

Encapsidation of the Flavivirus Kunjin Replicon RNA by Using a Complementation System Providing Kunjin Virus Structural Proteins *in trans*

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Kunjin virus (KUN) replicon RNA was encapsidated by a procedure involving two consecutive electroporations of BHK-21 cells, first with KUN replicon RNA C20DXrep (with prME and most of C deleted) and about 24 h later with a recombinant Semliki Forest virus (SFV) replicon RNA(s) expressing KUN structural proteins. The presence of KUN replicon RNA in encapsidated particles was demonstrated by its amplification and expression in newly infected BHK-21 cells, detected by Northern blotting with a KUN-specific probe and by immunofluorescence analysis with anti-NS3 antibodies. No infectious particles were produced when C20DXrep RNA and recombinant SFV RNAs were electroporated simultaneously. When the second electroporation was performed with a single SFV replicon RNA expressing the KUN contiguous prME genes and the KUN C gene together but under control of two separate 26S subgenomic promoters (SFV-prME-C107), a 10-fold-higher titer of infectious particles was achieved than when two different SFV replicon RNAs expressing the KUN C gene (SFV-C107) and prME genes (SFV-prME) separately were used. No SFV replicon RNAs expressing KUN structural proteins were encapsidated in secreted particles. Infectious particles pelleted by ultracentrifugation of the culture fluid from cells sequentially transfected with C20DXrep and SFV-prME-C107 RNAs were neutralized by preincubation with monoclonal antibodies to KUN E protein. Radioimmunoprecipitation analysis with anti-E antibodies of the culture fluid of the doubly transfected cells showed the presence of C, prM/M, and E proteins in the immunoprecipitated particles. Reverse transcription-PCR analysis showed that the immunoprecipitated particles also contained KUN-specific RNA. The encapsidated replicon particles sedimented more slowly than KUN virions in a 5 to 25% sucrose density gradient and were uniformly spherical, with an ~35-nm diameter, compared with ~50 nm for KUN virions. The results of this study demonstrate for the first time packaging of flavivirus RNA *in trans*, and they exclude a role in packaging for virtually all of the structural region. Possible applications of the developed packaging system include the definition of the packaging signal(s) in flavivirus RNA as well as the amino acid motif(s) in the structural proteins involved in RNA encapsidation, virion assembly, and secretion. Furthermore, it could facilitate the development of a noninfectious vaccine delivery system based on encapsidation of a noncytopathic flavivirus replicon expressing heterologous genes.

Kunjin virus (KUN) is an endemic Australian flavivirus that occasionally causes febrile illness or mild encephalitis in humans. The KUN genome consists of positive-stranded RNA of 11,022 nucleotides (13) containing one long open reading frame coding for three structural (C, prM, and E) and seven nonstructural (NS) proteins in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (8). Recently we described the preparation of the first flavivirus replicon (self-replicating) RNAs with deletions in the structural region and deletions and insertions in the 3' untranslated region (UTR) (15). Using KUN replicon RNA with an insertion in the 3' UTR of the neomycin gene under control of the internal ribosomal entry site of encephalomyelocarditis virus RNA, we were able to establish BHK cell lines persistently expressing this replicon RNA (15). Importantly, replication of KUN replicon RNAs either after transfection into BHK cells or in a cell line persistently expressing the KUN replicon did not produce any cytopathic effect; this distinguishes KUN replicons from the cytopathic replicons of other positive-stranded RNA viruses such as Semliki Forest virus (SFV), Sindbis virus (SIN), and polio-

virus, which are widely used as vectors for expression of heterologous genes *in vitro* (for references, see reference 15) and *in vivo* (10, 12, 42). The success of these expression systems was based mainly on the ability to produce high-titer stocks of pseudoinfectious particles containing replicon RNA packaged by structural proteins. In SFV and SIN alphavirus expression systems, this was achieved by cotransfection of replicon RNA with a defective helper RNA(s) expressing structural genes but lacking the packaging signal (6, 22). The replicon RNA expression provides the enzymes for RNA replication and transcription of both RNAs, whereas the helper alphavirus RNA supports the production of structural proteins for packaging of replicon RNA via expression of its subgenomic region.

Flaviviruses and picornaviruses differ from the alphaviruses SFV and SIN by their genome structure (structural genes situated at the 5' end of the genome) and by the absence of synthesis of subgenomic RNA (31, 35, 38). There are no data to date on packaging of flavivirus RNA. Packaging of poliovirus replicon RNA was successfully achieved either by superinfection of replicon-transfected cells with a helper poliovirus (25, 26) or by transfection of replicon RNA into cells previously infected with recombinant vaccinia virus expressing structural proteins (4, 27). Although infectious particles containing encapsidated poliovirus replicon RNA were produced under these conditions, additional purification of particles was

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required in order to separate them from recombinant vaccinia virus.

The goal of these studies was to develop a KUN replicon packaging system for possible future use in defining the flavivirus RNA packaging signal(s) and in expression of heterologous genes, taking advantage of the noncytopathic nature and persistence of replication of the KUN replicon. Successful packaging of KUN replicon RNA was achieved when an SFV replicon RNA(s) expressing KUN structural proteins was electroporated at least 12 h after electroporation of KUN replicon RNA. A single recombinant SFV RNA expressing the KUN prME and KUN C proteins together but under control of two separate 26S promoters was more efficient in packaging experiments than were two SFV RNAs expressing KUN prME and KUN C separately. We demonstrated that the resulting secreted infectious particles were uniformly spherical with an ~35-nm diameter and contained replicating KUN replicon RNA encapsidated by KUN structural proteins C, prM/M, and E. These findings represent the first demonstration of the packaging of flavivirus RNA *in trans* and may prove to be useful for identification of the packaging signal(s) and for development of a vaccine delivery system based on expression from a noncytopathic flavivirus replicon.

MATERIALS AND METHODS

Cells. BHK21 cells were grown in Dulbecco's modification of minimal essential medium (Gibco BRL) supplemented with 10% fetal bovine serum at 37°C in a CO₂ incubator.

Construction of the plasmids. (i) **C20DXrep.** The KUN replicon cDNA construct C20DXrep was constructed from the previously described C20rep (with the structural gene sequence except for the first 20 codons deleted) (15) by replacing an *SphI*²⁴⁶⁷-*XhoI*¹¹⁰²¹ fragment representing the sequence coding for the entire nonstructural region and the 3' UTR with the corresponding fragment from a stable full-length KUN cDNA clone, FLSDX. Details of clone FLSDX will be described elsewhere; its RNA transcripts have a specific infectivity of about 10⁴ PFU/μg, which is about 100,000-fold more infectious than those from our previously described KUN full-length cDNA clone pAKUN (13).

(ii) **SFV-C107.** An SFV replicon construct expressing the KUN core (C) gene was obtained by cloning of the *BglII*-*BamHI* fragment, representing the sequence commencing with the last 7 nucleotides of the KUN 5' UTR followed by the sequence coding for the first 107 of the 123 amino acids of the KUN C protein, from the plasmid pCINeoC107 (14) into the *BamHI* site of the SFV replicon expression vector pSFV1 (Gibco BRL) (Fig. 1). This sequence approximates the size of the mature form of C (comprising the first 105 amino acids) after the carboxy-terminal hydrophobic sequence has been cleaved by the viral protease (33). The resulting translation product of the cloned fragment is likely to contain an extra six amino acids relative to mature C, comprising two native KUN amino acids (GG) downstream of cleavage site plus four amino acids (IPGN) derived from the SFV vector (Fig. 1).

(iii) **SFV-prME.** The KUN prME sequence was PCR amplified from another highly efficient full-length KUN cDNA clone, FLBSDX, modified from FLSDX (see "C20DXrep" above), by using appropriate primers with incorporated initiation and termination codons and flanked by *BglII* sites. To minimize the number of possible mismatches which could occur during PCR amplification, we used high-fidelity *Pfu* DNA polymerase (Stratagene) in our PCRs. The amplified fragment was digested with *BglII* and cloned into the *BamHI* site of the SFV replicon expression vector pSFV1 to obtain the SFV-prME construct (Fig. 1).

(iv) **SFV-prME-C107.** An SFV replicon construct expressing both the KUN prME and KUN C genes was obtained by cloning the *MscI*-*SpeI* fragment from the SFV-C107 plasmid containing the SFV 26S subgenomic promoter, KUN C sequence, and SFV 3' UTR into the SFV-prME vector digested with *SmaI* and *SpeI* (Fig. 1). Thus, the final double subgenomic promoter construct SFV-prME-C107 should produce SFV replicon RNA which upon transfection into BHK cells will direct synthesis of two subgenomic RNAs expressing the KUN prME and KUN C genes.

RNA transcription and transfection. KUN replicon RNA transcripts were prepared from C20DXrep plasmid DNA linearized with *XhoI* and from SFV plasmids linearized with *SpeI* by using SP6 RNA polymerase essentially as described previously (13, 15). Electroporation of RNAs into BHK21 cells was performed as described previously (15). Briefly, 10 to 20 μg of *in vitro*-transcribed RNAs was electroporated into 2 × 10⁹ BHK21 cells in 400 μl in a 0.2-cm cuvette (Bio-Rad) by using the Bio-Rad Gene Pulser apparatus.

IF analysis. Replication of KUN replicon RNA C20DXrep after initial electroporation and after infection of BHK cells in packaging experiments was monitored by immunofluorescence (IF) analysis with antibodies to KUN NS3

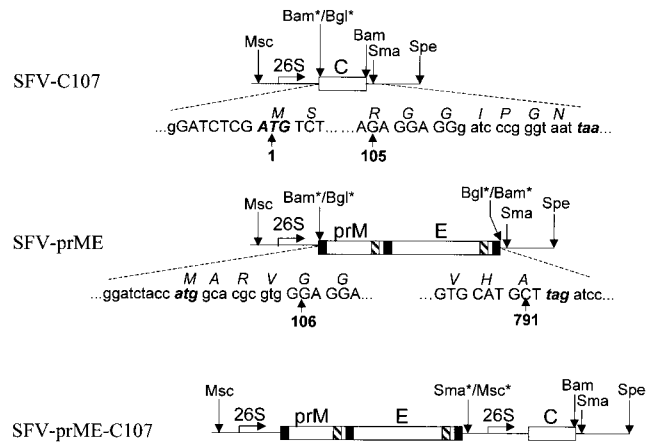


FIG. 1. Schematic representation of the recombinant SFV constructs. The solid line in all constructs represents the segment of the SFV replicon genome flanking the multiple cloning site; open boxes show the inserted KUN structural genes for C, prM, and E as indicated; 26S shows the position of the subgenomic SFV promoter; and the filled and hatched boxes in the KUN prM and E genes represent hydrophobic signal and anchor sequences, respectively (8). Uppercase letters in the nucleotide sequences show authentic KUN nucleotides; lowercase letters show nucleotides derived from the pSFV1 vector or encoded in the primers used for PCR amplification of KUN genes. Boldface and italicized letters show initiation (ATG) and termination (taa and tag) codons. Italicized letters above codons in the nucleotide sequence represent corresponding amino acids. Numbers with arrows represent amino acid positions in the KUN polyprotein (8). Msc, Sma, Spe, Bam, and Bgl represent specific restriction sites. Asterisks indicate that these restriction sites were destroyed during the cloning procedure (see Materials and Methods).

protein as described previously (15). Expression of KUN E protein after electroporation with SFV-prME and SFV-prME-C107 RNAs was detected by IF with a 1/10 dilution of a pool of mouse monoclonal antibodies to KUN E protein, designated 3.91D, 10A1, and 3.67G (1), which were generously provided by Roy Hall, University of Queensland, Brisbane, Australia. Expression and nuclear localization of KUN C protein after electroporation with SFV-C107 and SFV-prME-C107 RNAs were monitored by IF analysis with rabbit polyclonal antibodies to KUN C protein as described previously (37). Cells were fixed in acetone to detect cytoplasmic IF and in formaldehyde and methanol for nuclear IF (37).

Metabolic labeling and RIP analysis. Metabolic labeling with [³⁵S]methionine-cysteine of electroporated BHK cells was performed essentially as described in the *SFV Gene Expression System Manual* (21) with some minor modifications. Briefly, cells at 18 h after the electroporation with SFV RNAs (with or without prior electroporation with KUN replicon RNA), were pulse-labeled with [³⁵S]methionine-cysteine either for 4 h or for 1-2 h followed by different periods of incubation (chase) in medium with an excess of unlabeled methionine-cysteine. Cell culture fluid was collected for analysis of secreted proteins by electrophoresis and radioimmunoprecipitation (RIP). Labeled cells were lysed in buffer containing 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 2 mM EDTA, the nuclei were removed by low-speed centrifugation, and the lysate supernatant was used for parallel analysis with the culture fluid.

For RIP analysis, labeled cell culture fluids were first filtered through a 0.45-μm-pore-size filter (Sartorius AG, Gottingen, Germany) and digested with RNase A (20 μg per ml) for 30 min at 37°C to ensure the removal of membrane particulate material and naked RNA. Filtered and RNase-treated culture fluids, or untreated cell lysates, were then mixed with 1/20 volume of the pooled anti-E monoclonal antibodies (see above) or with rabbit anti-C antibodies and incubated overnight at 4°C with constant rotation in microcentrifuge tubes. Protein A-Sepharose beads were then added to a final concentration of about 1%, and incubation was continued for another 1 h at 4°C. After three washes with RIP assay buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid sodium salt, 0.1% sodium dodecyl sulfate [SDS]) and one wash with phosphate-buffered saline (PBS), the beads were resuspended in the SDS-gel sample buffer, boiled for 5 min, and subjected to electrophoresis in an SDS-polyacrylamide gel. After electrophoresis, the gels were dried and exposed to X-ray film.

Northern blot hybridization. Five micrograms of total RNA, isolated by using Trizol reagent (Gibco BRL) from BHK21 cells infected with culture fluid collected from cells doubly transfected with C20DXrep RNA and SFV RNAs expressing KUN structural proteins, was electrophoresed for Northern blotting as described previously (15). The hybridization probes were ³²P-labeled cDNA

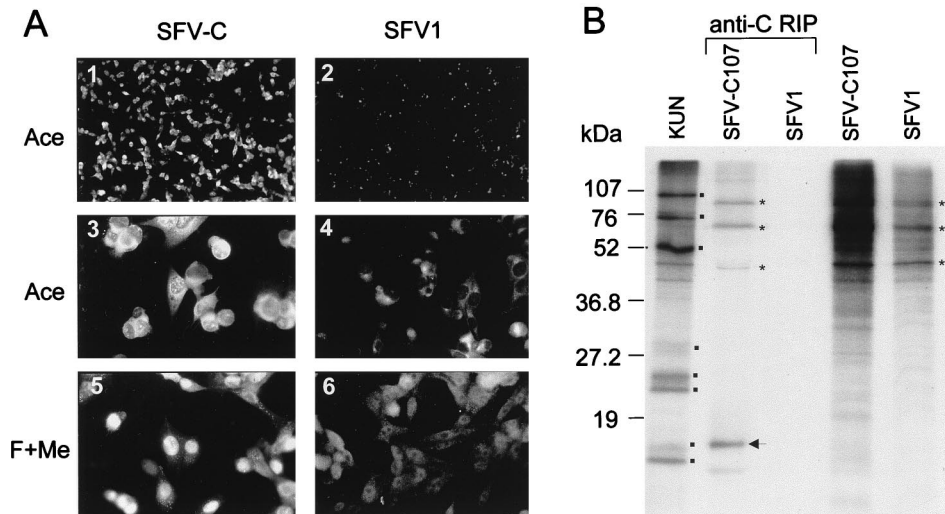


FIG. 2. Expression of KUN C protein by the recombinant SFV-C107 replicon. (A) IF analysis of BHK21 cells at 18 h after transfection with SFV-C107 RNA (panels 1, 3, and 5) by using KUN anti-C antibodies or of cells transfected with the control SFV1 RNA prepared from pSFV1 vector (panels 2, 4, and 6). Cells in panels 1 and 2 were photographed at a lower magnification than those in panels 3 to 6. Ace, acetone fixation; F+Me, formaldehyde-methanol fixation (for details of fixation, see reference 37). (B) RIP analysis with antibodies to C protein (anti-C) of SFV-C107- and SFV1-transfected BHK21 cells. Cells in 60-mm-diameter culture dishes at 18 h after transfection were labeled with 50 μ Ci of [³⁵S]methionine-cysteine per ml for 4 h. Labeled cell lysates for RIP were prepared as described in Materials and Methods, and samples were electrophoresed in an SDS-15% polyacrylamide gel. Sample volumes were 1 μ l of 500 μ l of lysate for SFV-C107, 0.5 μ l of 300 μ l of lysate for SFV1, and 10 μ l of 30 μ l total recovered after RIP of 160 μ l of both SFV-C107 and SFV1 (anti-C) cell lysates. Dots indicate the locations of KUN proteins NS5, NS3, E, NS4B, prM, NS2A, C, and NS4A/NS2B (from top to bottom) in the radiolabeled KUN-infected cell lysate. An arrow shows the position of KUN C protein. Asterisks show the locations in gels of strongly labeled SFV and cell proteins. Numbers represent sizes of low-range prestained Bio-Rad protein standards. This figure and Fig. 3 to 8 were prepared by scanning all the original data (slides and autoradiograms, etc.) on an Arcus II scanner (Agfa) with FotoLook software (Agfa) for Macintosh at a 150-lpi resolution, followed by assembling of the montages with Microsoft PowerPoint 97 software and printing on Epson Stylus Color 800 printer at a 720- to 1,440-dpi resolution with Epson photo quality ink jet paper.

fragments representing the 3'-terminal 761 nucleotides of the KUN genome, including the 3' UTR (see Fig. 5B and 6), and 446 nucleotides of the SFV NSP2 region (see Fig. 5C).

Preparation of encapsidated particles and determination of their titer. For all infections with encapsidated particles, cell culture fluid was membrane filtered and treated with RNase A as described above. To prepare partially purified particles, treated culture fluids from transfected cells were clarified by centrifugation at 16,000 \times g in a microcentrifuge for 15 min at 4°C, and the particles were pelleted from the resulting supernatant fluid by ultracentrifugation at 40,000 rpm for 2 h at 4°C in the AH650 rotor of a Sorvall OTD55B centrifuge. The pellets were resuspended in 50 μ l of PBS supplemented with RNase A (20 μ g per ml), left to dissolve overnight at 4°C, and then used for infection of BHK21 cells, for reverse transcription-PCR (RT-PCR) analysis, or for sedimentation analysis. To determine the titer of encapsidated particles, BHK21 cells on 1.3-cm² coverslips were infected with 100 or 200 μ l of serial 10-fold dilutions of cell culture fluid or of resuspended pelleted material for 1.5 h at 37°C. The fluid was then replaced with 1 ml of Dulbecco's minimal essential medium supplemented with 2% fetal bovine serum; cells were incubated for 24 h at 37°C in a CO₂ incubator and then subjected to IF analysis with anti-NS3 antibodies as described above. The infectious titer of packaged particles was calculated by using the following formula: titer (infectious units [IU]) per 2 \times 10⁶ initially transfected cells = $N \times (SW/SIA) \times 10^9 \times (V/VI)$, where N is the average number of anti-NS3-positive cells in the image area, calculated from five image areas in different parts of the coverslip; SW is the surface of the well in a 24-well plate (200 mm²); SIA is the surface of the image area (1.25 mm², using defined magnification parameters, calculated according to the manual for the Wild MPS46/52 photoautomat [Wild Leitz, Heerburg, Germany]); V is the total volume of the culture fluid (usually 3 to 5 ml per 60-mm-diameter dish) collected from the population of 2 \times 10⁶ initially electroporated BHK21 cells; VI is the volume used for infection of the coverslips (usually 100 to 200 μ l); and 10⁹ is the dilution factor.

RESULTS

The SFV replicon vector was chosen for the expression of KUN structural proteins for packaging because of the high level of cytoplasmic expression of heterologous proteins, rapid construction of recombinant RNA molecules, and absence of infectious SFV viral contaminants in the culture fluid of transfected cells (22). It was therefore essential initially to insert KUN structural genes into the SFV replicon and demonstrate

a suitable level of their expression. Our original intention was to express the entire open reading frame coding for the KUN structural proteins C, prM, and E in the SFV replicon expression vector pSFV1. Unfortunately, numerous attempts to clone a *Bgl*II-*Bgl*II PCR fragment representing the KUN C-prM-E region into the pSFV1 vector resulted in recovery of clones containing the KUN cDNA fragment inserted only in the orientation reverse to that required for expression directed by the SFV 26S subgenomic promoter (results not shown). To overcome this problem we decided to prepare two separate SFV constructs expressing the KUN C gene and KUN prME genes, respectively (Fig. 1).

Expression of the KUN C gene by the recombinant SFV-C107 replicon. Electroporation of SFV-C107 RNA into BHK21 cells resulted in expression of KUN C protein in almost 100% of cells as judged by IF with antibodies to KUN C protein (Fig. 2A, panel 1). KUN C protein expressed in SFV-C107 RNA-transfected cells was localized in the cytoplasm (Fig. 2A, panel 3 [acetone fixation]) and also in the nuclei (Fig. 2A, panel 5 [formaldehyde-methanol fixation]). These results are in complete agreement with our recently reported results on the nuclear localization of KUN C protein in KUN-infected cells and in a stable cell line expressing KUN C protein from pCINeoC107 plasmid DNA (37). Difficulties were experienced in identification of KUN C protein in radiolabeled lysates of SFV-C107-transfected cells (Fig. 2B), probably due to the presence of only five methionine and no cysteine residues in the whole C amino acid sequence. We therefore performed immunoprecipitation of the radiolabeled lysates with anti-C antibodies. A labeled band migrating slightly slower than KUN C protein was apparent in the lysates of SFV-C107- but not in those of SFV1-transfected cells (compare SFV-C and SFV1 in Fig. 2B). This slower migration of the C protein in SFV-C107-transfected cells may be due to the presence of an extra six

amino acids added during plasmid construction (Fig. 1). A number of higher-molecular-weight bands were also observed in the RIP samples from SFV-C107- but not from SFV1-transfected cells (Fig. 2B). It is possible that this coprecipitation occurred because of some nonspecific interactions of KUN C protein with SFV or cell proteins. Nevertheless, we have demonstrated expression of KUN C protein from the recombinant SFV replicon, and we focused next on expressing prME genes.

Expression of KUN prME genes by the recombinant SFV-prME replicon. It was shown in a number of flavivirus studies that expression of a prME cassette either from recombinant vaccinia viruses, or from a DNA expression vector, or from SIN virus replicons resulted in the secretion of subviral particles containing correctly processed E and M proteins (for references, see reference 32). Importantly, expression of both full-length prM and full-length E, including all the hydrophobic domains (signal and anchor sequences in Fig. 1), was required for proper maturation and secretion of subviral particles (2, 19, 28). Therefore, we included the full-length prME sequence plus the preceding signal sequence in our SFV-prME construct (see Materials and Methods) (Fig. 1). The construct was partially sequenced, and the identity to the published KUN sequence (7) of all the cleavage sites in the prM-E region responsible for proper processing of the prM, pr, M, and E proteins was confirmed (data not shown).

When SFV-prME RNA was electroporated into BHK21 cells, nearly 100% of cells were found to be positive in IF analysis with monoclonal antibodies to KUN E protein at 18 h after electroporation (Fig. 3A, panel 1). To confirm expression of the KUN prM and E proteins in transfected cells and to detect secretion of prME into the tissue culture fluid, we labeled transfected cells with [³⁵S]methionine-cysteine for 1 h, followed by incubation for increasing chase periods. A strongly labeled band corresponding to KUN E protein was apparent in both culture fluid and cell lysates of SFV-prME-transfected cells at all times (Fig. 3B). A labeled band corresponding to KUN prM protein was detected in cell lysates, and another band corresponding in migration to the predicted molecular weight of KUN pr protein was detected in the culture fluid of transfected cells (Fig. 3B). An apparent increase in the intensity of labeling of the E and pr proteins in the culture fluid (Fig. 3B) and a concomitant decrease in the intensity of labeling of the E and prM proteins in the cell lysates (Fig. 3B) were observed during the chase period. Interestingly, secreted pr protein was also detected in the culture fluid of cells infected with other flaviviruses (24, 30), and protein migrating similarly was detected in the culture fluid of cells infected with recombinant vaccinia virus expressing Japanese encephalitis virus prME genes (18). Presumably, the pr and M proteins were cleaved from their precursor prM by furin or a similar cell protease during its secretion from cells (for a review, see reference 17). Because of its small size (~8 kDa), extracellular M protein probably ran off the gel during electrophoresis and therefore could not be detected. The identity of the expressed KUN E and coprecipitated prM proteins detected in the culture fluid and cells (Fig. 3B) was confirmed by RIP with KUN anti-E antibodies (data not shown). These results demonstrated a correct processing of KUN prME polypeptide in cells transfected with SFV-prME RNA and the secretion of E, pr, and possibly M proteins into the culture fluid.

Expression of all three KUN structural proteins by the recombinant SFV-prME-C107 replicon. Although we were able to package KUN replicon in cells by using transfection with two SFV RNAs expressing the prME and C genes separately (see results in the next section), the efficiency of packaging was rather low. In order to increase the efficiency of packaging and

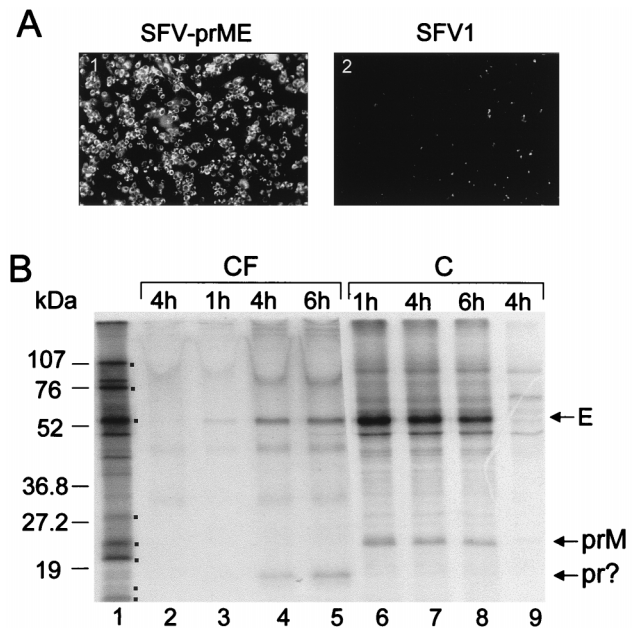


FIG. 3. Expression of the KUN prME genes by the recombinant SFV-prME replicon. (A) IF analysis of SFV-prME- and SFV1-transfected BHK21 cells at 18 h after transfection by using KUN monoclonal anti-E antibodies. (B) Results of pulse-chase labeling with [³⁵S]methionine-cysteine of SFV-prME-transfected BHK21 cells. Culture fluid (CF) and cell lysate (C) samples were collected during chase periods (see Materials and Methods). Lane 1, radiolabeled KUN cell lysate, with dots indicating KUN proteins as in Fig. 2B. Lanes 2 and 9, samples collected after a 4-h chase period from culture fluid and cells, respectively, after transfection with the control SFV1 RNA. Lanes 3, 4, and 5, culture fluid samples collected at 1, 4, and 6 h of chase, respectively; lanes 6, 7, and 8, corresponding chase samples from the cells. Ten microliters of a total of 700 μ l of culture fluid and 5 μ l of a total of 300 μ l of cell lysate samples were used for electrophoresis. The exposure time of the dried gel for cell lysates was 1 day, and that for culture fluids was 5 days. M, the low-molecular-mass cleavage product (~8 kDa) of prM, was not resolved (see text). Numbers represent sizes of the low-range prestained Bio-Rad protein standards.

to simplify the procedure, we prepared a single SFV replicon construct expressing the prME genes and the C gene simultaneously. Because of the difficulties experienced with cloning of the entire C-prM-E region into the pSFV1 vector (see the first section of Results) and also to avoid possible uncertainty regarding cleavage at the carboxy terminus of C in the absence of flavivirus protease NS2B-NS3 (3, 23, 34, 39–41), an SFV replicon expressing the prME and C genes under the control of two separate 26S promoters was prepared (Fig. 1, SFV-prME-C107).

IF analysis of SFV-prME-C107-electroporated BHK cells with anti-E and anti-C antibodies showed expression of both E and C proteins in nearly 100% of the cells by 18 h after transfection (results not shown). As expected, both E and C proteins were expressed in the same cell (compare dual IF labeling by anti-C and anti-E antibodies in Fig. 4A). In contrast, only about 40% of cells were dual labeled with these antibodies when two individual SFV RNAs expressing prME and C, respectively, were electroporated simultaneously (results not shown). When cells transfected with SFV-prME-C107 RNA were pulse-chased with [³⁵S]methionine-cysteine and the lysates were immunoprecipitated with KUN anti-E antibodies, both E and prM proteins were coprecipitated (Fig. 4B), as was observed after transfection of SFV-prME RNA (Fig. 3B). A gradual increase of immunoprecipitated labeled E protein in culture fluids and a corresponding decrease of immunoprecipitated labeled E and prM proteins in the cell lysates were

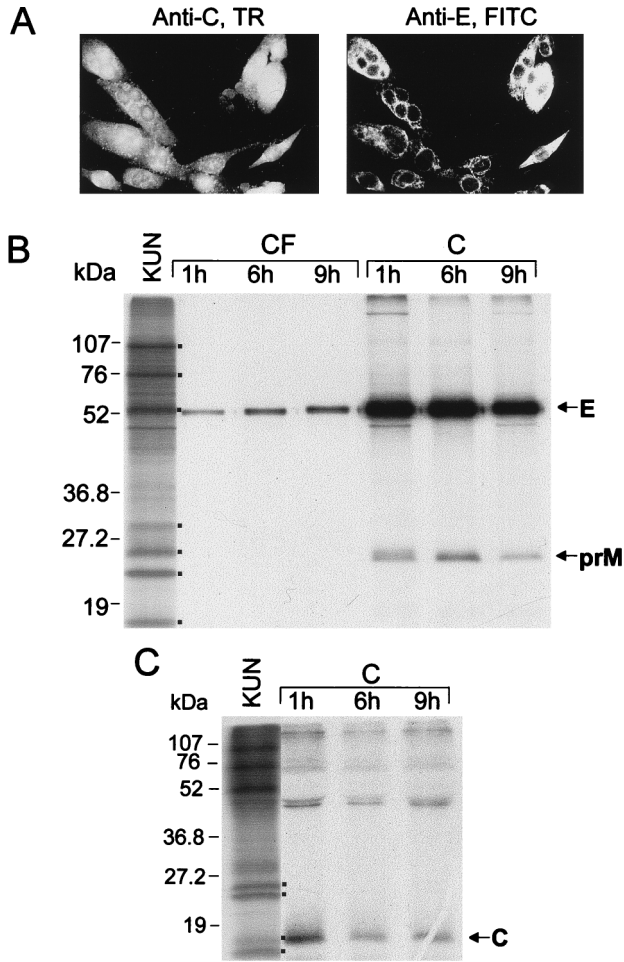


FIG. 4. Expression of all three KUN structural proteins by the recombinant SFV-prME-C107 replicon. (A) Dual IF analysis of the same field of BHK21 cells fixed in acetone at 18 h after transfection with SFV-prME RNA by using KUN anti-C and anti-E antibodies with Texas red (TR)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, respectively. (B and C) Cells at 18 h after transfection with SFV-prME-C107 RNA were pulsed with [³⁵S]methionine-cysteine for 1 h; subsequently, 300 μ l (from a total of 600 μ l) of cell lysates (lanes C) and 1 ml (from a total of 2 ml) of culture fluids (lanes CF) collected at different chase intervals (1, 6, and 9 h) were immunoprecipitated either with KUN anti-E antibodies (B) or with KUN anti-C antibodies (C) as described in Materials and Methods. Ten microliters (from a total of 30 μ l) of immunoprecipitated samples was electrophoresed in SDS-12.5% (B) and -15% (C) polyacrylamide gels. Dots in panel B indicate KUN proteins in the labeled KUN cell lysates as in Fig. 2B. Dots in panel C represent KUN proteins prM, NS2A, C, and NS4A/NS2B (from top to bottom) in the radiolabeled KUN-infected cell lysate. Numbers represent sizes of the low-range prestained Bio-Rad protein standards.

observed during the chase period (Fig. 4B). Immunoprecipitation of the labeled cell lysates with anti-C antibodies confirmed expression of C protein in transfected cells and showed a decrease in the amount of precipitated C during the chase period (Fig. 4C), probably involving some degradation of C protein during a prolonged chase period as was observed previously in KUN-infected cells (36). The results of RIP analysis of culture fluid, not treated with detergents, with anti-C antibodies were negative (results not shown), indicating that no free C protein was secreted into culture fluid of SFV-prME-C107-transfected cells. In a later experiment (see Fig. 7C, lane 2), particles secreted from cells transfected only with SFV-prME-C107 RNA were purified, and only E and prM were

coprecipitated with anti-E antibodies; again, no secreted C was detected.

Overall, the immunofluorescence and labeling patterns in cells transfected with SFV-prME-C107 RNA were very similar to those observed in cells transfected with two different RNAs expressing prME and C proteins separately (compare the results in Fig. 4 with those in Fig. 2 and 3), suggesting that proper processing and maturation of all three KUN structural proteins occurred when they were expressed from the recombinant SFV replicon.

Packaging of the KUN replicon RNA into pseudoinfectious particles by the KUN structural proteins expressed from the recombinant SFV replicons. Because with KUN replicon construct C20rep we were able to successfully transfect only ~10% of cells (15), it was essential to use a KUN replicon with greater transfection efficiency for attempted packaging in doubly transfected cells (i.e., cells transfected with the KUN replicon and recombinant SFV replicons expressing KUN structural proteins). Therefore, we prepared a new replicon construct, C20DXrep (see Materials and Methods), with significantly improved efficiency of transfection in BHK21 cells. Using an anti-NS3 antibody to detect amplification of KUN replicon RNA, we demonstrated that up to about 80% of 2×10^6 to 4×10^6 BHK21 cells could be routinely electroporated with ~10 μ g of C20DXrep RNA (16). The improved replicon C20DXrep therefore was used in all packaging experiments.

Initial cotransfection experiments showed that simultaneous transfection of C20DXrep RNA and SFV RNAs expressing KUN structural proteins did not produce detectable infectious particles (results not shown). Under these conditions, KUN replicon RNA was poorly amplified (very few cells were positive by IF with anti-NS3 antibodies), whereas SFV RNAs replicated efficiently (there was strong IF with anti-E or anti-C antibodies in nearly 100% of cells) (results not shown). Therefore, a delay of 12 h or longer between electroporations was used in subsequent experiments in order to allow KUN replicon RNA to accumulate before electroporation of SFV RNAs. In preliminary experiments we compared the relative efficiencies of packaging of KUN replicon RNA with the single SFV-prME-C107 RNA or the two SFV RNAs, SFV-prME and SFV-C107, by monitoring yields by IF and Northern blot analyses. BHK cells were infected with the cell culture fluid collected at 26 h after electroporation with SFV RNAs of cells electroporated 27 h previously with C20DXrep RNA. The single SFV-prME-C107 RNA was more efficient, producing a large increase in the number of IF-positive cells and in synthesis of KUN-specific RNA (Fig. 5B). The titer, expressed as infectious units per milliliter (see Materials and Methods), was $\sim 2 \times 10^5$, or 10-fold greater than the result when two SFV RNAs were used. The difference in the production of infectious particles is probably due mainly to a lower proportion of cells simultaneously expressing both prME and C proteins after cotransfection of two SFV RNAs (40%) compared to transfection of a single SFV RNA (nearly 100%). Noticeably, the efficiency of the electroporation of SFV RNAs and subsequent expression of KUN structural proteins from these vectors were not apparently affected by the previous electroporation and replication of C20DXrep RNA, as judged by dual IF analysis with anti-NS3 and anti-E or anti-C antibodies (data not shown). As expected, no infectious particles containing packaged KUN replicon RNA were detected when only SFV-prME RNA was used in packaging experiments with C20DXrep RNA (panel 3 in Fig. 5A), demonstrating that coexpression of C protein is absolutely essential for the formation of the secreted infectious particles.

Significantly, no evidence of transmission of replicating SFV

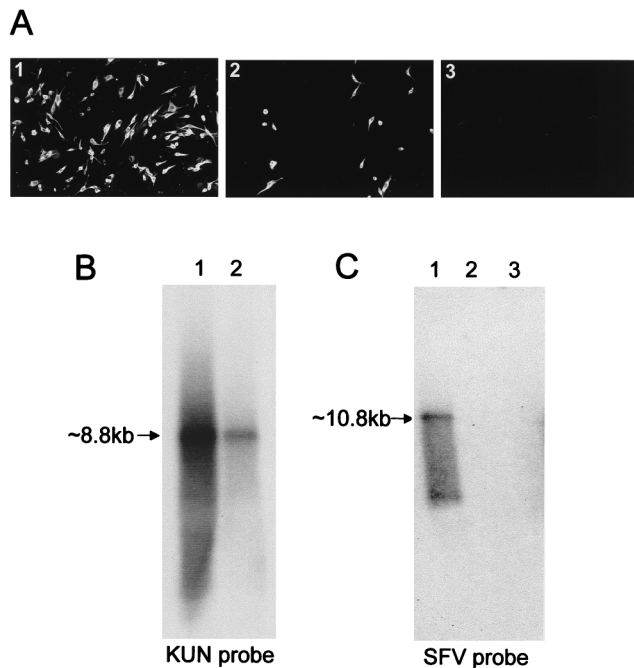


FIG. 5. Packaging of KUN replicon RNA by KUN structural proteins expressed from the recombinant SFV replicons. (A) IF analysis with KUN anti-NS3 antibodies of BHK21 cells infected with the undiluted culture fluid collected from BHK21 cells at 26 h after transfection with SFV-prME-C107 RNA (panel 1), SFV-prME and SFV-C RNAs (panel 2), or SFV-prME RNA (panel 3) of cells transfected 27 h previously with C20DXrep RNA. Panels 1 and 2 represent selected fields of coverslips enriched in positive cells. (B and C) Northern blot analysis of RNAs isolated from BHK21 cells infected as described for panel A, using labeled KUN-specific (B) and SFV-specific (C) cDNA probes (see Materials and Methods). Lane 1 in panel B and lane 2 in panel C correspond to the cells in panel 1 of panel A. Lane 2 in panel B and lane 3 in panel C correspond to the cells in panel 2 of panel A. Lane 1 in panel C represents 5 ng of *in vitro*-synthesized SFV-prME-C107 RNA. The arrows in panels B and C indicate the positions of RNAs of about 8.8 kb for KUN replicon RNA and about 10.8 kb for SFV-prME-C107 RNA, determined relative to migration in the same gel of ethidium bromide-stained λ DNA digested with *Bsr*EII (New England Biolabs).

RNAs was obtained by IF analysis with anti-E and anti-C antibodies of cells infected with the culture fluid collected from cells transfected with any of the recombinant SFV RNAs either with or without previous transfection with C20DXrep RNA (data not shown). This assay is very sensitive, because a very low number of packaged recombinant SFV replicon RNAs able to express KUN C or E protein would have been detected by the IF analysis following the manifold amplification of any SFV RNAs after such delivery. Similar (negative) results were also obtained by Northern blot analysis with an SFV-specific probe of RNA isolated from cells infected with the particles collected from cells sequentially transfected with KUN replicon and SFV replicon RNAs (Fig. 5C, lanes 2 and 3). The packaging observed after these sequential transfections was therefore clearly specific for KUN replicon RNA.

In order to optimize the conditions for efficient packaging of C20DXrep RNA in cotransfection experiments with SFV-prME-C107 RNA, various time points between electroporations (Fig. 6A) and between the second electroporation and harvesting of the infectious particles (Fig. 6B) were examined. Initially, optimization of the time between the two electroporations was established with a fixed time for collection of the infectious particles. Equal amounts of cells were seeded onto cell culture dishes after the first electroporation with C20DXrep RNA, and cells were subsequently electroporated

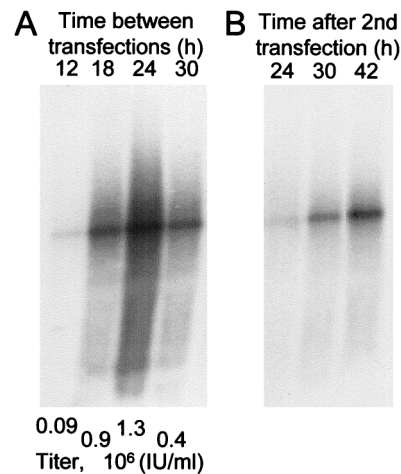


FIG. 6. Optimization of conditions for packaging of KUN replicon RNA. Northern blot analysis of BHK21 cells infected with filtered and RNase-treated culture fluid samples collected after sequential transfections with KUN replicon C20DXrep RNA and SFV-prME-C107 RNA is shown. Samples of total RNA used for Northern blotting were isolated from cells at 24 h after infection with samples of the culture fluid collected either at a fixed time (24 h) after the second transfection and with different time intervals between transfections (A) or at different times after the second transfection and a fixed time (30 h) after the first transfection (B). Titrations in panel A represent the amounts of infectious units contained in the corresponding samples of culture fluids used for these infections. The probe in both panels was a radiolabeled cDNA fragment representing the last 761 nucleotides of the KUN genome.

with SFV-prME-C107 RNA after a further 12, 18, 24, or 30 h of incubation. Culture fluid was then harvested from each dish at 24 h after the second electroporation, and serial dilutions were used to infect BHK21 cells for titration by IF analysis with anti-NS3 antibodies. As the period between electroporations was extended from 12 to 30 h, an increase of infectious particles was observed, with the highest titer of 1.3×10^6 IU per ml ($\sim 2 \times 10^6$ particles per 10^6 cells) corresponding to the 24-h interval (Fig. 6A). Northern blot analysis of total RNA from infected cells with a labeled KUN-specific cDNA probe confirmed this optimal time interval of 24 h (Fig. 6A). When $\sim 10 \mu\text{g}$ of *in vitro*-transcribed full-length FLSDX RNA (similar to the amount used for C20DXrep RNA [see Materials and Methods]) was electroporated into BHK cells, the titer of infectious particles accumulated in the culture fluid by 45 h, as determined by IF assay of foci in newly infected BHK cells with anti-NS3 antibodies, was $\sim 10^7$ IU per ml (data not shown). Thus, the yield of KUN virions after electroporation of transcribed full-length RNA was only ~ 10 -fold higher than the yield of encapsidated replicon particles obtained by dual transfections with KUN and SFV replicon RNAs (see above).

In a separate experiment BHK cells were electroporated with SFV-prME-C107 RNA at 30 h after electroporation with C20DXrep RNA and seeded into one 60-mm-diameter culture dish. Single aliquots of the culture fluid (1 ml of a total of 3 ml) were then collected after 24, 30, and 42 h of incubation after the second electroporation, and the culture fluid was adjusted to the original volume by adding fresh medium. Collected aliquots were then used to infect BHK cells, and total cell RNA recovered from these infected cells at 24 h was then analyzed for relative amounts of amplified KUN replicon RNA by Northern blot hybridization. The gradual increase in amplified KUN replicon RNA from 24 to 42 h after the second electroporation with SFV-prME-C107 RNA (Fig. 6B) was in accord with an observed increase in released infectious parti-

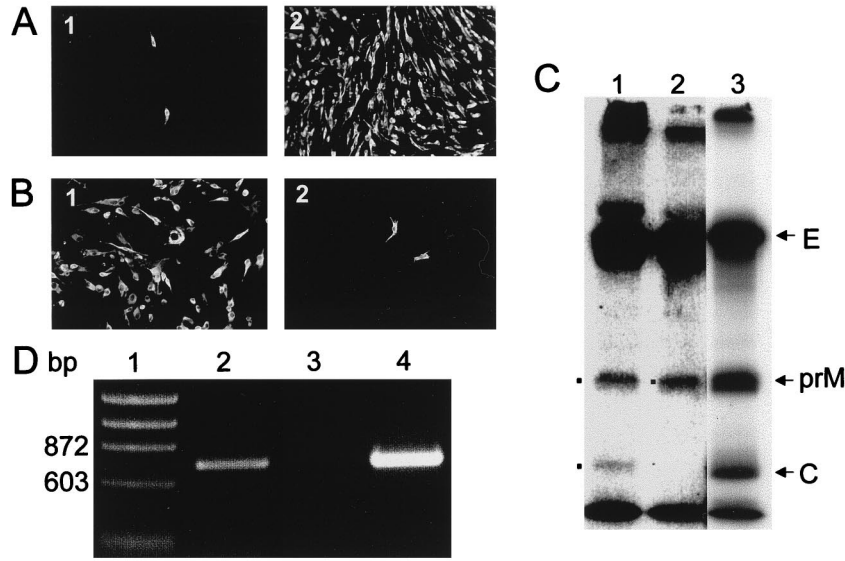


FIG. 7. Characterization of infectious particles. (A) Neutralization of infectivity of encapsidated particles, released from cells transfected sequentially with C20DXrep and SFV-prME-C107 RNAs (as in Fig. 6). Panels 1 and 2 show IF with anti-NS3 antibodies of cells infected with culture fluid collected at 24 h after transfections and incubated for 1 h at 37°C with anti-E monoclonal antibodies or culture medium, respectively. (B) IF analysis with anti-NS3 antibodies of cells infected with equal proportions of resuspended pellet (panel 1; 2 µl from a 50-µl total volume) or supernatant fluid (panel 2; 200 µl from a 5-ml total volume) from the culture fluid collected from cells transfected as described for panel A and subjected to ultracentrifugation (see Materials and Methods). (C) RIP analysis with anti-E antibodies of culture fluids from cells sequentially transfected with C20DXrep and SFV-prME-C107 RNAs (lane 1), transfected with SFV-prME-C107 RNA alone (lane 2), or infected with KUN virus (lane 3). Dots show bands corresponding to C and prM in lane 1 but a corresponding band for only prM in lane 2. (D) RT-PCR analysis with KUN-specific primers of RNAs extracted from the anti-E immunoprecipitates in panel C; lanes 2 to 4 are equivalent to lanes 1 to 3 in panel C. Note that immunoprecipitates were treated with RNase A (20 µg per ml) for 30 min at 37°C prior to isolation of RNA in order to ensure the absence of free RNA in the RIP samples. RNA from the treated RIP samples was isolated by digestion with proteinase K (100 µg per ml) in the presence of 0.5% SDS for 30 min at 37°C followed by phenol-chloroform extraction and ethanol precipitation. Lane 1, φX174 replicative-form DNA digested with *Hae*III (New England Biolabs).

cles assayed by IF analysis of newly infected cells with anti-NS3 antibodies (data not shown). Further incubation of cells was abandoned in this experiment because of the severe cytopathic effects which had occurred by 42 h after electroporation of SFV-prME-C107 RNA. However, in other experiments longer incubation (68 h) of cells after the second electroporation with recombinant SFV RNA(s) resulted in reduction of the titer of accumulated infectious particles from surviving cells (data not shown).

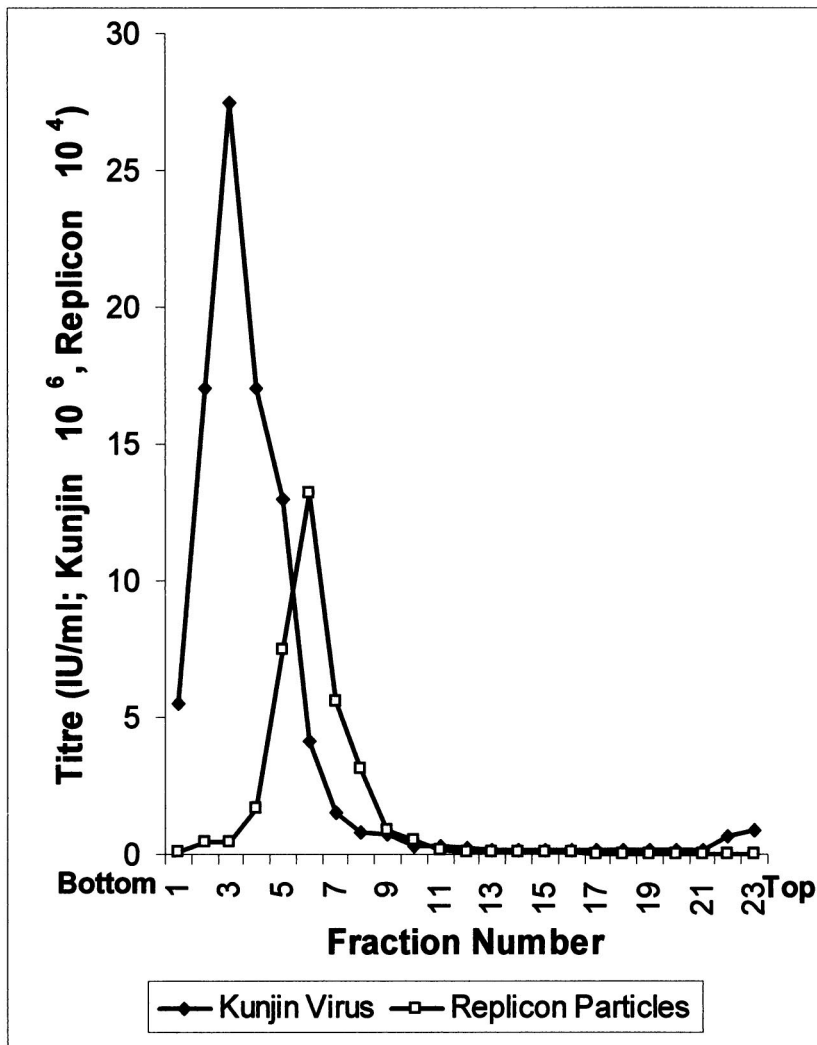
In an attempt to improve the efficiency of the packaging system, we prepared an SFV-prME-C105 construct in which the 3' sequence of the C gene was engineered so that the resulting translation product, C, would terminate precisely at Arg¹⁰⁵ (Fig. 1), identically to the mature form of KUN C protein (29). When SFV-prME-C107 and SFV-prME-C105 RNAs were used in parallel in packaging experiments with C20DXrep RNA, the titers of infectious particles released in the culture fluid of transfected cells were virtually identical (~9 × 10⁵ infectious particles per ml for both). These results indicate either that C protein expressed from SFV-prME-C107 RNA was processed correctly (cleaved at Arg¹⁰⁵) by KUN NS2B-NS3 protease expressed from C20DXrep RNA or that the presence of an additional carboxy-terminal six amino acids in C107 protein (Fig. 1) did not interfere with packaging.

Characterization of the infectious particles. Having established that the infectivity of particles secreted into the culture fluid of cells transfected with C20DXrep and SFV-prME-C107 RNAs was filterable and RNase resistant, we then performed a virus neutralization test to confirm that the particles were packaged by KUN structural proteins. A 1-h incubation of this culture fluid at 37°C with a 1/10 dilution of antibodies to KUN E protein with known neutralizing activity (1) resulted in almost complete loss of transmissible infectivity measured by IF

(panel 1 in Fig. 7A), while no loss of infectivity was observed in the control sample incubated under similar conditions in the absence of antibodies (panel 2 in Fig. 7A). Similar results were obtained when incubations with antibodies were performed at room temperature or at 4°C (data not shown). We then established the particulate nature of the particles by showing that they can be concentrated by pelleting. A clarified culture fluid of cotransfected cells was subjected to ultracentrifugation (see Materials and Methods), and resuspended pellet and supernatant were used to infect BHK cells. Analysis by IF with anti-NS3 antibodies 24 h later showed that almost all of the infectious particles were present only in the pelleted material (compare panels 1 and 2 in Fig. 7B).

In order to identify the proteins and to detect the presence of KUN replicon RNA in the released infectious particles, they were immunoprecipitated with anti-E antibodies in the absence of detergents from the culture fluid of cells sequentially transfected with C20DXrep and SFV-prME-C107 RNAs and radiolabeled with [³⁵S]methionine-cysteine. Half of the immunoprecipitated sample was used for separation of proteins in an SDS-polyacrylamide gel; radioautography of the gel showed the presence of the E, prM, and C proteins in the immunoprecipitates (Fig. 7C, lane 1). These same proteins were also resolved in a parallel experiment using culture fluid from KUN-infected cells (Fig. 7C, lane 3). E and prM proteins, but no C protein, were immunoprecipitated by anti-E antibodies from culture fluid of cells transfected only with SFV-prME-C107 RNA (Fig. 7C, lane 2). It was concluded that a specific interaction between KUN replicon RNA and KUN C protein was required for assembly and secretion of infectious particles. Note that secreted flaviviruses often contain significant amounts of uncleaved prM (see, for example, reference 24), as observed in Fig. 7C.

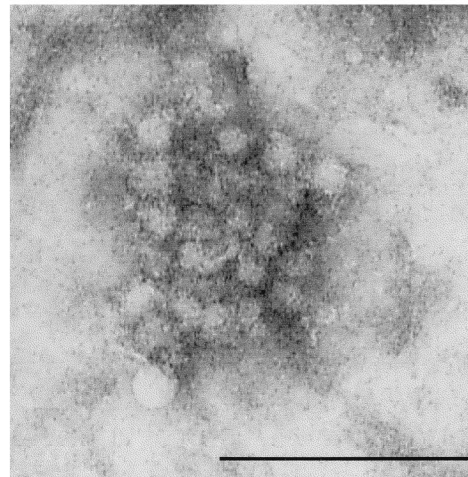
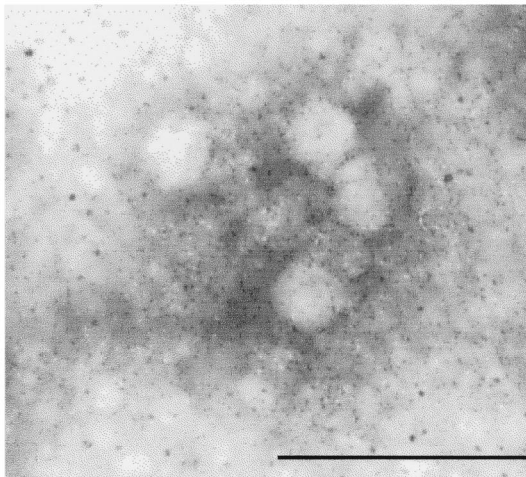
A



B

KUN virions

Replicon particles



RNA extracted from the remainder of the immunoprecipitates pretreated with RNase A (see the previous paragraph) was reverse transcribed and PCR amplified with KUN-specific primers. As expected, a DNA fragment of the predicted size (~700 bp, NS2A region) was observed in the RT-PCR product from packaged replicon and KUN virus prepared similarly (Fig. 7D, lanes 2 and 4). No RT-PCR product was obtained from RNA extracted from the immunoprecipitate of the culture fluid collected from cells transfected with SFV-prME-C107 RNA alone (Fig. 7D, lane 3) or with C20DXrep RNA alone (data not shown). These results established that replicon RNA was encapsidated in the packaged particles assayed previously for infectivity and KUN proteins (Fig. 7A to C).

Further characterization of the packaged particles containing replicon RNA was performed by sedimentation analysis. In parallel with KUN virions (both concentrated by ultracentrifugation) they were sedimented through 5 to 25% sucrose density gradients. Fractions of 0.5 ml were collected, diluted, and assayed for infectivity by IF assay with anti-NS3 antibodies at 18 h for KUN virions or at 24 h for replicon particles (see the legend to Fig. 8A). The maximum infectivity for replicon particles was concentrated in fractions 5 to 7, with a peak titer of $\sim 1.3 \times 10^5$ IU/ml (fraction 6), while infectious KUN virions were concentrated mostly in fractions 2 to 4, with a peak titer of $\sim 2.8 \times 10^7$ IU/ml (fraction 3) (Fig. 8A). These three fractions from each gradient were combined, incubated with anti-E antibodies to aggregate virions and encapsidated particles, and concentrated by ultracentrifugation for electron microscopy (for experimental details, see the legend to Fig. 8B). As might be expected from the gradient sedimentation results (Fig. 8A), particles containing encapsidated replicon RNA were smaller than KUN virions, i.e., ~ 35 nm in diameter compared to the ~ 50 -nm diameter of virion particles (Fig. 8B). Both replicon and virion particles appeared to be spherical and uniform in size; surface details were not resolved, probably because of attachment of some anti-E antibody molecules (Fig. 8B). Taken together, the results described in this section demonstrate that the particles produced by the dual replicon packaging system are in fact virus-like particles containing KUN replicon RNA encapsidated by the KUN structural proteins C, prM/M, and E.

DISCUSSION

We have developed a packaging system allowing encapsidation of KUN replicon RNA by cotransfection with SFV replicon RNA(s) expressing KUN structural proteins. To our knowledge this is the first demonstration of packaging of a flavivirus RNA *in trans*, and it establishes that essential packaging signals do not reside in the structural region of the genome. Although KUN replicon RNA was packaged after sequential transfection with SFV RNAs SFV-C107 and SFV-prME, the yield of infectious particles was rather low (Fig. 5A

and B). A greatly improved yield was obtained when a single recombinant SFV replicon RNA, SFV-prME-C107, expressing both the KUN C and prME genes together but under control of separate 26S SFV subgenomic promoters, was prepared; this SFV RNA was used in all further encapsidation experiments. The less efficient production of packaged particles when two SFV RNAs (SFV-C107 and SFV-prME) were used was possibly due mainly to a significantly lower proportion of cells simultaneously expressing both the prME and C proteins (see Results). In addition, the lower yield of packaged particles could also be due to a difference in replication rates of the two SFV replicons within the same cell, especially if equimolar amounts of the KUN structural proteins were required for packaging (Fig. 5A and B).

Further optimization of conditions for encapsidation of C20DXrep RNA revealed that a time delay of about 24 h between transfections of KUN replicon RNA C20DXrep and recombinant SFV replicon RNA SFV-prME-C107 was required to obtain the maximum yield of encapsidated infectious particles another 24 h later ($\sim 1.3 \times 10^6$ particles per ml, or $\sim 2 \times 10^6$ particles per 10^6 transfected cells [Fig. 6A]), which was comparable to that obtained by using SFV replicons as helpers for packaging of defective Moloney murine leukemia virus particles ($\sim 7 \times 10^6$ particles per 10^6 transfected cells [20]). Interestingly, the yield of infectious SIN particles per milliliter obtained by cotransfection of two SIN RNAs (replicon RNA and helper RNA expressing SIN structural genes) varied from 3.9×10^6 (9) to 5×10^7 to 8×10^7 (5), representing 0.5 to 5% of the yield obtained after transfection of full-length infectious SIN RNA (5). These ratios are similar to the ratios observed for the packaged KUN replicon RNA and transfected full-length FLSDX RNA ($\sim 10\%$) (see Results). It is important to emphasize in this context that the yields of infectious virus, viral RNA, and proteins per cell produced by flaviviruses are notoriously low compared to those of alphaviruses. For example, in comparative assays the rate of synthesis of KUN RNA was 10 to 100 times less than the rate of SFV RNA synthesis (7).

In the present cotransfection experiments, it is likely that a rapid switch-off of cellular and possibly KUN nonstructural protein synthesis mediated by replicating SFV RNA (22) inhibited the initiation of KUN RNA replication, since only a minor proportion of KUN replicon-positive cells and no infectious particles secreted in the culture fluid were detected when C20DXrep and recombinant SFV RNAs were transfected simultaneously (see the penultimate section of Results). In contrast, replication of SFV RNAs was not noticeably affected by coreplicating KUN RNA (see the penultimate section of Results). Previously we showed that the maximum level of synthesis and accumulation of KUN replicon RNA occurred at about 24 h after electroporation (15). The observed increase of the titer of infectious particles as the period between transfection

FIG. 8. Sedimentation and electron microscopy analyses of KUN replicon and virion particles. (A) Sedimentation profiles of virions and replicon particles in parallel sucrose density gradients. Particles were collected from culture fluids of BHK cells either at 35 h after sequential transfections with C20DXrep and SFV-prME-C107 RNAs or at 24 h after infection with KUN virus and were concentrated by ultracentrifugation as described in Materials and Methods. The pelleted particles were resuspended in 300 μ l of PBS-0.1% bovine serum albumin overnight at 4°C and clarified by centrifugation at $16,000 \times g$ in a microcentrifuge for 10 min. The supernatant was overlaid on top of a 12-ml 5 to 25% sucrose density gradient which was centrifuged at 38,000 rpm for 70 min at 20°C in an SW41 rotor. Fractions of 0.5 ml were collected from the bottom of the gradient and diluted 1:2 (replicon particles) or 1:100 (KUN virions) for infectivity assays by IF on coverslip cultures of BHK cells at 24 h (replicon particles) or at 18 h (KUN virions) after infection, using anti-E antibodies; titers of infectious particles were determined as described in Materials and Methods. (B) Electron micrographs of virions and encapsidated replicon particles stained with uranyl acetate. Fractions 5 to 7 of replicon particles in panel A, and fractions 2 to 4 of KUN virions, were pooled and incubated with a 1/20 dilution of anti-E antibodies for 1 h at 20°C, followed by 2 h of incubation at 4°C with constant rotation. Particles were then again concentrated by ultracentrifugation as described above, and pellets were resuspended in 175 μ l of PBS-0.1% bovine serum albumin overnight at 4°C. Resuspended particles were then sonicated in a Transsonic 700/h sonicating water bath (CAMLAB, Cambridge, United Kingdom) for 1 min and pelleted onto a carbon-coated Formvar grid by centrifugation in an 18° fixed-angle A-100 rotor in a Beckman Airfuge for 1 h at 80,000 rpm. Grids were stained with 4% uranyl acetate, and particles were visualized by electron microscopy. Bars, 200 nm.

tions was increased from 12 to 24 h (Fig. 6A) indicated a direct correlation between (i) the amount of presynthesized KUN RNA and/or KUN nonstructural proteins present in cells at the time of transfection of SFV replicon RNA and (ii) the final production of infectious particles. However, we did not directly monitor the replication of KUN replicon RNA after transfection of SFV replicon RNA in these experiments. Alternatively, a certain threshold of synthesized KUN RNA and proteins, or cell factors (KUN-induced membranes?), may be required for efficient replication of KUN replicon RNA in the presence of replicating SFV replicon RNA.

Comparison of the sedimentation characteristics of the encapsidated replicon particles and KUN virions by centrifugation through a 5 to 25% sucrose gradient showed that infectious replicon particles sedimented more slowly (Fig. 8A), suggesting that they are possibly smaller. This notion was confirmed when the particles concentrated from the peak gradient fractions were negatively stained and visualized by electron microscopy, the diameters being ~35 nm for replicon particles and ~50 nm for virions (Fig. 8B). The difference in size is probably due to the smaller size of the encapsidated replicon RNA (8,775 versus 11,022 nucleotides) and the fact that fewer protein molecules are required for encapsidation. Similarly, defective interfering particles of the alphaviruses SIN and SFV containing RNAs with deletions were also found to be smaller than virions (5, 11).

There are two major applications of the KUN replicon packaging system which we are exploring further. The first involves the development of a new vaccine delivery system based on the encapsidation of noncytopathic KUN replicons expressing genes of other medically important viruses, and we have a number of candidate genes for these studies. It should be noted that the use of a heterologous packaging system such as that presented here eliminates the possibility of contamination of packaged replicons with infectious virus, which appears to be a major concern with the use of homologous packaging systems such as SFV and SIN in which homologous recombination between the two RNAs occurs (21, 29). A second application is based on the use of recombinant SFV replicon RNAs expressing KUN structural proteins and containing fragments of KUN replicon RNA for rapid mapping of any packaging signal(s) in the KUN RNA fragments. It is important in this regard to emphasize the observed specificity of packaging of KUN replicon RNA; no SFV replicon RNAs were transmitted by the secreted particles (Fig. 5C; see Results). In view of our previous data on the strength of interaction between the KUN C protein and KUN UTRs (14), it was not surprising that no packaging of KUN replicon RNA to produce infectious particles occurred when C was not supplied by an SFV replicon (Fig. 5A, panel 3), indicating the essential role of C in assembly of particles.

Our results represent a significant advance in the area of flavivirus virion assembly and secretion. In summary, it is now possible to introduce mutations into the KUN replicon RNA and into genes for the structural proteins and to assess their effect on the assembly and release of virus-like particles. Thus, the dual system (KUN replicon plus SFV vector for expression of KUN structural proteins) should allow rapid mapping of the packaging signal(s) in flavivirus RNA and the amino acid motif(s) in the structural proteins involved in RNA encapsidation, virion assembly, and secretion. It can also facilitate the development of a noninfectious and noncytopathic vaccine delivery system.

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