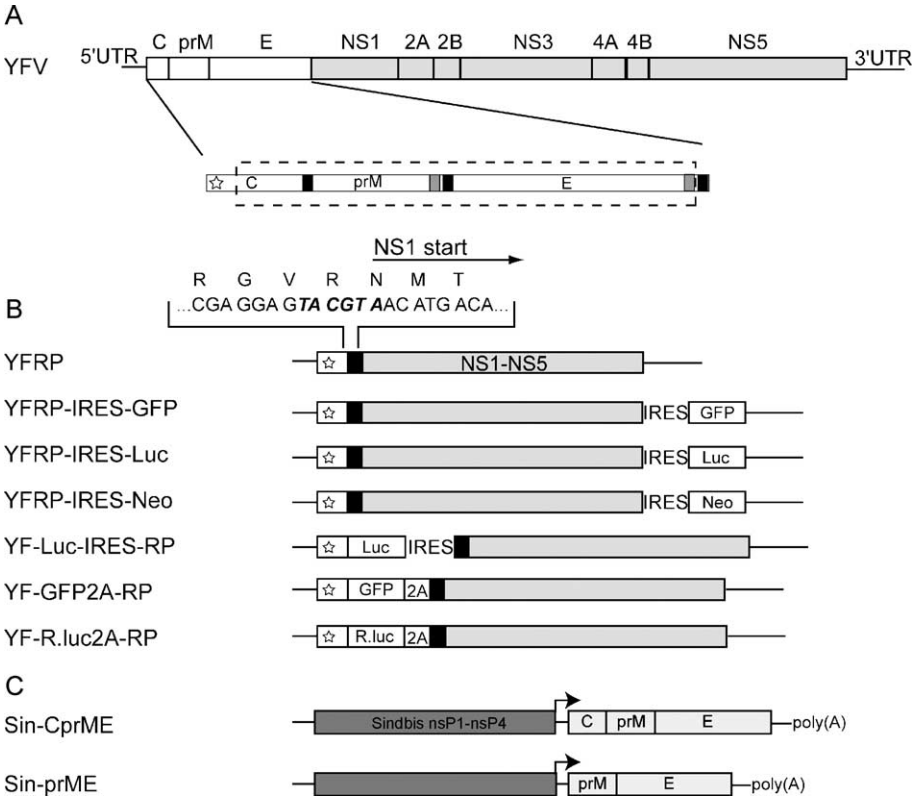


Fig. 1. Organization of the YFV genome and replicon constructs. (A) Schematic representation of the YFV genome with open boxes denoting regions coding for the structural (white) and nonstructural (light gray) proteins that are translated as a polyprotein. The 5′ and 3′ untranslated regions (UTRs) are also depicted. The structural protein coding region is shown is greater detail. The star indicates the presence of the RNA cyclization sequence that is located within the coding sequence for the capsid protein. Black and dark gray boxes indicate signal and stop transfer sequences, respectively. Dotted box indicates the region of the structural protein coding sequence that was deleted to construct YFV replicons. (B) Schematic representations of YFV replicon constructs. The sequence at the junction between the retained capsid and the NS1 signal sequence is shown, with the unique *Sna*BI restriction site that was introduced at the junction indicated in bold italics. Light gray box denotes YFV nonstructural protein coding sequence. Black box in YFV replicon constructs indicates NS1 signal sequence. Lines indicate 5′ and 3′ UTRs. IRES, encephalomyocarditis virus internal ribosome entry site; GFP, green fluorescence protein; Luc, firefly luciferase; R.luc, *Renilla* luciferase; Neo, neomycin phosphotransferase II; 2A, 17 amino acid residue autoproteolytic peptide from foot and mouth disease virus. (C) Schematic representation of the SINV replicon constructs that were used to express YFV structural proteins. The coding sequences for the SINV nonstructural proteins nsP1–nsP4 are denoted by a dark gray open box. The YFV structural proteins (white open box) are expressed from the SINV subgenomic promoter (black arrow).

powerful tools for dissecting various aspects of flavivirus genome replication. Indeed, replicons of WNV have been recently described that utilized an inserted *Renilla* luciferase (R.luc) reporter gene to determine the effects of mutations and deletions within the 3′ NTR on genome replication (Lo et al., 2003). We sought to develop an analogous system based on YFRP that could be used to study various aspects of YFV, in which detection of reporter gene expression could be used as a fast and easy alternative to more laborious methods of assessing genome replication.

At least three different strategies have been described to allow expression of reporter genes from flavivirus replicons. Dicistronic replicons were first reported for KUN in which an encephalomyocarditis virus internal ribosome entry site (EMCV IRES) was used to direct internal translational initiation of a reporter gene that was inserted into an upstream region of the 3′ UTR (Khromykh and Westaway, 1997). We constructed a set of similar replicons, each derived from YFRP (Fig. 1), that allowed expression of GFP (YFRP-IRESGFP in Fig. 1), Luc (YFRP-IRESLuc in Fig. 1), or Neo (YFRP-IRESNeo in Fig. 1). A second strategy involved the in-frame fusion of the reporter gene following the 21 codons encoding the N-terminus of the capsid protein that were retained in YFRP. To ensure proper processing of the reporter gene from the signal sequence of NS1, two different approaches were pursued. In the first approach, the EMCV IRES was inserted immediately after the stop codon of the reporter gene allowing internal translational initiation at the start of the NS1 signal sequence (YF-LucIRES-RP in Fig. 1). A second approach involved insertion of the foot and mouth disease virus 2A coding sequence (2A) between the C-terminus of the reporter gene and the NS1 signal sequence (YF-GFP2A-RP and YF-R.luc2A-RP, Fig. 1). The 2A protein is a 17 amino acid residue autoproteolytic peptide that has been shown to mediate efficient cleavage of C-terminally fused heterologous protein sequences (Percy et al., 1994). YFV replicons lacking the 2A protein sequence, between the reporter gene and the NS1 signal sequence,

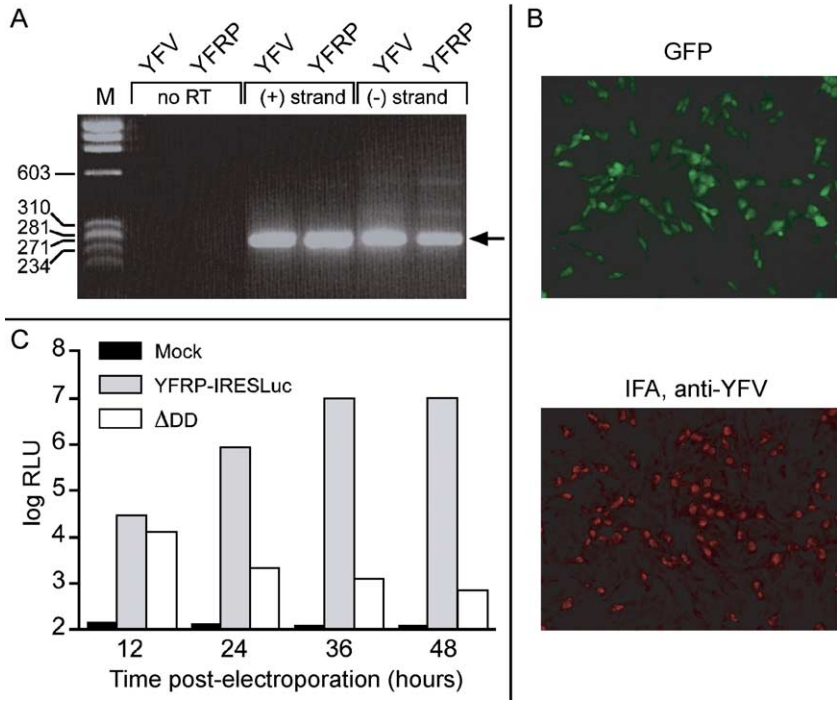


Fig. 2. Replication of YFV replicons. (A) Detection of RNA replication by RT-PCR. Cytoplasmic RNA isolated 24 h post-electroporation from BHK cells electroporated with in vitro-transcribed YFV or YFRP RNA was subjected to standard RT-PCR to detect the accumulation of plus (+) and minus (−) strand. Arrow denotes the expected size for the RT-PCR product (~250 bp). M, φX174 DNA-*Hae*III-digested DNA marker (size of fragments shown in base pairs). (B) Replication of YF-GFP2A-RP. BHK cells electroporated with in vitro-transcribed YF-GFP2A-RP RNA were visualized by either GFP fluorescence or by the expression of YFV-specific proteins visualized by immunofluorescence assay (IFA) using an anti-YFV serum antibody at 24 h post-electroporation. The same field of cells is shown for both images. (C) Replication of YFRP-IRESLuc. Firefly luciferase activity was detected in cytoplasmic extracts prepared from BHK cells electroporated with in vitro-transcribed YFRP-IRESLuc or replication-incompetent YFRP-IRESLuc (ΔDD) RNA at 12, 24, 36, and 48 h post-electroporation. Firefly luciferase activity expressed in Raw Luciferase Units (RLU).

were found to be deficient in reporter gene expression, and this was the result of a severe defect in genome replication (data not shown). In contrast, reporter gene expression was readily observed from either dicistronic (IRES containing) or monocistronic (2A protein containing) YFV replicons (see below).

GFP was readily detectable in BHK cells by fluorescence microscopy as early as 24 h after transfection with either YFRP-IRESGFP or YF-GFP2A-RP replicon RNAs (Fig. 2B, top image). Moreover, replication was confirmed indirectly by the accumulation of YFV-specific proteins that were detected by immunofluorescence staining of transfected cells using a mouse anti-YFV serum (Fig. 2B, lower image). However, at equivalent time points, lower levels of GFP fluorescence from YFRP-IRESGFP than from YF-GFP2A-RP replicons were consistently observed (data not shown). One possibility was that translation of the GFP protein from the IRES might be less efficient than the cap-dependent translation of GFP in YF-GFP2A-RP. It was also possible that insertion of the IRES-GFP cassette into the 3′ UTR of YFRP might adversely effect RNA replication, resulting in an overall decrease in the level of GFP expression. This latter possibility is supported by similar observations reported previously for KUN and WNV replicons (Khromykh and Westaway, 1997; Shi et al., 2002).

The construction and characterization of WNV replicons expressing R.luc have been previously described, which allowed differentiation of translated input replicon RNA from translation of replicon RNA resulting from genome replication (Lo et al., 2003). The highly sensitive and robust nature of luciferase as a reporter gene and its demonstrated utility in WNV prompted us to construct an analogous set of YFV replicons. YFV replicons were constructed that allowed expression of either Luc or R.luc. YFV replicons that expressed Luc were dicistronic, utilizing an IRES to initiate either translation of Luc (YFRP-IRESLuc) or the YFRP replication proteins (YF-Luc-IRES-RP). A monocistronic YFV replicon, analogous to YF-GFP2A-RP, was used to express R.luc (YF-R.luc2A-RP). To test these replicons, BHK15 cells were electroporated with in vitro-transcribed replicon RNA and subsequently assayed for luciferase activity at 12, 24, 36, and 48 h post-electroporation (Fig. 2C). The level of Luc activity in BHK15 cells electroporated with YFRP-IRESLuc RNA increased significantly from 12 to 36 h before reaching a maximum value at 36 h post-electroporation. To demonstrate that the observed time-dependent increase in Luc activity was the result of replicon genome replication, a replication-defective YFRP-IRESLuc replicon was constructed. This was done by deletion of the conserved GDD motif within the RNA-dependent RNA polymerase (RdRP) domain of flavivirus NS5. A similar

mutation of the GDD motif has been previously shown to have a lethal effect on flavivirus RNA replication (Khromykh et al., 1998a, 1998b). In contrast to YFRP-IRESLuc RNA, BHK15 cells electroporated with YFRP-IRESLuc(ΔDD) RNA demonstrated a time-dependent decrease in Luc activity. Similarly, a time-dependent increase in R.luc activity was observed in cells transfected with YF-R.luc2A-RP replicon RNA but not in cells transfected with YF-R.luc2A-RP(DD) replicon RNA (data not shown).

To confirm that the time-dependent increase in reporter gene activity was the result of RNA replication of the replicon in transfected cells, we utilized a SYBR Green I dye-based comparative Ct method quantitative RT-PCR assay to determine the relative amounts of replicon RNA in cells transfected with either wt YF-R.luc2A-RP or YF-R.luc2A-RP(ΔDD) replicon RNAs (data not shown). Transfection of cells with equivalent amounts of either wt or ΔDD replicon in vitro transcribed RNA resulted in similar levels of R.luc activity at 6 h post-transfection, which indicated that both replicon RNAs were transfected into cells with comparable efficiency. However, as early as 12 h post-transfection, a 10- to 20-fold reduction in the level of ΔDD replicon RNA was detected relative to the level of wt replicon RNA in transfected cells. The observed reduction in the relative levels of replicon RNA at 12 h post-transfection corresponded to a 20- to 30-fold decrease in R.luc activity of cells transfected with ΔDD replicon RNA compared to wt replicon RNA-transfected cells. Moreover, at 36 h post-transfection, a 300- to 400-fold increase in the level of wt replicon RNA was detected relative to the levels of wt replicon observed at 12 h post-transfection. In contrast, the level of ΔDD replicon RNA decreased from 12 to 36 h post-transfection, as did the level of R.luc activity detectable in ΔDD replicon RNA-transfected cells. These results indicated that the time-dependent increase in R.luc activity observed in wt YF-R.luc2A-RP-transfected cells was indeed the result replicon RNA replication.

### *Persistent replication of YFV replicons in BHK cells*

The establishment of a population of cells in which flavivirus replicons persistently replicate under antibiotic selection has been previously described (Khromykh and Westaway, 1997; Pang et al., 2001; Shi et al., 2002). Such cells have been used in complementation experiments to provide functional NS proteins in *trans* to replication-defective KUN replicons that lacked one or more functional NS proteins. *Trans*-complementation analysis represents a powerful genetic tool for dissecting the function of viral proteins. In KUN, systematic *trans*-complementation analysis across nearly the entire nonstructural coding region revealed the intriguing possibility that certain nonstructural proteins are necessary in *cis* for both genome replication and virus assembly (Khromykh et al., 2000; Liu et al., 2002). Unfortunately, a comprehensive *trans*-complementation analysis of the sort described for KUN has thus far not been reported for any other member of the flavivirus genus. Yet, a comprehensive *trans*-complementation analysis of other members of the flavivirus genus would be necessary to distinguish features of the flavivirus lifecycle that can be generalized for all flaviviruses from those features that may be unique to certain members. Therefore, we sought to develop a replicon-based system that could be used to perform a systematic *trans*-complementation analysis of YFV.

To establish a population of cells that could be used for *trans*-complementation studies of YFV, BHK cells were electroporated with YFRP-IRESNeo RNA, allowed to recover for a period of 12 h, and then subjected to antibiotic selection with media containing G418. Nearly complete cell death of mock-electroporated cells occurred within 5–7 days, while BHK cells transfected with YFRP-IRESNeo replicon persisted after subsequent multiple passages under G418 selection. IFA using anti-YFV antibodies indicated that after multiple passages between 80% and 90% of the resulting cells expressed detectable levels of YFV-specific proteins (data not shown). In addition, the resulting cell population (BHK-REP) demonstrated little or no cytopathic effects with growth rates similar to parental BHK cells. Interestingly, transfection of BHK-REP cells with infectious full-length YFV RNA failed to produce plaques, despite nearly identical levels of virus production in these cells compared to BHK cells (data not shown). The apparent noncytopathic nature of YFRP-IRESNeo is consistent with analogous replicons of KUN and WNV that have been previously described (Khromykh et al., 1998a, 1998b; Shi et al., 2002), and may represent a general adaptation mechanism by the replicon to support persistent flavivirus replication.

### *Trans-complementation of NS1 and NS3*

To determine whether BHK-REP cells could support *trans*-complementation, replication-defective replicons were constructed by the introduction of large internal in-frame deletions within the NS proteins. NS1 is an ~350 amino acid residue protein that is translocated into the lumen of the endoplasmic reticulum during translation of the flavivirus polyprotein (Falgout et al., 1989). NS1 is found both secreted from infected cells and in association with flavivirus replication complexes (Flamand et al., 1999; Westaway et al., 1997). Although an enzymatic activity has not been described for NS1, a role in early minus strand synthesis has been suggested, possibly through a mechanism that involves recruitment of replication components to sites of RNA replication via interactions with other nonstructural protein including NS4A (Lindenbach and Rice, 1999). NS3 is a 620 amino acid residue multidomain protein with several associated enzymatic activities including protease (N-terminal ~170 residues) (Chambers et al., 1990), helicase, and nucleoside triphosphatase (sequences

C-terminal of protease) (Li et al., 1999). NS3 in association with NS2B functions as the viral protease (Chambers et al., 1991).

*Trans*-complementation of NS1 has been demonstrated previously for both YFV (Lindenbach and Rice, 1997) and KUN (Khromykh et al., 1999), and thus serves as a positive control in the *trans*-complementation studies described below. However, *trans*-complementation of NS3 has been reported only for KUN (Khromykh et al., 2000). It was found that for *trans*-complementation to occur, KUN replicons required the first 178 residues of NS3 (entire protease domain) in *cis*, since large deletions encompassing both the protease and helicase domains of NS3 could not be *trans*-complemented. Interestingly, while KUN replicons containing large C-terminal deletions of the helicase domain of NS3 were *trans*-complemented, the resulting replicons were defective in virus assembly, suggesting a possible role for full-length NS3 protein in *cis* for virus assembly (Liu et al., 2002).

A 200 amino acid residue deletion within NS1 (ΔNS1) was introduced into YF-R.luc2A-RP by in-frame ligation that resulted in retention of the first 72 and the last 81 codons of NS1. Construction of a larger in-frame deletion within NS1 (260 amino acid residues) has been previously described and was found to be lethal for YFV genome replication, yet was readily *trans*-complemented. Two additional YF-R.luc2A-RP replicons were also constructed, and these contained large in-frame deletions within NS3. The first of these replicons, ΔNS3.1, contained a deletion of ~390 amino acid residues within the C-terminal helicase domain of NS3, in which the first 178 residues (protease domain of NS3) and the last 53 C-terminal residues of NS3 were retained. *Trans*-complementation of a KUN replicon harboring a nearly identical deletion of NS3 has been described previously (see results of repdNS1.1/3.3 *trans*-complementation in Liu et al., 2002). To test whether the protease domain of NS3 was required in *cis* for *trans*-complementation of YFV replicons, a larger deletion within NS3 was constructed (ΔNS3.2), which deleted all but the first eight residues of the protease domain and the last 53 C-terminal residues of the helicase domain (~90% of NS3 deleted). As mentioned above, KUN replicon containing a deletion of both the protease and helicase domains of NS3 could not be *trans*-complemented (see results of repdNS1.1/NS3.6 *trans*-complementation in Liu et al., 2002).

To test *trans*-complementation of ΔNS1 and ΔNS3 YF-R.luc2A-RP replicons, BHK-REP cells were electroporated with either wild type (wt), ΔNS1, or ΔNS3 YF-R.luc2A-RP replicon RNAs and subsequently assayed for R.luc activity from 12 to 72 h post-electroporation (Fig. 3). The level of R.luc activity in BHK-REP cells electroporated with either wt, ΔNS1, or ΔNS3 replicons exhibited a time-dependent increase in R.luc activity that was consistent with successful *trans*-complementation of these replicons in BHK-REP cells. Indeed, for BHK-REP cells electroporated with ΔNS1 replicon, the levels of R.luc activity observed were nearly equivalent to BHK-REP cells electroporated with wt YF-R.luc2A-RP replicon. However, *trans*-complementation of NS3 in BHK-REP cells occurred with somewhat lower efficiency than NS1 and failed to attain wild-type or ΔNS1 levels of R.luc activity at any of the observed time points post-electroporation. In addition, although successful *trans*-complementation was observed for both ΔNS3 replicons, significantly higher levels of *trans*-complementation were observed for NS3.1 than for NS3.2.

### *Packaging of replicons*

Liu et al. (2002) previously reported that despite the ability of certain replication-defective KUN RNAs to be *trans*-complemented, KUN RNAs lacking full-length NS3 could not be packaged into virus particles by structural

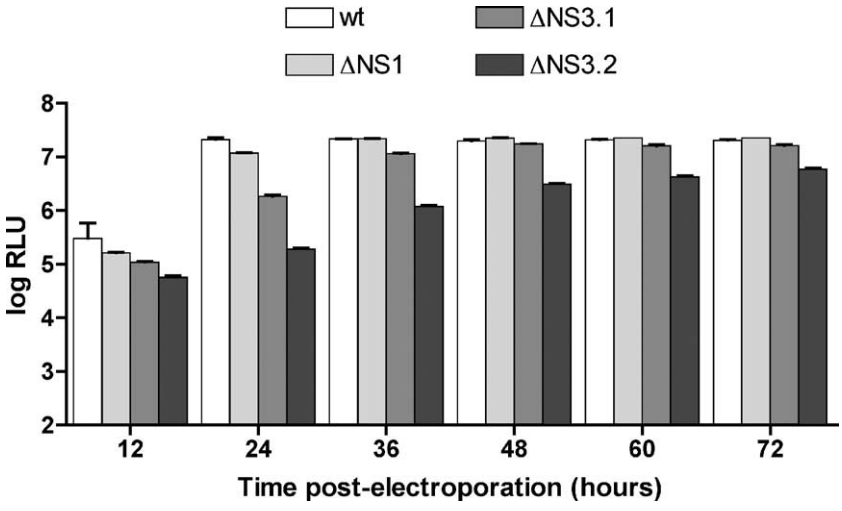


Fig. 3. *Trans*-complementation of NS1 and NS3. BHK-REP cells were used to *trans*-complement replication-incompetent YF-R.luc2A-RP replicons ΔNS1, ΔNS3.1, and ΔNS3.2. BHK-REP cells electroporated with in vitro-transcribed wt, ΔNS1, ΔNS3.1, and ΔNS3.2 YF-R.luc2A-RP RNAs were assayed for R.luc activity at 12, 24, 36, 48, 60, and 72 h post-electroporation. R.luc activity expressed in Raw Light Units (RLU).

proteins provided either in *cis* or in *trans*. As a result of this observation, it was suggested that full-length NS3 is required in *cis* for the process of flavivirus assembly. To investigate this intriguing possibility further, a system was devised to allow packaging of replicon RNA into PIPs by expressing the YFV structural proteins in *trans*.

Encapsidation of flavivirus replicon RNA into PIPs was previously demonstrated using an alphavirus-based replicon to express the flavivirus structural proteins in *trans* in cells initially transfected with a flavivirus replicon (Khromykh et al., 1998a, 1998b). Alphaviruses are members of the *Togaviridae* family of positive-sense RNA viruses and utilize a subgenomic promoter to drive expression of their structural proteins (Strauss and Strauss, 1994). Replicons of several alphaviruses have been reported in which heterologous proteins are expressed in place of the structural proteins using the subgenomic promoter (Frolov et al., 1996). Thus, a full-length cDNA plasmid of Sindbis virus (SINV), the prototype alphavirus, was used to construct SINV replicons that express the structural proteins of YFV via the SINV subgenomic promoter.

The sequence encoding either CprME or prME of YFV was inserted in place of the SINV structural proteins, immediately downstream of the subgenomic promoter (Sin-CprME and Sin-prME, respectively, in Fig. 1). The replicon Sin-CprME includes the entire structural polyprotein of YFV, while Sin-prME excludes the first 101 residues of the capsid protein. The hydrophobic C-terminus of the capsid protein (amino acid residues 102–121) was retained at the N-terminus of prME in Sin-prME to ensure proper translocation and processing of the prME polyprotein. IFA using an anti-YFV E protein-specific antibody on BHK cells transfected with either Sin-CprME or Sin-prME replicon RNAs revealed high levels of E protein expression that were readily detectable as early as 10 h post-transfection (Fig. 4A).

We next determined whether SINV replicons could be used to package YFV replicons into PIPs. BHK cells were first electroporated with either YF-GFP2A-RP or YF-R.luc2A-RP replicons, followed by a second electroporation with either Sin-CprME or Sin-prME replicons. To allow sufficient amplification of the initially transfected YFV replicon RNAs, a delay of 24 h was used prior to electroporation of cells with SINV replicons. Following the second electroporation, media that contained PIPs were harvested and used to infect naive BHK cells. Infection of BHK cells with PIPs containing YF-GFP2A-RP yielded readily observable GFP fluorescence at 24 h postinfection (Fig. 4B). In contrast, infection of BHK cells with PIPs that were generated by using Sin-prME to package YF-GFP2A-RP failed to produce GFP fluorescent BHK cells. The titer of YF-GFP2A-RP-containing PIPs was determined by count-

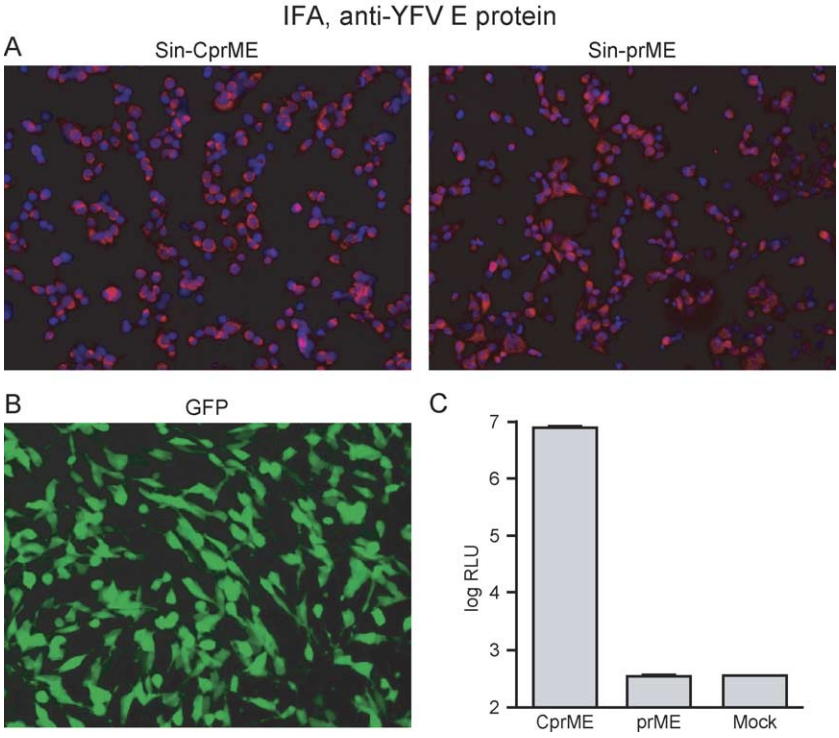


Fig. 4. Expression of YFV structural proteins and packaging of YFV replicons into pseudo-infectious particles (PIPs). (A) IFA with anti-YFV E antibody at 12 h post-transfection of BHK cells electroporated with in vitro-transcribed SINV replicon RNAs that express the YFV structural polyprotein with (Sin-CprME) or without (Sin-prME) the YFV C protein. (B) Production of YF-GFP2A-RP and (C) YF-R.luc2A-RP PIPs by double electroporation (see Materials and methods). GFP fluorescence of BHK cells 24 h postinfection with YF-GFP2A-RP PIPs (~1 × $10^6$ PIPs/ml). R.luc activity of BHK cells 24 h postinfection with YF-R.luc2A-RP PIPs generated by double electroporation with Sin-CprME (CprME) or Sin-prME (prME) replicon RNAs. R.luc activity expressed in Raw Light Units (RLU). Mock values of R.luc activity were obtained from uninfected BHK cells.

ing the number of GFP fluorescent cells within a defined area of infected BHK cells (see Materials and methods). Titers of YF-GFP2A-RP PIPs routinely approached ~1–2 × $10^6$ PIPs per ml, and this was consistent with the level of packaging previously reported for KUN replicons (Khromykh et al., 1998a, 1998b). Packaging experiments were also performed using YF-R.luc2A-RP and Sin-CprME or Sin-prME. The results of these experiments are shown in Fig. 4C and confirm the essential role of the capsid protein in packaging, since the use of Sin-prME failed produce detectable levels of YF-R.luc2A-RP-containing PIPs (Fig. 4C).

*Packaging of trans-complemented replicons*

We next determined whether Sin-CprME could be used in BHK-REP cells to package *trans*-complemented YFV replicons into PIPs. BHK-REP cells were electroporated with wt, ΔNS1, or ΔNS3 YF-R.luc2A-RP replicon RNAs and then assayed for R.luc activity 36 h post-electroporation to confirm successful *trans*-complementation (Fig. 5, white bars). At 36 h, BHK-REP cells initially transfected with YFV replicons were then electroporated with Sin-CprME to initiate packaging of *trans*-complemented replicon RNAs into PIPs. At 12 h following the second electroporation, media-containing PIPs were harvested and used to infect naive BHK-REP cells. At 36 h postinfection, the infected BHK-REP cells were then assayed for R.luc activity (Fig. 5, gray bars). As expected, packaging of wt and ΔNS1 replicons in BHK-REP cells was readily detected. Unexpectedly, packaging of ΔNS3.1 replicon was also detected, but at lower levels than either wt or ΔNS1 replicons in BHK-REP cells. In addition, packaging of ΔNS3.2 replicon was detected in BHK-REP cells, although packaging of this replicon occurred at significantly lower levels than ΔNS3.1. The packaging of replication-defective replicons was not likely to be the result of a recombination event with the YFRP-IRESNeo present in BHK-REP cells, since infection of BHK cells with replication-defective PIPs failed to result in levels of R.luc activity that were above mock-infected BHK cells (data not shown).

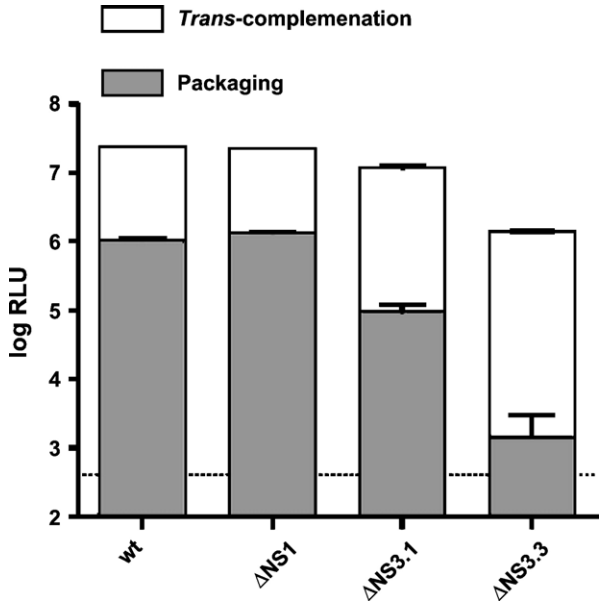


Fig. 5. Packaging of *trans*-complemented YFV replicon RNAs. BHK-REP cells were electroporated with replication-competent (wt) or replication-incompetent (ΔNS1, ΔNS3.1, and ΔNS3.2) YF-R.luc2A-RP replicon RNAs. The level of *trans*-complementation at 36 h post-electroporation for each replicon is shown in white bars. At 36 h post-electroporation, *trans*-complemented RNAs were then packaged into PIPs by electroporation with Sin-CprME replicon RNA. At 12 h following the second electroporation, PIPs were harvested and used to infect naïve BHK-REP cells. At 36 h postinfection, cells were assayed for R.luc activity (gray bars). R.luc activity expressed in Raw Light Units (RLU). Dotted line denotes mock value of R.luc activity that was obtained from extracts of uninfected BHK-REP cells.

## Discussion

We have constructed a series of YFV-based replicons that encode various reporter genes, the expression of which was found to be dependent on the amplification of input replicon RNA via RNA replication. In addition, a population of cells containing a persistently replicating YFV replicon RNA under G418 selection was used to *trans*-complement replication-defective replicons lacking functional NS1 or NS3 proteins. In general, ΔNS3 replicons were less efficiently *trans*-complemented than ΔNS1 replicons in BHK-REP cells. It is possible that the introduced deletion in NS3 might effect processing and translocation of the polyprotein in such a way that *trans*-complementation occurs less efficiently. Another possibility is that certain protein sequences within NS3 that are required for flavivirus replication act more efficiently in *cis* than in *trans*. In a model proposed for the formation of the flavivirus replication complex, Khromykh et al. (1999) suggested that interactions between NS3 and nascent NS5, which occur in *cis* during translation of the viral polyprotein, are involved in the recruitment of positive strand viral RNA template to membrane sites of RNA replication. Kapoor et al. (1995) found that the region of NS3 that interacts with NS5 lies within its C-terminal helicase domain. Since nearly the entire helicase domain has been deleted in ΔNS3.1, the presumed interaction with NS5 is prevented in *cis* and this might result in the observed decrease in *trans*-complementation levels between ΔNS3.1 and ΔNS1 replicons in BHK-REP cells. Liu et al. (2002) previously described *trans*-complementation analysis of KUN replicons containing large deletions within NS3. It was found that *trans*-complementation required the presence of the protease domain of NS3 in *cis*, since only deletions in the C-terminal helicase domain of NS3 could be *trans*-complemented. In contrast, we have found that the protease domain of NS3 is not absolutely required in *cis* for *trans*-complementation of YFV replicons. Indeed, *trans*-complementation was

observed for a large deletion that included both the protease and the helicase domains of NS3. However, significantly lower levels of *trans*-complementation were observed in the absence of the protease domain in *cis*, suggesting a possible role, but not an absolute requirement, for this region of NS3 in *cis*. One possibility is that the protease domain of NS3 may initially function in *cis*, perhaps mediating its own cleavage during or soon after translation of the flavivirus polyprotein. This initial *cis* cleavage of the polyprotein, while apparently not absolutely required for *trans*-complementation of the ΔNS3.2 replicon, may facilitate subsequent polyprotein processing events that are required for the observed, more efficient *trans*-complementation of the ΔNS3.1 replicon.

To facilitate our future investigations of virus assembly, SINV replicons expressing the YFV structural proteins were constructed and used to package YFV replicons into PIPs. The presence of the capsid protein was found to be absolutely required for packaging. Moreover, the ability to efficiently package YFV replicons indicates that any potential RNA sequences within YFV genome, which might function as packaging signals, are not contained within the YFV structural protein coding sequence. In addition, the demonstration that replication-defective YFV replicons could be packaged into PIPs, when these replicons were *trans*-complemented in BHK-REP cells, indicates that neither full-length NS1 nor NS3 protein was required in *cis* for YFV virion assembly. The ability of ΔNS3 YFV replicon RNAs to be packaged into PIPs reported here contradicts the purported necessity of full-length NS3 in *cis* for KUN virus assembly (Liu et al., 2002). It should be noted that phylogenetically YFV and KUN are quite distantly related mosquito-borne flaviviruses and thus it is conceivable that the mechanisms by which these two viruses carry out the process of assembly may have diverged such that KUN but not YFV requires NS3 or sequences within its coding region in *cis* for virus assembly. However, a possible role of NS3 (presumably in *trans*) in YFV virus assembly was recently described (Kummerer and Rice, 2002). Interestingly, mutations in NS2A that blocked infectious particle assembly and release could be compensated for by second-site mutations in the helicase domain of NS3. Although the mechanism by which NS3 (and NS2A) might be involved in virus assembly remains obscure, the involvement of the NS proteins in the process of flavivirus assembly seems certain.

The replicons described here have broad applications for future investigation of various aspects of YFV genome replication and virus assembly. In particular, further *trans*-complementation analysis of the remaining YFV nonstructural proteins, which is currently underway, should enable a better understanding of the role of these proteins in the process of virus assembly. In addition, the ability to package YFV replicons with structural proteins provided in *trans* should facilitate understanding the nature of the apparent coupling between RNA replication and virus assembly and the presumably related mechanism by which the flavivirus genome RNA is specifically packaged into virus particles.

## Materials and methods

### *Cell culture*

BHK15 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS). BHK-REP cells were maintained in Dulbecco's modification of MEM (DMEM) containing 10% FBS and 0.8 mg/ml G418 (Invitrogen). All cells were grown in 37 °C incubators in the presence of 5% $CO_2$.

### *Replicon constructs*

The following replicon cDNA plasmids were verified with restriction mapping or DNA sequencing analysis. Replicons of YFV were derived from pACNR/FLYF, a full-length cDNA clone of YFV 17D kindly provided by C. M. Rice (Bredenbeek et al., 2003). To facilitate construction of the YFV replicon (YFRP) cDNA, a unique *Bsp*EI restriction site was introduced into the non-YFV-derived DNA sequence of pACNR/FLYF using standard overlapping PCR techniques. All replicons (Fig. 1) were generated by PCR-directed mutagenesis using appropriately designed sets of primers and standard techniques. YFRP was constructed by deletion of nucleotides 179–2382 using PCR and a three-fragment in-frame ligation that resulted in the introduction of a unique *Sna*BI restriction site. Deletion of nucleotides 179–2382 corresponds to a deletion of amino acid residue 22 of the capsid protein to amino acid 469 of the E protein, which immediately precedes the signal sequence for NS1.

### *YF-GFP2A-RP and YF-R.Luc2A-RP*

The GFP2A sequence was PCR amplified from the plasmid C20DX/GFP/2Arep, kindly provided by Varnavski and Khromykh (1999), using forward and reverse primers that incorporated a *Sna*BI restriction site and was then cloned into the *Sna*BI restriction site of YFRP. To construct YF-R.luc2A-RP, *Renilla* luciferase (R.luc) was PCR amplified from pRL-CMV (Promega Corp., Madison, WI) using a forward primer that incorporated a *Sna*BI restriction site and a reverse primer that contained a *Bgl*II restriction site. The FMDV 2A sequence was PCR amplified from YF-GFP2A-RP (described above) using a forward primer that corresponded to the start of the FMDV 2A sequence and that included a *Bgl*II restriction site and a reverse primer that spanned the downstream *Kpn*I restric-

tion site in YF-GFP2A-RP. The resultant PCR products were then inserted into YFRP that was digested with *Sna*BI and *Kpn*I restriction enzymes, and the resulting plasmid was designated YF-R.luc2A-RP.

*YFRP-IRES-GFP, YFRP-IRES-Luc, and YFRP-IRES-Neo*

To construct the following dicistronic replicons, which utilize an EMCV IRES to direct cap-independent translation of a reporter gene, a unique *Nsi*I restriction site was introduced at the start of the 3′ NTR immediately following the UGA termination codon of NS5 in YFRP using standard overlapping PCR mutagenesis to create YFRP-*Nsi*I. To construct YFRP-IRES-GFP, an IRES-GFP DNA cassette was PCR amplified from pIRES2-EGFP (Clontech, San Jose, CA) using forward and reverse primers that each included an *Nsi*I restriction site and was then cloned into the *Nsi*I restriction site of YFRP-*Nsi*I. To construct YFRP-IRES-Luc, the EMCV IRES was amplified by PCR from pIRES2-EGFP using a forward primer containing an *Nsi*I restriction site and a reverse primer that incorporated an *Nco*I restriction site. Fire-fly luciferase (Luc) was amplified by PCR from pGL-Basic (Promega Corp.) using a forward primer containing an *Nco*I restriction site and a reverse primer that incorporated an *Nsi*I restriction site. The resultant PCR products were then inserted into the *Nsi*I restriction site of YFRP-*Nsi*I by standard techniques. YFRP-IRES-Neo was constructed in a similar fashion as YFRP-IRES-Luc. The neomycin phosphotransferase II gene (Neo) was obtained from pIRES2-EGFP by PCR.

*YF-IRES-RP and YF-Luc-IRES-RP*

To construct YF-IRES-RP, EMCV IRES was PCR amplified from pIRES2-EGFP using a forward primer that contained a *Sna*BI restriction site and a reverse primer that incorporated an *Nco*I restriction site. A DNA fragment from the start of the NS1 signal sequence to a downstream *Mlu*I restriction site in YFRP was PCR amplified using a forward primer that incorporated an *Nco*I site and a reverse primer that spanned the *Mlu*I restriction site in YFRP. The resultant PCR products were then inserted into YFRP treated with *Sna*BI and *Mlu*I restriction enzymes using standard techniques. To construct YF-Luc-IRES-RP, Luc was PCR amplified from pGL-Basic using forward primer and reverse primers that included a *SnaB*I restriction site and then inserted into the *SnaB*I restriction site of YF-IRES-RP.

*YF-R.luc2A-RP ΔNS1 and ΔNS3 replicons*

An internal deletion of 600 nts resulting in an in-frame deletion of 200 codons within the NS1 protein coding sequence was introduced by ligation of T4 DNA polymerase (New England Biolabs, Beverly, MA) blunt-ended YF-R.luc2A-RP vector digested with *Bss*HII and *Kpn*I restriction enzymes. An in-frame C-terminal deletion of 393 codons within the helicase domain of NS3 (ΔNS3.1) was introduced into YF-R.luc2A-RP by PCR techniques, which resulted in the creation of a novel in-frame *Pst*I restriction site at the site of ligation with the addition of novel Leu and Gln residues at the fusion site. ΔNS3.1 retains the first 177 codons of the N-terminal protease domain and last 53 codons of the C-terminal helicase domain of NS3. Using a similar strategy, ΔNS3.2 was constructed, which resulted in an in-frame deletion of 563 codons with retention of the first eight codons of the N-terminal protease domain and last 53 codons of the C-terminal helicase domain of NS3.

*Sin-CprME and Sin-prME replicons*

SINV-based replicons that expressed the YFV structural proteins were constructed from pToto64, a full-length cDNA clone of SINV that has been previously described (Owen and Kuhn, 1996). Using the appropriate set of primers, the coding sequence for the entire YFV structural protein sequence (CprME, nts 119–2351, YFV numbering) was obtained by PCR amplification from a full-length cDNA plasmid of YFV derived from pACNR/FLYF. Using standard overlapping PCR techniques, the start codon of CprME was inserted immediately downstream of the SINV subgenomic promoter. The 3′ end of the CprME coding sequence was fused in-frame to the UGA codon that normally terminates translation of the SINV structural proteins. The final SIN-CprME replicon results in a complete exchange of the SINV structural protein coding sequence with that from YFV. Likewise, Sin-prME, a SINV replicon that expresses YFV prME (nts 423–2351, YFV numbering) was constructed using overlapping PCR techniques.

*In vitro transcription and transfections*

RNA transcripts of YFV and SINV replicons were generated by in vitro transcription using SP6 RNA polymerase (Amersham Biosciences, Piscataway, NJ) from DNA templates linearized by digestion with *Xho*I and *Sac*I restriction enzymes, respectively, and that were subsequently purified using GFX columns (Amersham Biosciences). For RT-PCR experiments, following transcription, reactions were treated with 1 unit (per 40 μl transcription reaction) of RNase-free DNase (Ambion Inc., Austin, TX) for 30 min at 37 °C to degrade the DNA template. For transfection of BHK15 and BHK-REP cells, subconfluent monolayers of cells grown in T-75 culture flasks (~$1.5 \times 10^7$ cells) were harvested by trypsinization and washed twice with phosphate-buffered saline (PBS) before final resuspension in 400 μl PBS. The resulting cells were combined with ~10 μg of in vitro transcribed RNA, placed in a 2-mm gap cuvette (BioRad, Hercules, CA), and electroporated (two pulses at settings of 1.5 kV, 25 μF, and 200 Ω) using a GenePulser

II apparatus (BioRad). Following a 5-min recovery at room temperature, cells were resuspended in MEM supplemented with 10% FBS. Following electroporation of BHK-REP cells, media were replaced at 12 h post-electroporation with MEM supplemented with 10% FBS and 0.8 mg/ml G418.

*Luciferase assays*

For preparation of cell extracts for luciferase assay, BHK15 or BHK-REP cells were washed with PBS and lysed by the addition of cell culture lysis buffer (Promega Corp.), for assay of Luc, or *Renilla* cell lysis buffer (Promega Corp.), for assay of R.luc, followed by incubation of cells on a shaker at room temperature for 10 min. Cell extracts were then harvested and stored at −80 °C. Prior to assay, frozen extracts were thawed and then homogenized by brief vortexing. Luciferase assays were initiated by mixing 10 μl of prepared cell extract (~1 μg total protein) with 50 μl of the appropriate Luc or R.luc substrate prior to measurement of luciferase activity using a Lumat luminometer (Berthold, Australia).

*RT-PCR analysis*

For RT-PCR analysis, cytoplasmic extracts were prepared 24 h post-electroporation from transfected BHK15 cells grown in 60-mm culture dishes. Cells were washed three times with PBS prior to incubation of cells on ice for 10 min in the presence of cell lysis buffer (150 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.6), and 0.2% NP-40). Cells were then harvested, vortexed briefly, and centrifuged for 5 min at 800 × *g* in a 4 °C microcentrifuge to remove nuclei. SDS was then added to the cleared supernatant to a final concentration of 1%, followed by phenol/chloroform extraction and ethanol precipitation. Precipitated RNA was dried using a speed vac and then resuspended in DEPC-treated $H_2O$. First strand synthesis was performed on cytoplasmic RNA preparations using Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA) following the manufacturer's recommendations. The resulting cDNA was then amplified by Pfx thermostable polymerase (Stratagene) using 30 cycles of 94 °C/30 s, 50 °C/30 s, 68 °C/30 s.

*Generation of PIPs and titration*

Packaging of YFV replicons into PIPs was done essentially as described previously (Khromykh et al., 1998a, 1998b). Briefly, BHK or BHK-REP cells were first electroporated with in vitro-transcribed YFV replicon RNA followed by a 24- to 36-h recovery period in T-75 cell culture flasks at 37 °C in DMEM containing with 10% FBS. Packaging of YFV replicons into PIPs was then initiated by electroporation of recovered cells with in vitro-transcribed SINV replicon RNAs that express the YFV structural proteins. At 12 h following the second electroporation, medium containing PIPs was harvested, centrifuged for 5 min at 3000 × *g* to remove cell debris, and stored at −80 °C. For titration of YF-GFP2A-RP PIPs, confluent monolayers of BHK or BHK-REP cells were infected at room temperature with PIP-containing medium. Following an attachment period of 1.5 h, the medium was replaced by DMEM containing 5% FBS. At 24–36 h postinfection, infected cells expressing GFP were visualized by fluorescence microscopy. GFP-expressing cells from five random fields were counted and used to calculate titers using the following formula: PIPs/ml = $N \times (S_{well}/S_{IA}) \times 10^n \times 1/V$, where $N$ is the average number of GFP-expressing cells within an image area, determined from five random fields; $S_{well}$ is the surface area of the cell culture dish (1000 $mm^2$ for a 6 well), $S_{IA}$ is the surface area of the image area (1.03 $mm^2$), $10^n$ is the dilution factor, and $V$ is the volume of inoculum. To assay packaging of YF-R.luc2A-RP PIPs, BHK or BHK-REP cells were infected in triplicate in 24-well cell culture dishes and assayed for R.luc activity at 24 or 36 h (for packaging of *trans*-complemented replicons) postinfection.

*Fluorescence analysis*

Expression of GFP in adherent living cells (washed with PBS) was visualized by fluorescence microscopy using an inverted fluorescence microscope affixed with a digital CCD camera for image capture (Nikon, Melville, NY). Accumulation of YFV-specific proteins from replicating YFV replicons was evaluated by immunofluorescence assay. Cells in 24-well cell culture dishes were fixed with 100% methanol for 1 h at −20 °C. The fixed cells were then washed with PBS and incubated with a 1:100 dilution of polyclonal anti-YFV serum (ATCC) for 1 h at 37 °C. After incubation with primary antibody, cells were washed with PBS and incubated for 1 h at 37 °C with a 1:200 dilution of anti-mouse antibody conjugated to Texas red (Pierce, Rockford, IL). The treated cells were then washed with PBS prior to visualization by fluorescence microscopy. For detection of YFV E protein expression from SINV replicon-expressing YFV structural proteins, cells in 24-well cell culture dishes were treated as above using a 1:200 dilution of a monoclonal anti-YFV E protein antibody (Chemicon, Temecula, CA) as the primary antibody and a Texas red-conjugated anti-mouse secondary antibody at a dilution of 1:400 for the secondary antibody.

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