JOURNAL OF VIROLOGY, Nov. 2007, p. 11737–11748 Vol. 81, No. 21
0022-538X/07/$08.00+0 doi:10.1128/JVI.01112-07

Production of Pseudoinfectious Yellow Fever Virus with a Two-Component Genome▽

Alexandr V. Shustov,[1] Peter W. Mason,[1,2,3] and Ilya Frolov[1]*

Department of Microbiology and Immunology,[1] Department of Pathology,[2] and Sealy Center for Vaccine Development, University of Texas Medical Branch, 301 University Blvd., Galveston, Texas 77555[3]

Received 22 May 2007/Accepted 16 August 2007

Application of genetically modified, deficient-in-replication flaviviruses that are incapable of developing productive, spreading infection is a promising means of designing safe and effective vaccines. Here we describe a two-component genome yellow fever virus (YFV) replication system in which each of the genomes encodes complete sets of nonstructural proteins that form the replication complex but expresses either only capsid or prM/E instead of the entire structural polyprotein. Upon delivery to the same cell, these genomes produce together all of the viral structural proteins, and cells release a combination of virions with both types of genomes packaged into separate particles. In tissue culture, this modified YFV can be further passaged at an escalating scale by using a high multiplicity of infection (MOI). However, at a low MOI, only one of the genomes is delivered into the cells, and infection cannot spread. The replicating prM/E-encoding genome produces extracellular E protein in the form of secreted subviral particles that are known to be an effective immunogen. The presented strategy of developing viruses defective in replication might be applied to other flaviviruses, and these two-component genome viruses can be useful for diagnostic or vaccine applications, including the delivery and expression of heterologous genes. In addition, the achieved separation of the capsid-coding sequence and the cyclization signal in the YFV genome provides a new means for studying the mechanism of the flavivirus packaging process.

The *Flavivirus* genus of the family *Flaviviridae* contains a variety of important human and animal pathogens. In nature, flaviviruses circulate between vertebrate hosts and arthropod vectors mainly represented by a large number of mosquito and tick species. Almost 40 members of this genus, classified into four distinct antigenic complexes, are capable of causing human disease.

The flavivirus genome is a single-stranded RNA of positive polarity of almost 12 kb. It encodes a single polypeptide that is co- and posttranslationally processed by cellular and viral proteases into the viral structural proteins C, prM/M, and E that form infectious viral particles and the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 that form the enzyme complex required for replication of the viral genome (27). The flavivirus genome mimics the structure of cellular messenger RNAs by having a 5′ methylguanylate cap but differs from the cellular RNA templates due to the absence of a 3′-terminal poly(A) sequence.

In flavivirus virions, a single copy of viral genomic RNA is packaged by the C (capsid) protein into a nucleocapsid surrounded by a lipid envelope with embedded dimers of E and M proteins (23). The mechanism of interaction between the nucleocapsid and the envelope is not completely understood yet, but it appears to be less specific than, for instance, the alphavirus nucleocapsid-envelope interaction, and the flavivirus virions can be efficiently formed by capsid and envelope proteins derived from the viruses that belong to distant antigenic complexes (5, 11, 28, 31, 35). Moreover, the presence of nucleocapsid is not an absolute requirement for particle assembly, and their formation and release from the cells can be achieved by expression of only prM and E from a wide variety of vectors. These so-called subviral particles (SVPs) contain no RNA or capsid protein (29), but have the envelope proteins organized into an icosahedral, lipid-containing structure (41). The prM/E-embedded SVPs are capable of inducing an efficient immune response that protects animals against a future infection with the replication-competent viruses (17, 18, 20, 37). These SVPs can be produced from viral or DNA vectors. The lack of nucleocapsid-packaged replication-competent RNA makes the application of SVPs as potential vaccines very advantageous but requires the development of a new means for the large-scale production or delivery of the expression constructs. The prM/E-expressing cassettes can be designed on the basis of viral and nonviral vectors (1, 6, 7, 14, 15, 21, 22, 29, 33, 36, 39). In the case of viral vectors (19, 36), there is always a concern of either the development or preexistence of an immune response to the viral vector used. The DNA-based cassettes encoding these genes under control of efficient RNA polymerase II-based promoters appear to be preferred. However, their application in clinical practice remains questionable. Therefore, vaccination against flaviviruses is still mainly achieved by using either inactivated or live-attenuated vaccines (INVs and LAVs, respectively).

Recent studies have suggested that flavivirus structural proteins are dispensable for RNA genome replication. These proteins can be either completely or partially deleted, and such RNAs (replicons) remain self-replicating and capable of expressing not only the nonstructural but also the remaining structural and/or additional heterologous genes (13). For ex-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1019. Phone: (409) 772-2327. Fax: (409) 772-5065. E-mail: ivfrolov@utmb.edu.

▽ Published ahead of print on 22 August 2007.

ample, the flavivirus genomes lacking functional capsid gene but having the other structural genes intact were synthesized in vitro and used directly for immunization (2, 16). Their replication led to the SVP production and ultimately induced a protective immune response. Application of the modified flaviviruses that are incapable of developing productive, spreading infection is a new means of designing safe and effective in producing protective immunity vaccines (2, 16). However, their application probably requires an improvement in the delivery of the in vitro-synthesized RNAs into the cells susceptible for RNA replication. This can be achieved by using the natural approach, by packaging these defective genomes into infectious particles composed of viral structural proteins.

In a previous study, we demonstrated that flavivirus genomes, incapable of expressing capsid protein and therefore deficient in causing spreading infection, could be packaged into infectious viral particles in the capsid-producing cell lines (30). Packaging was achieved either by transfection of the in vitro-synthesized RNA into the capsid-producing cells or by infecting such cells with the previously packaged capsid-deficient genomes. However, upon infection of the naive, noncapsid-producing cells in vitro or in vivo, these replication-deficient viruses, termed preudoinfectious viruses (PIVs), developed only a single round of infection. The infected cells produced only SVPs lacking a nucleocapsid and a viral genome. These SVPs induced an efficacious immune response, which protected animals against future infection with the replication-competent flaviviruses (30). Thus, the capsid-deficient viruses combined some of the critical characteristics of the live viruses (e.g., developed spreading and productive infection in the capsid-producing cell lines) with the safety of inactivated vaccines because of the inability of causing spreading infection in the naive cells both in vitro and in vivo. The capsid-producing cell lines were generated by using noncytopathic Venezuelan equine encephalitis virus (VEEV) replicons that did not interfere with replication of studied flaviviruses (West Nile and yellow fever viruses) and expressed capsid to a concentration sufficient for RNA packaging (32).

In the present study, we continued to further develop a transcomplementation approach for generating the replication-deficient, pseudoinfectious flaviviruses and succeeded in transforming a nonsegmented RNA^+ genome-containing YFV into the two-component genome PIV. Both of these genomes were deficient in the expression of at least one of the proteins required for productive replication (capsid or prM/E) but complemented one another's functions upon delivery into the same cell. This virus could be efficiently passaged in tissue culture (that is a prerequisite of large-scale production) and was capable of not only SVP production but also the expression of additional heterologous proteins.

MATERIALS AND METHODS

Cell cultures. The BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, MO). They were maintained at 37°C in alpha minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins.

Plasmid constructs. Standard recombinant DNA techniques were used for all plasmid constructions. Maps and sequences are available from the authors upon request. The parental low-copy-number plasmid pACNR/FLYF-17Dx containing infectious cDNA of YFV 17D strain genome was described elsewhere (4) and kindly provided by Charles M. Rice (Rockefeller University, New York, NY).

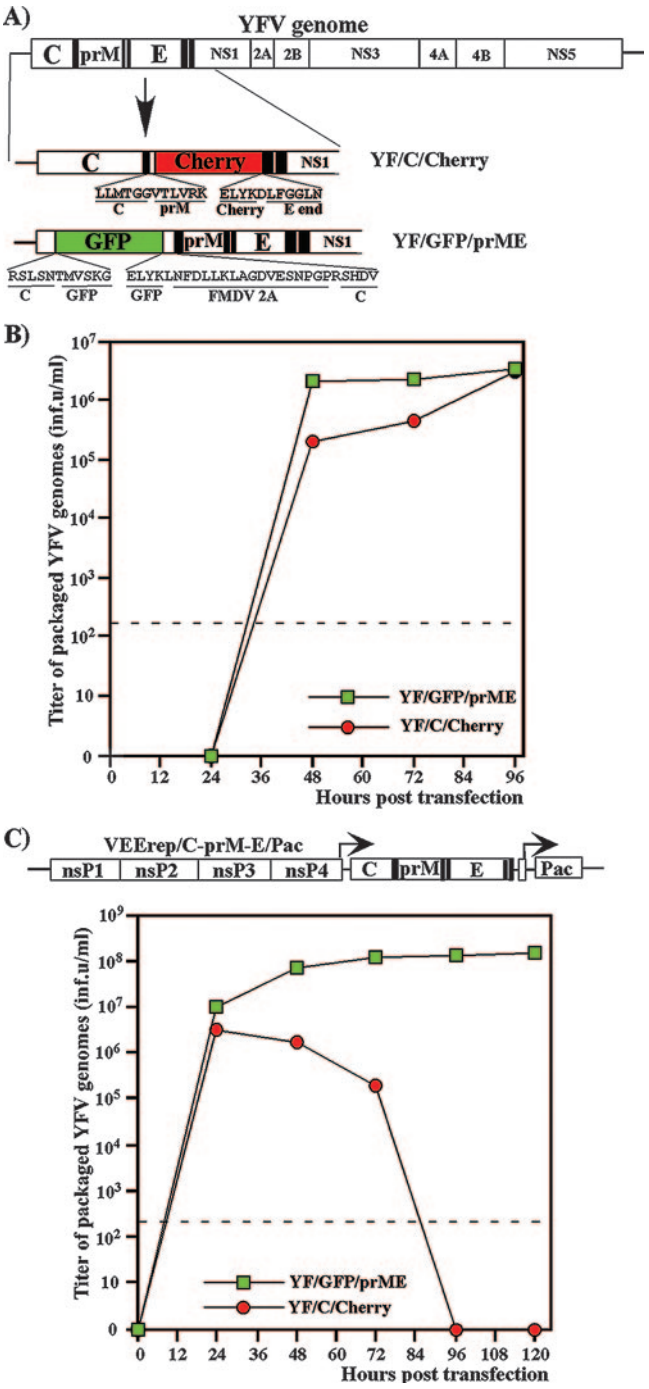


FIG. 1. Packaging of capsid- and prM/E-coding defective YFV genomes into infectious viral particles. (A) Schematic representation of the 5′-terminal sequences in the replication-deficient YFV genomes. The positions of signal peptides and transmembrane domains are indicated by filled boxes. (B) The release of the defective genome-containing viral particles from the cells cotransfected by the in vitro-synthesized RNAs. Media were replaced at the indicated time points and titers were determined as described in Materials and Methods. (C) Schematic representation of VEEV replicon encoding YFV C-prM-E and analysis of the release of packaged, defective YFV genomes. The in vitro-synthesized YFV genomes were transfected into the cells carrying VEErep/C-prM-E/Pac, and media were replaced at the indicated time points. Titers of packaged defective genomes were determined as described in Materials and Methods. Dashed lines indicate the limits of detection.

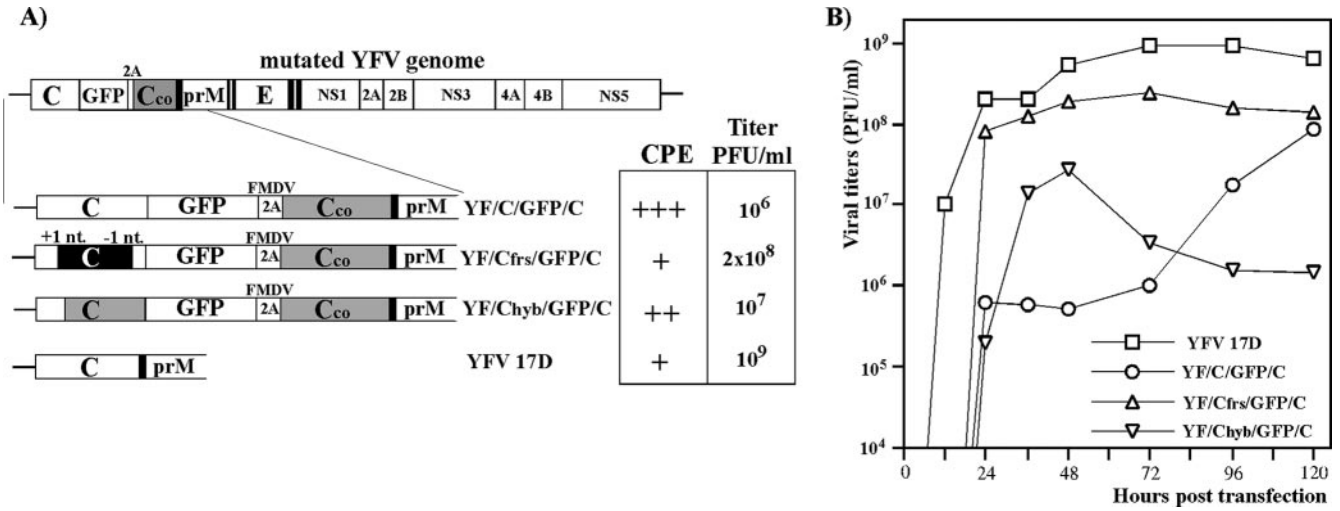


FIG. 2. Replication of YFV with duplicated capsid-specific sequence. (A) Schematic representation of recombinant YFV genomes. The codon-optimized, capsid-coding sequences are indicated in gray. The alternative ORF in the capsid of YF/Cfrs/GFP/C genome that results from introduction of two frameshift mutations is indicated by filled box. Titers and CPE development were evaluated at 72 h posttransfection of the in vitro-synthesized RNAs (see Materials and Methods for details). (B) Analysis of the release of recombinant viruses. The in vitro-synthesized RNAs were transfected into the cells, media were replaced at the indicated time points, and virus titers were determined by using a plaque assay as described in Materials and Methods. One of two reproducible experiments is presented.

pYF/GFP/prME contained a defective YFV genome (YF PIV), in which a fragment encoding aminoi acids (aa) 26 to 100 of the YF capsid gene was replaced by a codon-optimized green fluorescent protein (GFP) gene derived from pEGFP-N1 (Clontech). This plasmid was designed in our previous study (30), in which it was termed pYF/PIV. pYF/C/Cherry encoded the entire capsid protein, followed by prM signal peptide and 6 aa of prM, fused with the Cherry (one of the red fluorescent proteins)-coding sequence (40). The latter gene was fused in frame with the rest of the YF open reading frame (ORF) that started from the transmembrane domain of E protein (see Fig. 1A for details). Plasmid pYF/C/GFP/C contained the YFV genome, in which the 101-aa capsid-coding sequence was fused with GFP, followed by 2A protease of foot-and-mouth disease virus (FMDV 2A), codon-optimized capsid gene, and the rest of YF polyprotein prM-NS5-coding sequence. pYF/Cfrs/GFP/C and pYF/Chyb/GFP/C had essentially the same design (Fig. 2A), but in pYF/Cfrs/GFP/C, 1 nt was inserted after nt 202 and nt 422 was deleted, and in pYF/Chyb/GFP/C, the sequence between nt 201 and 422 was replaced by the synthetic gene encoding the same amino acid sequence but utilizing different codon usage. pYF/ΔC/GFP/C and pYF/C/ΔGFP/C were the derivatives of pYF/C/GFP/C, which contained deletions in the capsid- and GFP-coding sequences, respectively, that were identified in the selected deletion mutants (see Fig. 3A for details). pYF/GFP contained the YFV genome, in which the 5′ untranslated region (UTR) was followed by the ORF encoding 25 aa of YFV capsid, fused with GFP and FMDV

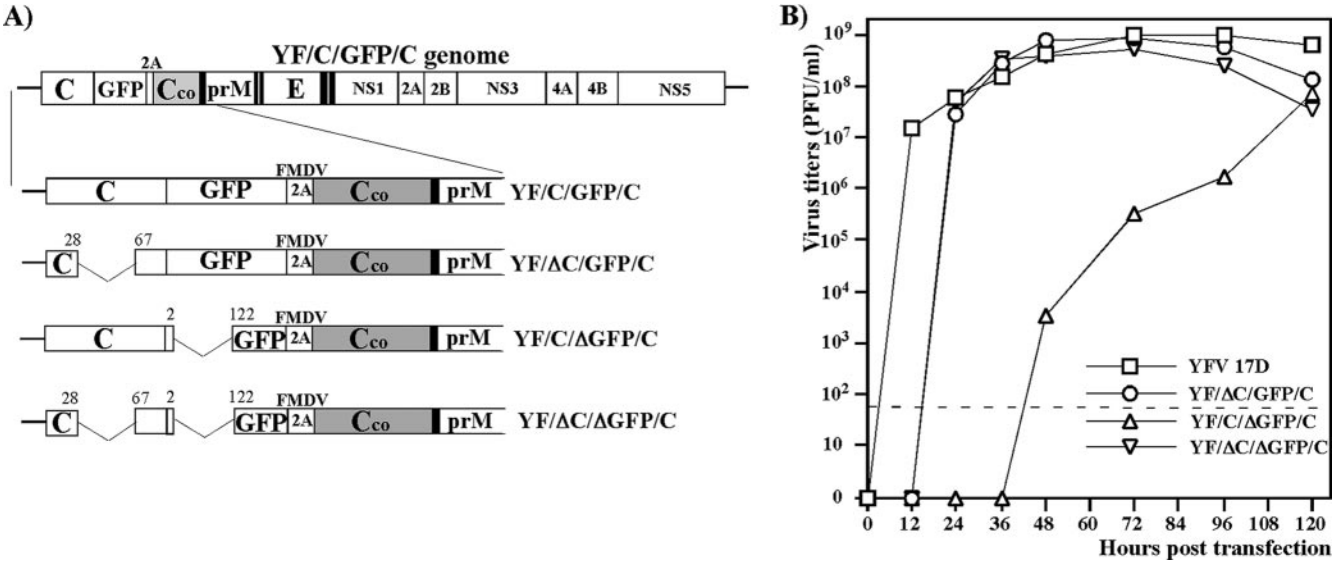


FIG. 3. Selection of YF/C/GFP/C genome-containing variants capable of efficient replication and identification of the adaptive mutations. (A) Schematic representation of the YF/C/GFP/C genome and the deletions identified in the efficiently replicating, less-cytopathic variants. Numbers indicate the positions of the deletions in the amino acid sequence of capsid and GFP proteins. (B) Replication of the reconstructed deletion mutants in BHK-21 cells. The in vitro-synthesized RNAs were transfected into the cells, and media were replaced at the indicated time points. Titers of the released viruses were determined in a plaque assay, as described in Materials and Methods. Dashed line represents the limit of detection. One of two representative, reproducible experiments is presented.

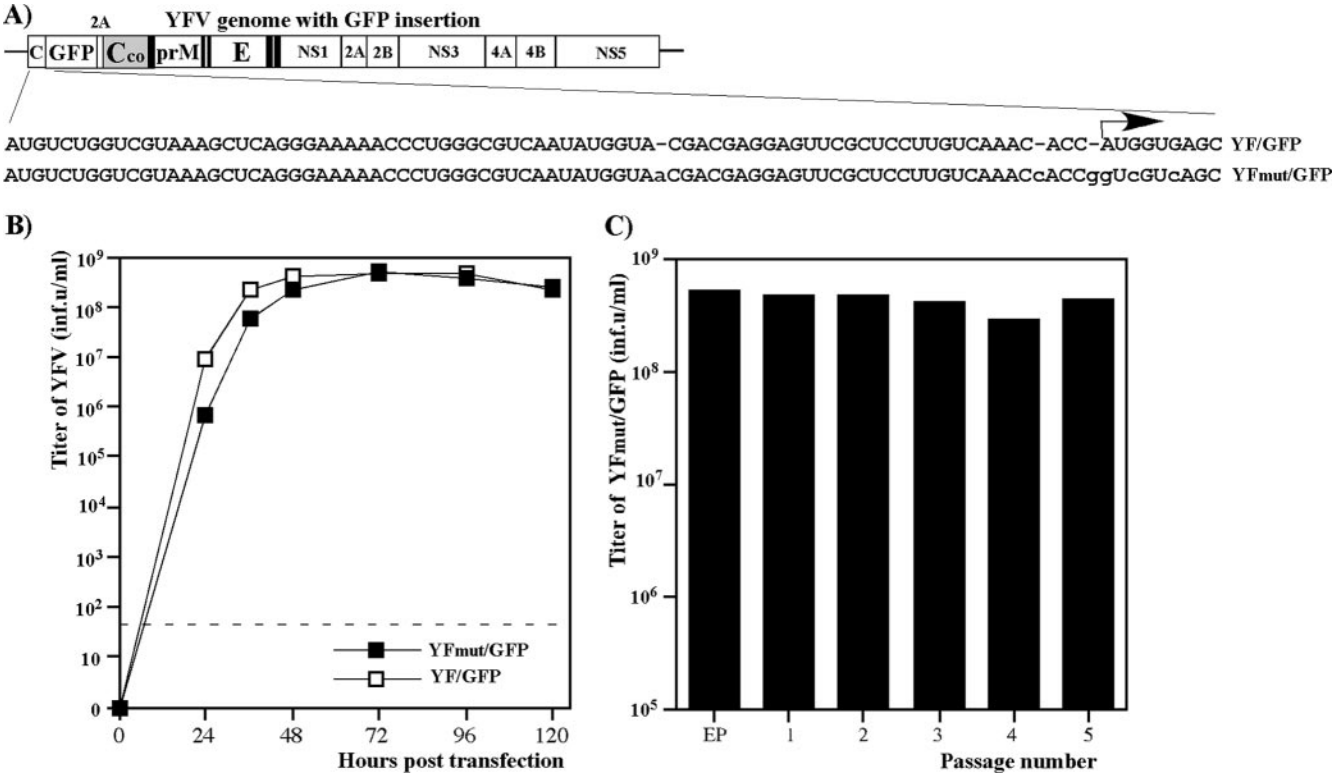
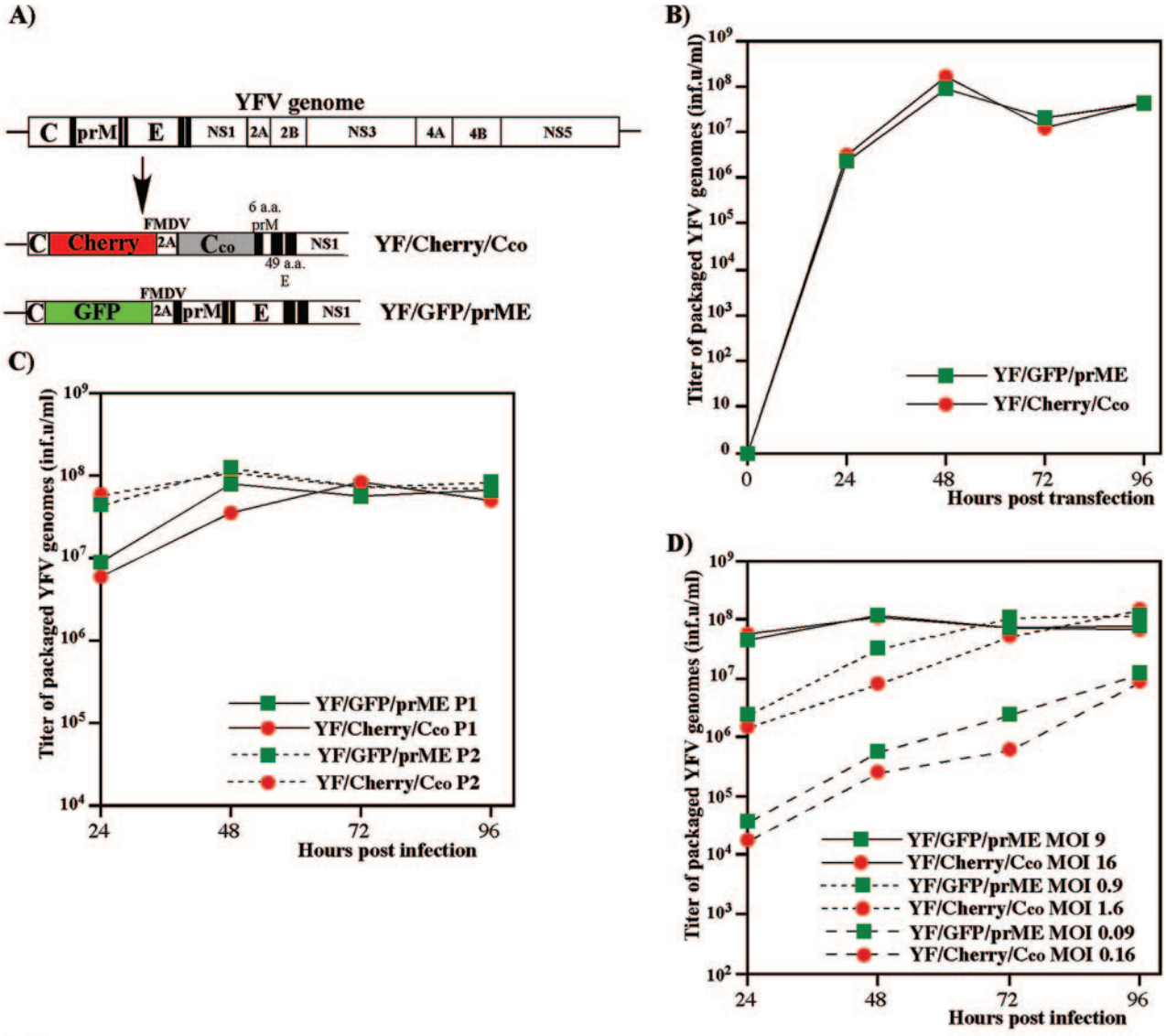


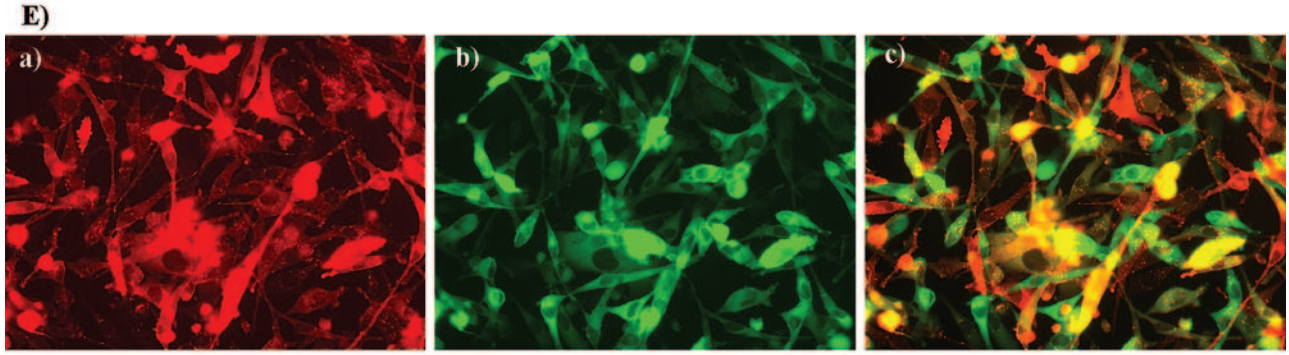
FIG. 4. Replication of the recombinant YFV genomes encoding a heterologous gene upstream of the polyprotein. (A) Schematic representation of the recombinant genome and sequence of the ORFs located upstream of the GFP gene. The codon-optimized capsid-coding sequence is indicated in gray. The arrow indicates the start of the GFP-coding sequence. The lowercase letters indicate mutations introduced into the capsid and GFP sequences. (B) Replication of designed YFV variants in BHK-21 cells. The in vitro-synthesized viral RNAs were transfected into the cells, and media were replaced at the indicated time points. Titers of released viruses were determined in a plaque assay. The dashed line represents the limit of detection. One of two representative, reproducible experiments is presented. (C) Titers of the recombinant YFmut/GFP virus after serial passaging in BHK-21 cells. See Materials and Methods for details.

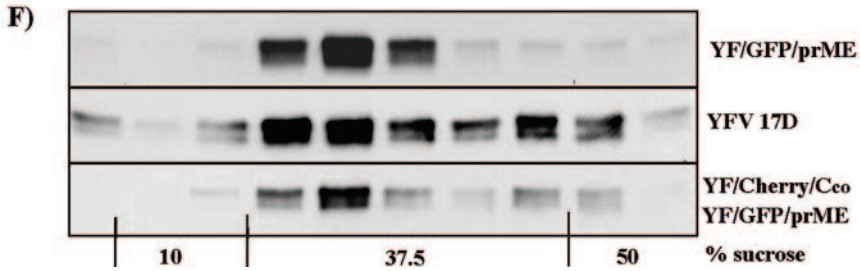
2A and the entire YFV polyprotein C-NS5, in which capsid gene was presented by the codon-optimized version (Fig. 4A). pYF/GFPmut had essentially the same design, but the fragment encoding 25 aa of capsid contained three 1-nt insertions and point mutations in the beginning of GFP-coding sequence (see Fig. 4A for details). pYF/Cherry/Cco contained a defective YFV genome, in which 75 nt of capsid-coding sequence was fused with Cherry gene, followed by sequence-encoding FMDV 2A protease, codon-optimized capsid with prM signal peptide, 6 aa of prM, 49 carboxy-terminal amino acids of E protein, and the rest of the YFV polypeptide (Fig. 5A). pYFrep/Cherry contained a YFV replicon in which the structural genes were replaced by a Cherry protein-coding sequence. At the amino terminus, Cherry was fused with 25 aa of YFV capsid, and at the carboxy terminus, it was followed by FMDV 2A, followed by NS1 signal peptide and the rest of the YFV polyprotein (Fig. 6A). VEErep/C-prM-E/Pac- and VEErep/Copt-prM-E/Pac-encoding plasmids have been described elsewhere (30). pVEErep/GFP-C-prM-E/Pac plasmid contained VEEV replicon, in which the subgenomic RNA encoded 25 aa of YFV capsid, fused with GFP, followed by FMDV 2A protease, codon-optimized YFV capsid, and prM/E genes. The second subgenomic promoter was driving the expression of puromycin acetyltransferase (Pac). All of the recombinant viral genomes and replicons were cloned under the control of the SP6 RNA polymerase promoter.

RNA transcriptions. Plasmids were purified by centrifugation in CsCl gradients. Before the transcription reaction, the YFV genome or replicon-containing plasmids were linearized by XhoI. Plasmids with VEEV replicons were linearized by MluI. RNAs were synthesized by SP6 RNA polymerase in the presence

FIG. 5. Analysis of two-component genome virus replication. (A) Schematic representation of the YFV capsid- and prM/E-coding genomes that are capable of transcomplementation during replication in the same cell. The codon-optimized, capsid-coding gene is indicated in gray. (B) Release of defective genome-containing viral particles from cells cotransfected by in vitro-synthesized RNAs. Media were replaced at the indicated time points, and titers of the infectious viral particles, containing each of the genomes, were determined as described in Materials and Methods. (C) Replication of the two-component genome YFV during the next rounds of passaging in BHK-21 cells at an MOI of ~10 i.u./cell. Media were replaced at the indicated time points, and titers of the released infectious particles, containing each of the genomes, were determined as described in Materials and Methods. (D) Replication of the two-component genome YFV after infecting cells at different MOIs. Media were replaced at the indicated time points, and titers of the released infectious particles were determined as described in Materials and Methods. (E) Replication of both defective genomes in the infected cells. BHK-21 cells were infected with two-component genome YFV at an MOI of ~1 i.u./cell, and replication of the genomes was evaluated at 48 h postinfection. Panel a represents cells containing replicating YF/Cherry/Cco, panel b represents cells with a replicating YF/GFP/prME genome, and panel c is an overlay. (F) Analysis of infectious virus and VLP release from the cells transfected with different YFV-specific RNAs. BHK-21 cells were transfected with the indicated RNAs. At 24 h posttransfection, media were replaced by serum-free media that was harvested 24 h later. Particles were pelleted by ultracentrifugation and further analyzed on the discontinuous sucrose gradients as described in Materials and Methods. The presence of YFV-specific proteins in the fractions was detected by Western blotting with a D1-4G2 MAb that recognizes viral E protein.


A)
YFV genome
C
prM
E
NS1
2A
2B
NS3
4A
4B
NS5
FMDV
6 a.a.
prM
Cherry
2A
Cco
NS1
YF/Cherry/Cco
49 a.a.
E
FMDV
GFP
2A
prM
E
NS1
YF/GFP/prME
B)
Titer of packaged YFV genomes (inf.u/ml)
YF/GFP/prME
YF/Cherry/Cco
Hours post transfection
C)
Titer of packaged YFV genomes (inf.u/ml)
YF/GFP/prME P1
YF/Cherry/Cco P1
YF/GFP/prME P2
YF/Cherry/Cco P2
Hours post infection
D)
Titer of packaged YFV genomes (inf.u/ml)
YF/GFP/prME MOI 9
YF/Cherry/Cco MOI 16
YF/GFP/prME MOI 0.9
YF/Cherry/Cco MOI 1.6
YF/GFP/prME MOI 0.09
YF/Cherry/Cco MOI 0.16
Hours post infection


E)
a)
b)
c)


F)
YF/GFP/prME
YFV 17D
YF/Cherry/Cco
YF/GFP/prME
10
37.5
50
% sucrose

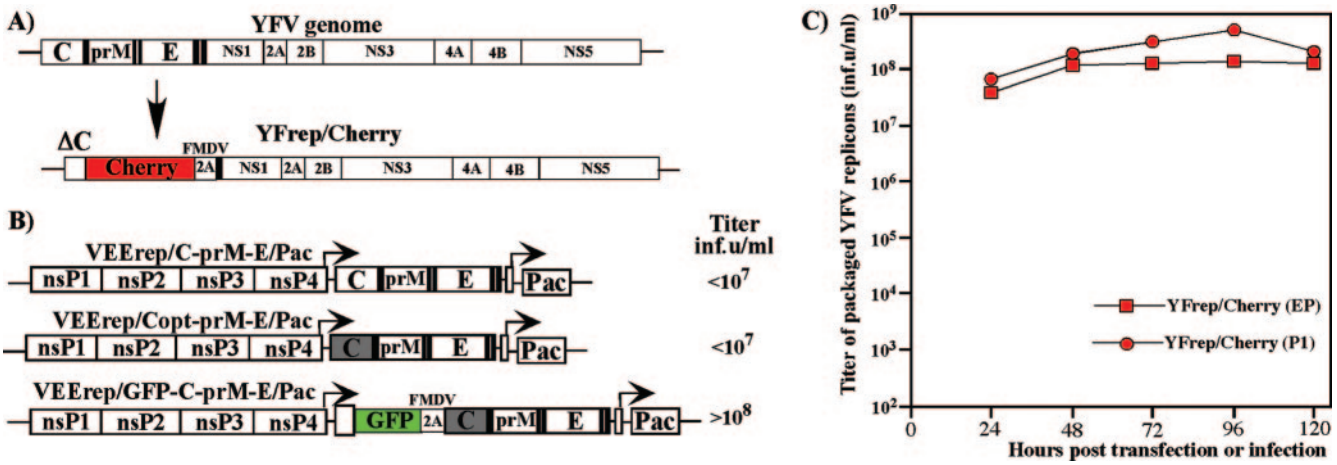


FIG. 6. Packaging of YFV replicon lacking all of the structural genes in the packaging cell line. (A) Schematic representation of YFV replicon, encoding fluorescent marker, Cherry, instead of the structural proteins. (B) Schematic representation of the previously described VEE replicons encoding C-prM-E and its new version. Titers of packaged YFrep/Cherry in the packaging cell lines developed using both of the VEEV replicons are indicated. (C) Release of the infectious, YFrep/Cherry genome-containing viral particles from the VEErep/GFP-C-prM-E/Pac-containing cells transfected with the indicated YF replicon or infected with the latter particles at the next passage. Media were replaced at the indicated time points, and titers of the released packaged replicons were determined as described in Materials and Methods.

of cap analog as described elsewhere (3). The yield and integrity of transcripts were analyzed by gel electrophoresis under nondenaturing conditions. Aliquots of transcription reactions were used for electroporation without additional purification.

RNA transfections. Electroporation of BHK-21 cells was performed using 8 μg of in vitro-synthesized YFV genome RNAs under previously described conditions (26). For establishing packaging cell cultures, Pur was added to the media to a concentration of 10 μg/ml at 24 h postelectroporation of 8 μg of in vitro-synthesized VEEV replicons. Transfection of in vitro-synthesized, defective YFV genome was performed 5 days later, when replicon-containing cells resumed efficient growth.

Measuring the titers of infectious viral particles containing defective YFV genomes. For measuring the titers of released virions containing different defective genomes, BHK-21 cells were seeded into six-well Costar dishes at a concentration of 5×10^5 cells/well. Four hours later, cells were infected with different dilutions of the samples and, after 1 h of incubation at 37°C in an 5% CO_2 incubator, 2 ml of αMEM supplemented with 10% FBS was added. The numbers of infected cells were estimated by counting GFP- and Cherry-positive cells under an inverted UV microscope after 36 h of incubation at 37°C. The fraction of infected cells was determined by counting GFP- and/or Cherry-positive cells in multiple defined areas of the microscopic field. Counts for different fields were averaged and used for calculation of the titers.

Titers of replication-competent viruses were determined by standard plaquing of the samples on BHK-21 cells (25). After 3 days of incubation at 37°C, monolayers were fixed by 2.5% formaldehyde and either stained with crystal violet orstudied under a fluorescent, dissecting microscope to evaluate the numbers of GFP-positive foci.

Passaging of viruses. Packaging cell lines were established by transfection of the in vitro-synthesized VEEV replicon RNAs, followed by Pur selection. These cell lines were either transfected by the in vitro-synthesized YFV replicon RNA as described above or infected with the previously packaged replicons. Samples were harvested at the time points indicated in the figures by replacing the media. Passaging of two-component genome YF viruses was performed by infecting BHK-21 cells at the MOIs indicated in the figures and figure legends. Samples were harvested at the time points indicated in the figures by replacing the media, and titers of particles, containing the defective genomes, were determined as indicated above. Replication-competent viruses were passaged by infecting naive BHK-21 cells with 100 μl of virus, harvested at the previous passage. Samples were harvested at 72 h postinfection, and titers were determined by plaque assay.

Analysis of YF SVP production. BHK-21 cells were transfected by 8 μg of in vitro-synthesized YFV 17D or YF/GFP/prME viral genomes or cotransfected with YF/Cherry/Cco and YF/GFP/prME genomes. After 24 h of incubation in 10 ml of αMEM supplemented with 10% FBS, the latter medium was replaced by 10 ml of serum-free medium VP-SF (Invitrogen). Incubation continued for 24 h, and then the media were harvested to analyze SVP release. The collected samples were clarified by the low-speed centrifugation (5,000 rpm, 10 min, 4°C) and then concentrated by ultracentrifugation through 2 ml of 10% sucrose, prepared on phosphate-buffered saline (PBS), in an SW41 rotor at 39,000 rpm and 4°C for 6 h. Pelleted material was further analyzed in a sucrose density gradient as previously described (38). Briefly, the 0.5-ml samples were loaded onto the discontinuous sucrose gradient (1 ml of 50%, 2.5 ml of 37.5%, and 1 ml of 10% sucrose prepared on PBS). Centrifugation was performed by using an SW55 rotor at 45,000 rpm and 4°C for 4 h. After fractionation, samples were diluted threefold with PBS, and SVPs were pelleted by centrifugation in TLA-55 rotor at 45,000 rpm and 4°C for 1 h in Optima MAX Ultracentrifuge (Beckman). Pellets were dissolved in the loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, lacking β-mercaptoethanol (to preserve binding to D1-4G2 monoclonal antibody [MAb]) and further analyzed by Western blotting. After protein transfer, the nitrocellulose membranes were processed by D1-4G2 MAb and horseradish peroxidase-conjugated secondary donkey anti-mouse antibodies purchased from Santa Cruz Biotechnology. Horseradish peroxidase was detected by using the Western blotting Luminol reagent according to the manufacturer's recommendations (Santa Cruz Biotechnology). Side-by-side gradient analyses were performed with YFV (2×10^8 PFU) subjected to the same procedures as described above.

Animal experiments. Litters of 2- to 4-day-old mice (outbred Swiss Webster; Harlan) were infected by the recombinant YFV/GFP at doses indicated in the text by the intracranial route (20 μl). Mice were monitored for 8 days for signs of disease and death. On day 8 all of the animals displayed severe signs of paralysis and were euthanized as required by the University of Texas Medical Branch animal core regulations, and virus titers in the brains were evaluated by plaque assay.

RESULTS

Capsid- and prM/E-expressing, defective YFV genomes strongly differ in replication efficiency. In the previous study, we developed a system for transcomplementation of the defects in YFV replication and packaging of defective genomes into infectious YF viral particles. To achieve this, we designed cell lines that contained VEEV replicons producing either YFV capsid or the entire structural polyprotein that complemented replication of the capsid-deficient YFV genomes (9, 30). However, the use of alphavirus replicons is not an absolute prerequisite for transcomplementation. A functional capsid can apparently be supplied by other cassettes capable of its production to a level sufficient for flavivirus genome packaging.

Therefore, we attempted to exclude any heterologous expression vectors from the packaging system and produce capsid from the second YFV genome that lacks the structural genes other than the capsid-coding one.

The PIV genome (YF/GFP/prME) contained a deletion of almost the entire capsid-coding sequence; the second, complementing, genome (YF/C/Cherry) had the prM/E-coding sequence deleted; and the capsid gene remained intact (see Materials and Methods for details). In these constructs, we intended to preserve the natural strategy of viral gene expression and processing of the synthesized proteins. To analyze the replication patterns of both genomes in tissue culture, we also cloned into their ORFs two different fluorescent proteins, GFP and Cherry (Fig. 1A). Both genomes were expected to be incapable of developing productive, spreading infection because of the inability to produce a complete set of structural proteins. However, they could produce all of the proteins required for viral particle formation while replicating in the same cell. The in vitro-synthesized RNAs were cotransfected into BHK-21 cells, and the expression of both markers, GFP and Cherry, confirmed their replication. Surprisingly, the titers of the released infectious viral particles containing either capsid- or prM/E-encoding genomes were lower than expected, close to 10^6 infectious units (i.u.)/ml, suggesting that the *trans*-complementation was inefficient (Fig. 1B). Comparison of the rates of GFP and Cherry expression indicated that the capsid-encoding genome replicated more efficiently than did its prM/E-producing counterpart (data not shown) and thus, strongly interfered with YF/GFP/prME RNA replication. After electroporation, the expression of Cherry reached detectable levels 18 to 24 h earlier than GFP expression did but, most importantly, YF/C/Cherry replication also caused cell death within 2 to 3 days posttransfection. Rapid CPE development was not a side effect of Cherry protein expression, because the same cassette in which Cherry was replaced by GFP also demonstrated high cytopathogenicity (data not shown).

In additional experiments, we compared the replication of the GFP- and Cherry-expressing genomes (YF/GFP/prME and YF/C/Cherry) in the previously designed cell line, in which the entire YFV structural polyprotein precursor, C-prM-E, was expressed from the VEEV replicon (30). In agreement with the data described above, the replication of capsid-expressing YF/C/Cherry genome had a deleterious effect on the cells, and essentially all of the transfected cells were dead within 96 h posttransfection (Fig. 1C), and the infectious virus particles were released to titers lower than those found in the media of the same cells transfected with the prM/E-expressing construct, YF/GFP/prME. As we previously described (30), the cells bearing YF/GFP/prME did not develop a cytopathic effect (CPE) (Fig. 1C) and continued to release packaged viral genomes during the subsequent cell passaging. Thus, these experiments indicated that either the presence of a capsid-coding sequence in the YF/C/Cherry genome or the expression of capsid protein itself (or both factors together) could strongly determine the cytopathogenicity of this replicating RNA. Therefore, this factor(s) created a profound difference in the replication of capsid- and prM/E-producing genomes that could be the reason for inefficient transcomplementation. Moreover, in the repeated experiments, transfected cells had probably died before they had a chance to release infectious viral particles at high titers.

Effect of capsid protein on replication of YFV genome RNA. To distinguish between the effects of capsid or capsid-coding sequence on the replication of defective YFV-specific RNA, we designed a set of recombinant YFV genomes in which the sequences encoding the entire polyprotein that includes all of the structural and nonstructural genes and the 5′-terminal sequences that contain the RNA promoter elements required for replication were functionally separated (Fig. 2A). To achieve this, we replaced a natural capsid gene in the polyprotein with its codon-optimized version (Cco) having a mutated cyclization sequence that was incapable of functioning in RNA replication. Then, upstream of Cco, we cloned the YFV 5′UTR, followed by sequence encoding the natural capsid without prM-specific signal peptide, fused with GFP and FMDV 2A protease genes. Thus, the upstream capsid gene in the YF/C/GFP/C genome contained a cyclization signal essential for RNA replication and the initiating methionine codon. The Cco amino acid sequence differed from that of the natural YFV capsid only by having a proline as a first amino acid because it was required for FMDV 2A-specific processing. This experimental system allowed us to perform a wide variety of manipulations in the 5′ terminus, including extensive modifications in the amino-terminal, natural capsid-coding part of the ORF, without affecting functional capsid protein (Cco) expression. Therefore, in another cassette, YF/Cfrs/GFP/C, the first capsid gene contained a 1-nt insertion after nt 202 of the genome and a 1-nt deletion after nt 421. These modifications were done in such a manner as to save the computer-predicted secondary structure of the 5′ end of the viral genome and the 3′ end of the negative-strand RNA; however, they changed the sequence of a 73-aa peptide that covers a large capsid fragment. In the third construct, YF/Chyb/GFP/C, the first capsid gene was a hybrid between the natural and codon-optimized sequences. It encoded a wild-type (wt) protein, but the RNA sequence downstream from the circularization signal, starting from nt 202, was different from that in the wt YFV genome. Thus, the 5′ termini of recombinant viral genomes (Fig. 2A) encoded one of three alternatives: (i) the natural capsid gene fused with GFP (YF/C/GFP/C); (ii) the almost natural RNA sequence (with only two frameshift mutations), but strongly modified protein (YF/Cfrs/GFP/C); or (iii) the modified RNA sequence, but natural protein (YF/Chyb/GFP/C). All three RNAs and the RNA of the YF 17D genome were synthesized in vitro, and equal amounts were transfected into BHK-21 cells. Analysis of infectious virus release demonstrated that only the construct expressing the mutated first capsid, YF/Cfrs/GFP/C, was capable of efficient replication to titers comparable to those achieved by the YFV 17D (Fig. 2B). However, the difference in replication rates was still noticeable. Both of the genomes expressing capsid with a wt amino acid sequence fused with GFP, namely, YF/Chyb/GFP/C and YF/C/GFP/C, demonstrated highly cytopathic phenotypes and low titers of released infectious virus in spite the fact that they expressed GFP to higher levels than did YF/Cfrs/GFP (data not shown). Taken together, the results of these experiments and those presented in the previous section indicated that the YFV capsid expressed outside of its natural position has a strong positive effect on the cytopathogenicity of the recombinant viruses and,

consequently, on their growth in tissue culture. In additional experiments, we also demonstrated that the cytotoxicity of the constructs did not depend on capsid expression in the GFP-fused or free form (data not shown).

Selection of YFV variants with reduced cytopathogenicity. In the experiments described above, the YF/C/GFP/C virus, containing two copies of a capsid gene demonstrated an unusual replication pattern (Fig. 2B) characterized by the very inefficient release of infectious virus within first 3 days posttransfection of the in vitro-synthesized RNA and the death of the majority of the cell population. However, a small percentage of the GFP-positive cells survived, continued to grow and, after 72 h posttransfection, produced virus more efficiently than during the early times (Fig. 2B). By day 5, virus titers approached 10^8 i.u./ml. These data suggested the possibility that adaptive mutations had accumulated in the viral genomes, and these could affect a viral, highly cytopathic phenotype and, consequently, lead to a prolonged, more efficient infectious virus release. To identify these adaptive changes, we analyzed the 5′UTR and the capsid- and GFP-encoding sequences in the genomes of the variants isolated from the six plaques, randomly selected from the samples harvested at late times postinfection (Fig. 2B). All of the plaques contained GFP-expressing viruses; however, reverse transcription-PCR analysis demonstrated that viruses continued to evolve even after plaque isolation. After amplification of the fragments, we detected the presence of four reproducible variants in all of the samples. Importantly, the same analysis of the sample, harvested directly after RNA transfection, revealed only one band, corresponding to the original cassette. This was an indication that heterogeneous PCR products did not result from the reverse transcription or PCRs. The detected DNA bands were isolated and cloned into the plasmid, and then insertions in multiple *Escherichia coli* clones were sequenced. The variants present in the plaques contained large, in-frame deletions in capsid (aa 29 to 66) or GFP (aa 3 to 121) genes or both deletions together (Fig. 3A). Interestingly, the deletions occurred between very short (UAAA) repeats, located in capsid sequence (in the loops of the computer-predicted secondary structure), and UGGUGA repeats in the GFP gene. These sequencing data were insufficient for a conclusive understanding of which deletion had a critical positive effect on virus replication. Therefore, both GFP- and capsid-specific deletions were cloned into the YF/C/GFP/C genome (Fig. 3A). The in vitro-synthesized RNAs were transfected into BHK-21 cells, and only the deletion in capsid demonstrated a positive effect on the yield of infectious virus release. The recombinants YF/ΔC/GFP/C and YF/ΔC/ΔGFP/C, but not the YF/C/ΔGFP/C, demonstrated growth rates that were similar to those of YFV 17D (Fig. 3B). Thus, the results of these experiments suggested that the modification of the first capsid-coding sequence might be a very efficient means of altering the replication efficiency of the virus and the construction of the variants capable of efficient propagation in tissue culture.

Development of YFV capable of expressing heterologous genes. The experiments with YF/ΔC/GFP/C suggested a possibility of modification of not only the YF/C/Cherry genome used in the transcomplementation experiments but also the wt YF 17D genome for making YFV capable of stable expression of the heterologous genes. Therefore, we designed two recombinant YFV genomes, YF/GFP and YFmut/GFP, in which the 75-nt fragment of the capsid-coding sequence was cloned upstream of the GFP and FMDV 2A genes, fused in-frame with the entire YFV polyprotein-coding sequence (Fig. 4A), containing a codon-optimized capsid gene. YF/GFP exhibited no any other changes in the 5′-terminal sequence and, in the YFmut/GFP, the additional modifications were as follows: (i) the UGGUGA sequence in GFP was replaced by UCGUCA that did not change the encoded protein sequence but modified one of the repeats found to be used during the deletion formation in the YF/C/ΔGFP and YF/ΔC/ΔGFP genomes and (ii) a short fragment between the cyclization sequence and GFP was modified by making three single-nucleotide insertions. The GFP-specific mutations were made to additionally decrease the possibility of recombination in the GFP gene, leading to deletions of the coding sequence, and the changes in the capsid-coding sequence were made to avoid possible recombination between the residual, 75-nt sequence in the beginning of the ORF with the codon-optimized capsid gene located downstream of the GFP. The in vitro-synthesized RNAs were transfected into BHK-21 cells, and essentially all of the cells demonstrated very similar GFP expression levels detectable within 18 h posttransfection, which suggested that both viruses were viable and did nor require additional adaptation for replication. The YF/GFP and YFmut/GFP viruses were less cytopathic than the wt YFV 17D, and the GFP-positive cells continued to grow until they reached a complete confluence. However, in spite of the reduced cytopathogenicity, both viruses were capable of efficient replication and accumulated in the medium to titers higher than 5×10^8 i.u./ml (Fig. 4B).

To evaluate the stability of the GFP insertion, one of the stocks of YFmut/GFP virus was blindly passaged five times in BHK-21 cells (see Materials and Methods for details), and we did not detect any significant change in the titers of the harvested samples (Fig. 4C). After five passages, 11% of the foci, formed under agarose cover, were GFP negative but stained by YFV-specific antibodies. These GFP-negative variants were still incapable of developing well-defined plaques, and thus the mutations likely accumulated in the GFP gene due to a lack of positive selection for functional protein but not as a result of the selection of better-replicating virus. The PCR-based analysis of the GFP-encoding region also did not detect fragments that were noticeably shorter than expected. In another experiment, we intracranially inoculated litters of 2- to 4-day-old mice with 1.6×10^6 and 1.6×10^4 i.u. of YF/GFP. All of the mice developed clinical signs of encephalitis and were euthanized at day 8 postinfection. The brains of the mice infected with high dose demonstrated the presence of the GFP-expressing virus at a concentration $2.46 \pm 0.68 \times 10^8$ i.u./g. These mouse-passaged viruses did not appear to have gained higher virulence since they neither expressed higher levels of GFP nor demonstrated more CPE in cell culture. Interestingly, in these samples, more than 97% of the virus continued to express GFP. These data suggested that the strategy of modifying the YFV genome, in which we separate the functional polyprotein-coding sequence and the 5′ promoter elements opens an opportunity for the stable expression of heterologous proteins as part of infectious YFV. YF/GFP and YFmut/GFP demonstrate very similar characteristics of replication; nevertheless, the YFmut-based vectors are probably more efficacious for studies that require long-term experiments and/or serial virus passaging.

Transcomplementation between two defective YFV genomes. Based on the data from the above-described experiments with YF/ΔC/GFP/C virus, we designed a defective-in-replication

YFV genome, YF/Cherry/Cco (Fig. 5A) that was capable of expressing the capsid gene and had the prM/E-coding sequence deleted. It encoded an YFV 5′UTR, followed by 25 aa of capsid, Cherry, FMDV 2A, Cco with a prM-specific signal peptide, and the carboxy-terminal fragment of E protein required for proper processing and compartmentalization of the following NS1-5 polyprotein. The in vitro-synthesized YF/Cherry/Cco and the transcomplementing counterpart, YF/GFP/prME, genomes were transfected into BHK-21 cells. They complemented each other's deficiencies in structural protein synthesis, and the cells efficiently released infectious viral particles having either capsid- or prM/E-encoding defective genomes with Cherry or GFP genes, respectively (Fig. 5B). Importantly, both genomes were packaged to very similar titers approaching 10^8 i.u./ml. These replicating genomes caused CPE inefficiently and readily established a persistent infection. The cells continued to grow and produced viruses not only within 4 to 5 days posttransfection but also after their passaging.

To test the possible large-scale production, virus stocks were further passaged in naive BHK-21 cells, and titers of particles containing each genome approached 10^8 i.u./ml (Fig. 5C). Moreover, it was not necessary to perform passaging at a high MOI. Cells infected at an MOI of ~1 i.u./cell released packaged genomes as efficiently as did cell infected at an MOI of ~10 i.u./cell (Fig. 5D). However, an additional decrease in MOI to ~0.1 i.u./cell resulted in noticeably lower virus titers. In the cell monolayers infected at an MOI of 1, we could detect the cells expressing only one marker (GFP or Cherry), but a large fraction of them (~50%) expressed both fluorescent proteins (Fig. 5E). In all of the experiments, titers of both packaged genomes were very close, and the MOI was calculated based on the titer of YF/GFP/prME-containing particles.

Analysis of virus density in the sucrose gradients demonstrated that cells transfected with YF/GFP/prME RNA released only low-density viral particles, corresponding to prM/E containing SVPs that lack a nucleocapsid and RNA. However, replication of two transcomplementing genomes led to the release of both low- and high-density particles demonstrating in the sucrose density gradient the same distribution as the samples of wt YFV 17D. These data additionally indicated that a virus with a two-component genome demonstrates characteristics similar to those of YFV 17D.

Packaging of the YFV replicons lacking structural genes. In our previous study (30), we developed a cell line expressing the YFV C-prM-E and Copt-prM-E cassette (encoding wt and codon-optimized capsids, respectively) from a persistently replicating VEEV replicon (30). These cell lines efficiently functioned in packaging the YF/GFP/prME-defective virus genome, and this activity indicated that capsid protein was produced and properly processed for genome incapsidation. However, the same cell lines were inefficient in packaging YF replicons encoding no structural proteins. As a result, titers of packaged replicons were always less than 10^7 i.u./ml. The reason for this low packaging level was not clear, but these data correlated with the previously published results of another study, in which Sindbis virus replicons producing YF C-prM-E cassette packaged similar YF replicons inefficiently as well (12). To test the possibility of packaging YF replicons to higher titers, we designed VEEV replicons encoding YFV C-prM-E in the same fusion protein as in the YF/GFP/Cco viral genome. One of the subgenomic RNAs encoded ORF that started with 25 aa of capsid protein, continued into the GFP gene, FMDV 2A protease, codon-optimized capsid, and prM/E coding sequence (Fig. 6B). The GFP sequence was left in the expression cassette to examine the possibility that VEEV replicons are copackaged into YFV-specific viral particles. The second subgenomic RNA drove the expression of the PAC gene, encoding puromycin acetyltransferase that makes cells resistant to translational arrest caused by the presence of puromycin in the medium. The in vitro-synthesized VEErep/GFP-C-prM-E/Pac RNA was transfected into BHK-21 cells, and a puromycin-resistant cell line was established within a few days of puromycin selection. The cells were then transfected with YFV replicon (YFrep/Cherry), in which all of the structural genes were replaced by a Cherry-coding sequence (Fig. 6A). As indicated in Fig. 6B, the cell line packaged the latter replicon to markedly higher titers and continued to produce infectious particles within a few days without development of profound CPE (Fig. 6C). The YF replicon-containing cells continued to grow and, finally, we terminated the experiments because the cells expressing both GFP and Cherry reached a confluence that caused their death. In multiple experiments, we never detected packaging of VEEV replicons into YFV structural proteins. An additional advantage of using the VEErep/GFP-C-prM-E/Pac-containing cell line lies in the possibility of using it for further passaging of YFV replicons (Fig. 6C). These cells could be infected with the previously packaged constructs, and this led to the development of a spreading infection and the release of replicon-containing particles to titers approaching 10^8 i.u./ml.

DISCUSSION

To date, flaviviruses remain a major public health concern. They are widely distributed in both hemispheres and cause a variety of human-associated diseases. However, safe and efficient vaccines have been produced against a handful of flavivirus infections. They are characterized as either live attenuated or inactivated. Approved vaccines were developed against JEV, TBEV, and YFV, but no licensed products have yet been produced against other flaviviruses, such as dengue virus and WNV. Live vaccines appear to be more efficient than the inactivated viruses or subunit vaccines; however, the obvious safety concerns remain because of the possibility of the reversion of attenuated viruses to a pathogenic phenotype. Application of inactivated vaccines usually requires multiple vaccinations and production of large amounts of material and the need for high-containment facilities to propagate the virulent viruses used for making inactivated products. Thus, although there are promising candidates for both types of flavivirus vaccines, there is no universal approach for their development.

One of the distinguishing features of flaviviruses is the ability of the envelope proteins to form so-called SVPs. Such particles can be efficiently produced by eukaryotic cells containing standard prM/E glycoprotein-expressing vectors (1, 29) or by the defective flavivirus genomes with a capsid gene deleted (2, 30). These SVPs lack genetic material and the entire nucleocapsid but function as efficient immunogens and induce a protective immune response against future infection with the replication-competent flaviviruses. The defective flavivirus genomes, lacking a capsid-coding sequence, can be either delivered into the cells in the RNA form (16) or packaged into infectious viral

particles using packaging cell lines (30), in which capsid is supplied in *trans* by, for example, the persistently replicating alphavirus replicons encoding flavivirus capsid gene under the control of the subgenomic promoter. Upon infection of the naive cells both in vitro and in vivo, these pseudoinfectious flaviviruses are capable of replication and SVP production but do not develop spreading, productive infection. Therefore, their application does not lead to disease development, and they represent an interesting intermediate between live and inactivated viruses.

Application of PIVs for vaccine purposes requires their large-scale production, and the development of the cell lines that package the defective genomes into infectious virions demonstrated this possibility (30). PIVs can be passaged in the packaging cell lines, but not in the naive cells, at an escalating scale. However, this is not the only means of their large-scale propagation. In the present study, we developed another approach that does not require the development of such cell lines but leads to efficient PIV production as well. Flavivirus genetic material was separated between two viral genomes capable of transcomplementing each other's deficiencies. Each of the originally designed defective YFV genomes encoded the entire RNA replicative machinery; one of them had a deletion of almost the entire capsid gene, and the second genome encoded no prM/E. To monitor the replication of each genome in tissue culture and to measure the titers of the infectious particles, the genomes encoded different fluorescent markers, GFP and Cherry. Their expression in the cells indicated the infection and replication of the particular genome. Upon delivery to the same cells, the YF/GFP/prME and YF/C/Cherry were expected to produce the entire set of viral structural proteins and, ultimately, be packaged into infectious virions. However, surprisingly, the initial attempts to establish productive replication were unsuccessful due to the high cytotoxicity of the capsid-producing, YF/C/Cherry, genome replication. It produced very high levels of fluorescent protein but also caused a robust CPE that resulted in a low-level release of the infectious viral particles. To further understand this phenomenon, we designed a YFV genome that encoded two copies of the capsid gene, and one of them could be exploited for extensive genetic manipulation. This YFV was also unusually cytotoxic and replicated to low titers. Later modifications of the capsid-coding sequence strongly indicated that the increase in cytotoxicity was caused by capsid protein itself (when it was expressed outside of the context of the C-prM-E cassette), rather than by possible changes in the RNA secondary structure (Fig. 2 and 3). Moreover, the YF/C/GFP/C virus with two copies of capsid gene in the genome could further evolve and develop variants adapted for growth to higher titers with lower levels of CPE development. To date, the exact mechanism of the effect of YFV capsid expression by YF/C/Cherry or YF/C/GFP/C viruses on CPE induction remains unclear, but the detailed investigation of this phenomenon was not the goal of the present study.

Sequencing of the YF/C/GFP/C variants adapted to a higher level of virus release provided us with a means of generating modified infectious viruses capable of the stable expression of additional heterologous proteins in vivo and in vitro. The designed infectious YFmut/GFP and YF/GFP viruses exhibited higher stability of insertions than that described for IRES/GFP cassettes cloned into 3′UTR of flavivirus genome (34). However, most importantly, the identified spontaneous deletions presented an opportunity to modify the originally designed, defective-in-replication YF/C/Cherry virus genome into YF/Cherry/Cco that had a different protein-coding strategy and was capable of efficient trans-complementation of the YF/GFP/prME replication. Cells cotransfected with the in vitro-synthesized RNA of both genomes produced viral particles, in which both capsid or prME-encoding genomes were present at the same concentration, and this unusual virus could be further passaged in naive cells at an escalating scale. Infection of cells at low MOIs unambiguously demonstrated that both genomes were packaged into separate viral particles, and therefore this YFV, having two genomes with complementary functions, cannot be termed a segmented genome virus (that is supposed to have all of the genome fragments packaged into the same virion) but is rather a two-component genome virus. Viruses of such types, with both genome segments packaged separately, were previously described in plants (24) and for Sindbis virus (8, 10). Further application of such viruses to immunization protocols might raise concern about the possible recombination between two genomes that could lead to the formation of an infectious, complete, replication-competent virus. Although this is a highly unlikely event, the capsid gene in the YF/Cherry/C RNA was represented by a synthetic, codon-optimized version, lacking a cyclization sequence. In multiple experiments with two-component genome YFV, we never detected the formation of infectious YFV with the unfragmented genome; however, it is possible to additionally reduce the possibility of recombination by using different pairs of cyclization sequences in the capsid- and prME-encoding genomes.

Interestingly, modification of the C-prM-E coding strategy in the not YFV genome-based constructs led to a dramatic increase in the packaging of the YFV vectors that encode no structural proteins at all. The cell lines producing 25 aa of C-GFP-Copt-prM-E from the persistently replicating VEErep/GFP-C-prM-E/Pac packaged YFV replicons to dramatically higher titers than those obtained in similar cell lines expressing the C-prM-E cassette only. Packaged YFV replicons were not only released to titers greater than 10^8 i.u./ml but could also be passaged in this packaging cell line without a decrease in titers. Thus, simple modification of C-prM-E coding subgenomic RNA by cloning 25 capsid-specific codons upstream of the structural polyprotein had a very strong positive impact on infectious particles release and might widen the number of YFV-based vectors for delivery and expression of heterologous genetic information. Our working hypothesis is that the unusual strategy of C-prM-E expression leads to a different compartmentalization of the translated structural proteins that promotes the infectious virions formation. However, this possibility requires more detailed investigation.

In conclusion, the results of the present study suggest several things. (i) YF PIV, which is capable of prM/E expression, can be passaged in tissue culture using another defective-in-replication, capsid-producing flavivirus genome as a helper (Fig. 7). (ii) During replication in the same cell, these two defective genomes produce a complete set of viral structural proteins and are efficiently packaged into separate infectious viral particles that can be characterized as a two-component genome virus (Fig. 7). As we

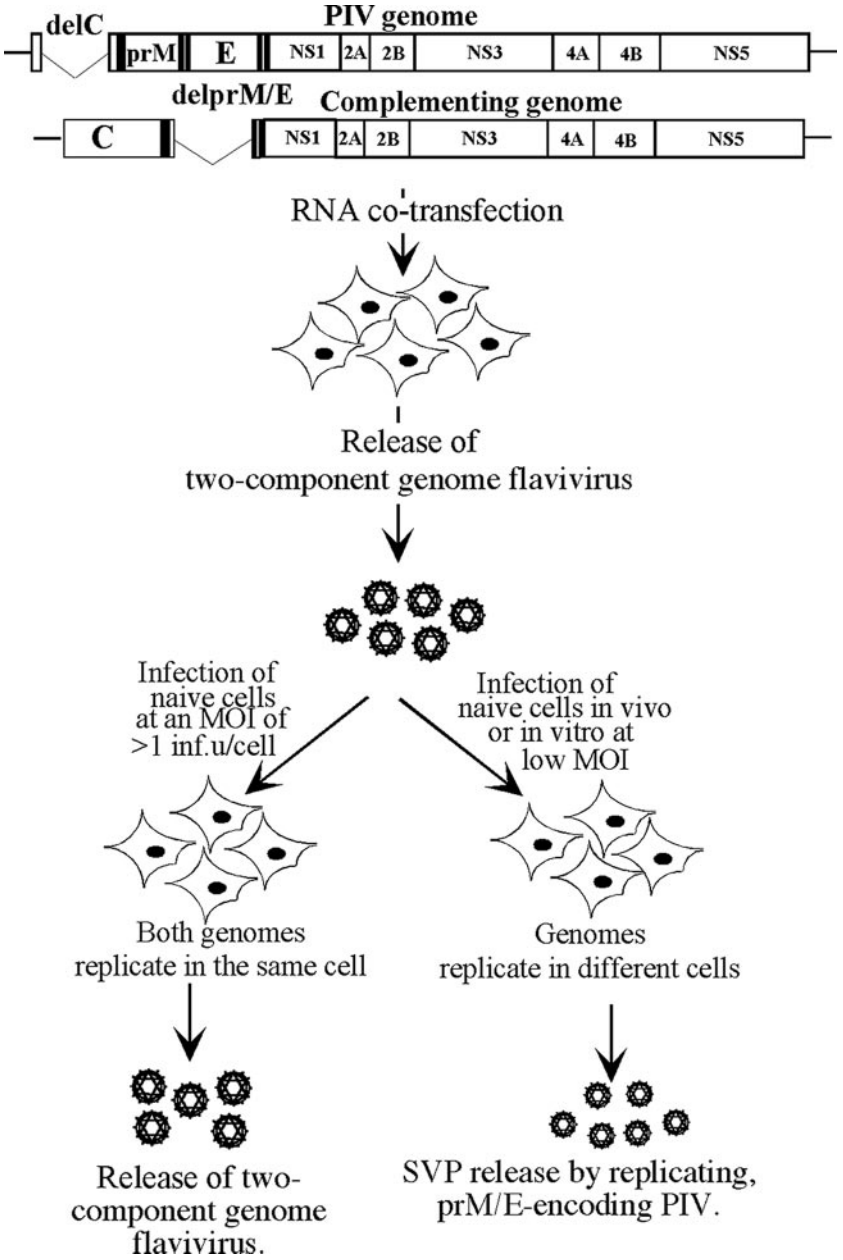


FIG. 7. Proposed replication strategies of the two-component genome virus at high and low MOIs. At a high MOI, both genomes, the PIV genome (encoding prM/E) and complementing genome (encoding capsid), are delivered to the same cell and produce a complete set of proteins required for virus replication. Cells produce a two-component genome virus that can be further passaged at an escalating scale. At a low MOI, cells receive only one of the genomes, and those infected with PIV produce SVPs containing no genetic material and nucleocapsid.

and other groups previously demonstrated, pseudoinfectious flaviviruses can serve as efficient immunogens, and thus the two-component genome virus might be used for the development of recombinant flavivirus-specific vaccines (2, 30). In additional experiments, the two-component genome WNV was viable and replicated to high titers (P. W. Mason, unpublished data); therefore, this strategy of pseudoinfectious virus production can probably be applied to other flavi- and pestiviruses and to hepatitis C virus. (iii) Expression of capsid from the YFV genome with the deleted prM/E genes required additional modification of the 5′-terminal sequence. Application of the same modifications to the replication-competent YFV led to the development of a virus that is capable of expressing additional genetic information, and designing of the same YFV C-prM-E expression cassettes in the VEEV replicons drastically improved trans-packaging of YFV-based vectors. (iv) Separation of the capsid-coding sequence and the promoter elements, either in the YFV genome or in the capsid-coding, defective-in-replication YFV genome provides an opportunity for expression of the structural genes derived from heterologous flaviviruses independently of the cyclization signal and represents a possible means for studying the mechanism of the packaging process.

ACKNOWLEDGMENTS

We thank Mardelle Susman for critical reading and editing of the manuscript.

This study was supported by Public Health Service grant AI053135 to I.F. and a grant from NIAID to P.W.M. through the Western Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research (NIH grant U54 AI057156).

REFERENCES

1. **Aberle, J. H., S. W. Aberle, S. L. Allison, K. Stiasny, M. Ecker, C. W. Mandl, R. Berger, and F. X. Heinz.** 1999. A DNA immunization model study with constructs expressing the tick-borne encephalitis virus envelope protein E in different physical forms. J. Immunol. **163:**6756–6761.
2. **Aberle, J. H., S. W. Aberle, R. M. Kofler, and C. W. Mandl.** 2005. Humoral and cellular immune response to RNA immunization with flavivirus replicons derived from tick-borne encephalitis virus. J. Virol. **79:**15107–15113.
3. **Bredenbeek, P. J., I. Frolov, C. M. Rice, and S. Schlesinger.** 1993. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. J. Virol. **67:**6439–6446.
4. **Bredenbeek, P. J., E. A. Kooi, B. Lindenbach, N. Huijkman, C. M. Rice, and W. J. Spaan.** 2003. A stable full-length yellow fever virus cDNA clone and the role of conserved RNA elements in flavivirus replication. J. Gen. Virol. **84:**1261–1268.
5. **Chambers, T. J., A. Nestorowicz, P. W. Mason, and C. M. Rice.** 1999. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. J. Virol. **73:**3095–3101.
6. **Colombage, G., R. Hall, M. Pavy, and M. Lobigs.** 1998. DNA-based and alphavirus-vectored immunization with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology **250:**151–163.
7. **Davis, B. S., G. J. J. Chang, B. Cropp, J. T. Roehrig, D. A. Martin, C. J. Mitchell, R. Bowen, and M. L. Bunning.** 2001. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J. Virol. **75:**4040–4047.
8. **Fayzulin, R., R. Gorchakov, O. Petrakova, E. Volkova, and I. Frolov.** 2005. Sindbis virus with a tricomponent genome. J. Virol. **79:**637–643.
9. **Fayzulin, R., F. Scholle, O. Petrakova, I. Frolov, and P. W. Mason.** 2006. Evaluation of replicative capacity and genetic stability of West Nile virus replicons using highly efficient packaging cell lines. Virology **351:**196–209.
10. **Geigenmuller-Gnirke, U., B. Weiss, R. Wright, and S. Schlesinger.** 1991. Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome. Proc. Natl. Acad. Sci. USA **88:**3253–3257.
11. **Guirakhoo, F., J. Arroyo, K. V. Pugachev, C. Miller, Z. X. Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran, K. Soike, M. Ratterree, and T. P. Monath.** 2001. Construction, safety, and immunogenicity in nonhuman primates of a chimeric yellow fever-dengue virus tetravalent vaccine. J. Virol. **75:**7290–7304.
12. **Jones, C. T., C. G. Patkar, and R. J. Kuhn.** 2005. Construction and applications of yellow fever virus replicons. Virology **331:**247–259.
13. **Khromykh, A. A., and E. G. Westaway.** 1997. Subgenomic replicons of the flavivirus Kunjin: construction and applications. J. Virol. **71:**1497–1505.
14. **Kochel, T., S. J. Wu, K. Raviprakash, P. Hobart, S. Hoffman, K. Porter, and C. Hayes.** 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. Vaccine **15:**547–552.
15. **Kochel, T. J., K. Raviprakash, C. G. Hayes, D. M. Watts, K. L. Russell, A. S. Gozalo, I. A. Phillips, D. F. Ewing, G. S. Murphy, and K. R. Porter.** 2000. A dengue virus serotype-1 DNA vaccine induces virus neutralizing antibodies and provides protection from viral challenge in *Aotus* monkeys. Vaccine **18:**3166–3173.
16. **Kofler, R. M., J. H. Aberle, S. W. Aberle, S. L. Allison, F. X. Heinz, and C. W. Mandl.** 2004. Mimicking live flavivirus immunization with a noninfectious RNA vaccine. Proc. Natl. Acad. Sci. USA **101:**1951–1956.
17. **Konishi, E., and A. Fujii.** 2002. Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. Vaccine **20:**1058–1067.
18. **Konishi, E., A. Fujii, and P. W. Mason.** 2001. Generation and characterization of a mammalian cell line continuously expressing Japanese encephalitis virus subviral particles. J. Virol. **75:**2204–2212.
19. **Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, and F. A. Ennis.** 1997. Poxvirus-based Japanese encephalitis vaccine candidates induce JE virus-specific $CD8^+$ cytotoxic T lymphocytes in mice. Virology **227:**353–360.
20. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology **188:**714–720.
21. **Konishi, E., M. Yamaoka, I. Kurane, and P. W. Mason.** 2000. A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine **18:**1133–1139.
22. **Konishi, E., M. Yamaoka, I. Kurane, and P. W. Mason.** 2000. Japanese encephalitis DNA vaccine candidates expressing premembrane and envelope genes induce virus-specific memory B cells and long-lasting antibodies in swine. Virology **268:**49–55.
23. **Kuhn, R. J., W. Zhang, M. G. Rossmann, S. V. Pletnev, J. Corver, E. Lenches, C. T. Jones, S. Mukhopadhyay, P. R. Chipman, E. G. Strauss, T. S. Baker, and J. H. Strauss.** 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell **108:**717–725.
24. **Lazarowitz, S. G.** 2001. Plant viruses, p. 533–998. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott-Raven Publishers, Philadelphia, PA.
25. **Lemm, J. A., R. K. Durbin, V. Stollar, and C. M. Rice.** 1990. Mutations which alter the level or structure of nsP4 can affect the efficiency of Sindbis virus replication in a host-dependent manner. J. Virol. **64:**3001–3011.
26. **Liljestrom, P., S. Lusa, D. Huylebroeck, and H. Garoff.** 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. J. Virol. **65:**4107–4113.
27. **Lindenbach, B. D., and C. M. Rice.** 2001. *Flaviviridae*: the viruses and their replication, p. 991–1041. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott-Raven Publishers, Philadelphia, PA.
28. **Lorenz, I. C., S. L. Allison, F. X. Heinz, and A. Helenius.** 2002. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J. Virol. **76:**5480–5491.
29. **Mason, P. W., S. Pincus, M. J. Fournier, T. L. Mason, R. E. Shope, and E. Paoletti.** 1991. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Virology **180:**294–305.
30. **Mason, P. W., A. V. Shustov, and I. Frolov.** 2006. Production and characterization of vaccines based on flaviviruses defective in replication. Virology **351:**432–443.
31. **Monath, T. P., K. McCarthy, P. Bedford, C. T. Johnson, R. Nichols, S. Yoksan, R. Marchesani, M. Knauber, K. H. Wells, J. Arroyo, and F. Guirakhoo.** 2002. Clinical proof of principle for ChimeriVax: recombinant live, attenuated vaccines against flavivirus infections. Vaccine **20:**1004–1018.
32. **Petrakova, O., E. Volkova, R. Gorchakov, S. Paessler, R. M. Kinney, and I. Frolov.** 2005. Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in mammalian cells. J. Virol. **79:**7597–7608.
33. **Phillpotts, R. J., K. Venugopal, and T. Brooks.** 1996. Immunization with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. **141:**743–749.
34. **Pierson, T. C., M. S. Diamond, A. A. Ahmed, L. E. Valentine, C. W. Davis, M. A. Samuel, S. L. Hanna, B. A. Puffer, and R. W. Doms.** 2005. An infectious West Nile virus that expresses a GFP reporter gene. Virology **334:**28–40.
35. **Pugachev, K. V., F. Guirakhoo, D. W. Trent, and T. P. Monath.** 2003. Traditional and novel approaches to flavivirus vaccines. Int. J. Parasitol. **33:**567–582.
36. **Pugachev, K. V., P. W. Mason, R. E. Shope, and T. K. Frey.** 1995. Double-subgenomic Sindbis virus recombinants expressing immunogenic proteins of Japanese encephalitis virus induce significant protection in mice against lethal JEV infection. Virology **212:**587–594.
37. **Qiao, M., M. Ashok, K. A. Bernard, G. Palacios, Z. H. Zhou, W. I. Lipkin, and T. J. Liang.** 2004. Induction of sterilizing immunity against West Nile Virus (WNV), by immunization with WNV-like particles produced in insect cells. J. Infect. Dis. **190:**2104–2108.
38. **Schalich, J., S. L. Allison, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J. Virol. **70:**4549–4557.
39. **Schmaljohn, C., L. Vanderzanden, M. Bray, D. Custer, B. Meyer, D. Li, C. Rossi, D. Fuller, J. Fuller, J. Haynes, and J. Huggins.** 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. **71:**9563–9569.
40. **Shaner, N. C., R. E. Campbell, P. A. Steinbach, B. N. Giepmans, A. E. Palmer, and R. Y. Tsien.** 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nat. Biotechnol. **22:**1567–1572.
41. **Zhang, Y., J. Corver, P. R. Chipman, W. Zhang, S. V. Pletnev, D. Sedlak, T. S. Baker, J. H. Strauss, R. J. Kuhn, and M. G. Rossmann.** 2003. Structures of immature flavivirus particles. EMBO J. **22:**2604–2613.