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Subgenomic Replicons of the Flavivirus Kunjin: Construction and Applications

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Several Kunjin virus (KUN) subgenomic replicons containing large deletions in the structural region (C-prM-E) and in the 3′ untranslated region (3′UTR) of the genome have been constructed. Replicon RNA ΔME with 1,987 nucleotides deleted (from nucleotide 417 [in codon 108] in the C gene to nucleotide 2403 near the carboxy terminus of the E gene, inclusive) and replicon RNA C20rep with 2,247 nucleotides deleted (from nucleotide 157 [in codon 20] in C to nucleotide 2403) replicated efficiently in electroporated BHK21 cells. A further deletion from C20rep of 53 nucleotides, reducing the coding sequence in core protein to two codons (C2rep RNA), resulted in abolishment of RNA replication. Replicon ΔME/76 with a deletion of 76 nucleotides in the 3′UTR of ΔME RNA (nucleotides 10423 to 10498) replicated efficiently, whereas replicon ΔME/352 with a larger deletion of 352 nucleotides (nucleotides 10423 to 10774), including two conserved sequences RCS3 and CS3, was significantly inhibited in RNA replication. To explore the possibility of using a reporter gene assay to monitor synthesis of the positive strand and the negative strand of KUN RNA, we inserted a chloramphenicol acetyltransferase (CAT) gene into the 3′UTR of ΔME/76 RNA under control of the internal ribosomal entry site (IRES) of encephalomyelocarditis virus RNA in both plus (ΔME/76CAT[+])- and minus (ΔME/76CAT[−])-sense orientations. Although insertion of the IRES-CAT cassette in the plus-sense orientation resulted in a significant (10- to 20-fold) reduction of RNA replication compared to that of the parental ΔME/76 RNA, CAT expression was readily detected in electroporated BHK cells. No CAT expression was detected after electroporation of RNA containing the IRES-CAT cassette inserted in the minus-sense orientation despite its apparently more efficient replication (similar to that of ΔME/76 RNA); this result indicated that KUN negative-strand RNA was probably not released from its template after synthesis. Replacement of the CAT gene in the ΔME/76CAT(+) RNA with the neomycin gene (Neo) enabled selection and recovery of a BHK cell culture in which the majority of cells were continuously expressing the replicon RNA for 41 days (nine passages) without apparent cytopathic effect. The constructed KUN replicons should provide valuable tools to study flavivirus RNA replication as well as providing possible vectors for a long-lasting and noncytopathic RNA virus expression system.

Kunjin virus (KUN) is an endemic Australian flavivirus sporadically causing encephalitis and is closely related to the other more pathogenic members of the *Flaviviridae* family, such as Murray Valley encephalitis (also endemic for Australia), West Nile, and Japanese encephalitis viruses. The KUN genome (Fig. 1A) is a positive-stranded RNA comprising 11,022 nucleotides (21) with one long open reading frame coding for three structural (C, prM, and E) and seven nonstructural (NS; NS1 to NS5) proteins (12). Recently, we described the preparation of a stable full-length cDNA copy of KUN RNA capable of producing an infectious RNA transcript in vitro (21), thus confirming the authenticity of the cDNA sequence in regard to correct gene expression and function. In order to extend a range of experimental tools in our ongoing studies on flavivirus RNA replication (8–11, 21, 22), we decided to prepare a KUN self-replicating RNA (subgenomic replicon) with a deletion in the region of the genome encoding structural components of the virion. A major advantage of using such a replicon is the isolation of RNA replication from virion assembly and maturation, thus allowing the opportunity for precise mapping of proteins, motifs, and RNA sequences directly involved in RNA replication.

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There are a number of positive-strand RNA animal viruses for which preparation of subgenomic replicons has been published, including Sindbis virus (4, 36, 43), Semliki Forest virus (SFV; 26), poliovirus (1, 13, 16, 19) and human rhinovirus 14 (28). Although no details of a flavivirus replicon have yet been published, successful preparation of several yellow fever virus (YF) subgenomic replicons was achieved by P. Bredenbeek and C. M. Rice (3). A useful application of replicons of the RNA viruses is the expression of heterologous genes. The advantages of using replicons of RNA viruses as expression vectors include wide host range, a high level of cytoplasmic expression, and rapid construction of recombinant RNA molecules. Thus a high level of transient expression of heterologous genes was achieved by using a Sindbis virus replicon (5, 15, 18, 36, 43), an SFV replicon (26, 37), and poliovirus replicons (2, 7, 31, 33, 34). Conditions have been optimized for efficient packaging of alphavirus SFV and Sindbis virus replicons by cotransfection with defective helper RNAs expressing structural proteins, resulting in the preparation of high-titer stocks of encapsidated replicons free of helper RNAs (5, 26). However, a major limitation of such expression systems for some applications is the development of delayed but still profound cytopathic effect during replication as observed for alphavirus replicons in mammalian cells (14), which in turn limits the period for continuous expression of foreign genes and also the possible use of such systems for vaccine purposes.

In this report we describe the preparation of KUN sub-

genomic replicons containing a number of deletions in the structural region and the 3′ untranslated region (3′UTR) of the KUN genome. Also prepared were dicistronic replicons with an insertion in the 3′UTR of the reporter gene chloramphenicol acetyltransferase (CAT) or the antibiotic resistance gene neomycin (Neo) which were expressed by internally initiated translation controlled by the encephalomyelocarditis virus internal ribosomal entry site (EMCV IRES; 42). Using a replicon expressing the Neo gene, a BHK21 cell culture continuously expressing this replicon was selected and maintained for 41 days without apparent cytopathic effect. These findings may prove to be useful for further understanding of the factors involved in flavivirus RNA replication as well as for the development of an alternative long-lasting and noncytopathic RNA virus expression system.

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 CO_2 incubator.

Construction of the plasmids. All deletion constructs (Fig. 1B to I) were prepared from the cDNA clones used in the construction of the plasmid pAKUN for generation of the infectious KUN RNA (Fig. 1A) (21, 30) by PCR-directed mutagenesis, using appropriate primers and conventional cloning. ΔME cDNA (Fig. 1B and G) and its derivatives (Fig. 1D and H to M) had deletions from nucleotides 417 to 2404, which represent loss of the signal sequence at the carboxy terminus of C (now reduced to 107 amino acids), deletion of prM and E, with the open reading frame resumed at codon 479 in E, preceding the signal sequence for NS1. C20rep (Fig. 1E) and C2rep (Fig. 1F) cDNAs represent progressive in-frame deletions in the coding sequence of C, leaving only 20 or 2 amino acids of C, respectively, with the open reading frame continued at codon 479 in E, as in ΔME. In ΔCME (Fig. 1C), all of the coding sequence was deleted from nucleotide 94 to 2379 inclusive, followed by the first nucleotide of a naturally occurring ATG codon in E (nucleotide 2380, codon 471). The nucleotide sequences flanking the deletions in all the described plasmid constructs were confirmed by sequencing analysis.

In ΔME/76 cDNA (Fig. 1D and H) and its derivatives (Fig. 1E and F) or in ΔME/352 cDNA (Fig. 1I), 76 or 352 nucleotides were deleted from the 3′UTR after nucleotide 10422, respectively, with a unique *Mlu*I restriction site inserted as shown (Fig. 1H and I). ΔME/76CAT(+) and ΔME/76CAT(−) cDNAs (Fig. 1J, H) were prepared by cloning an IRES-CAT cassette, PCR amplified from the plasmid pEMCV-CAT (42; kindly provided by P. Bredenbeek) and digested with *Mlu*I restrictase, into the *Mlu*I site of the plasmid ΔME/76. Recombinant plasmids with the insertions in both plus- and minus-sense orientations were isolated. Construct ΔME/352CAT(+) was obtained by cloning of the IRES-CAT cassette into the *Mlu*I-digested ΔME/352 plasmid (Fig. 1L). To construct ΔME/76Neo cDNA (Fig. 1M), the CAT gene in ΔME/76CAT(+) was replaced by the neomycin (Neo) gene (isolated from the plasmid pCIN4, a derivative of pCIN1 [35], kindly provided by S. Rees).

RNA transcription and transfection. All cDNA constructs were digested with *Xho*I to produce DNA templates for run-off in vitro RNA transcription as described previously (21). For electroporations, BHK21 cells were grown in T175 flasks (Nunc) to ~60 to 70% confluency, trypsinized, washed, and resuspended in diethyl pyrocarbonate-treated phosphate-buffered saline (PBS) to a concentration of 5×10^6 cells per ml. Four hundred microliters containing 2×10^6 cells was transferred to a 0.2-cm cuvette (Bio-Rad), mixed with ~10 to 20 μg of RNA (10 to 50 μl of transcription reaction mixture), and electroporated in the Bio-Rad Gene Pulser apparatus as described elsewhere (27).

Anti-NS3 antibody. Anti-NS3 antibody was prepared by immunizing an adult rabbit subcutaneously several times with recombinant baculovirus-expressed NS3 protein (23, 24) by methods that will be described elsewhere (20). The specificity of the antibody was confirmed by indirect immunofluorescence (IF) with mock (see Fig. 3B)- and KUN-infected cells (data not shown), and by radioimmunoprecipitation analysis of mock- and KUN-infected cell lysates (see Fig. 6C).

Northern blot analysis. Five to 10 μg of total RNA, isolated using Trizol reagent (Gibco BRL), was subjected to denaturing electrophoresis in a 1% agarose–2% formaldehyde gel followed by overnight transfer to a Hybond-N membrane (Amersham) as recommended by the manufacturer. Transfer membranes were air dried, subjected to UV radiation for 5 min and hybridized with specific probes in ExpressHyb solution (Clontech) essentially as described by the manufacturer. The probes were [^{32}P]-labelled KUN cDNA fragments representing the NS4B sequence (see Fig. 5A, C), the 3′UTR sequence (see Fig. 4A), the IRES-CAT sequence (see Fig. 5C), or the Neo sequence (see Fig. 6B).

CAT assay. The protocol was based on two recently published modifications (6, 25) which included the use of radiolabelled donor substrate ([^{14}C]-acetyl coenzyme A [CoA]) and unlabelled chloramphenicol, decreased ethanol concentration (from 20 to 1.25%), increased reaction volume, and increased time of preincubation of cell lysates at 65°C. The modifications were all directed at decreasing the high background observed in conventional CAT assays. Monolayers of BHK21 cells in 60-mm-diameter dishes at 48 h after electroporation with replicon RNAs were washed once with PBS, harvested, and subjected to three freeze-thaw cycles. Lysates were then incubated at 65°C for 15 min and spun for 2 min at maximum speed in the Microfuge, and 150 μl of the supernatants was used in CAT assays. The supernatants were incubated in a total volume of 400 μl with final concentrations of 0.2 M Tris-HCl (pH 8.0), 1.25 mM chloramphenicol (Sigma; 5 μl of the 65 mM stock solution in 100% ethanol), and 0.2 μCi of [^{14}C]-labelled acetyl CoA (NEN Products-Du Pont; 4 mCi/mM) overnight at 37°C. Half of the reaction mixture was then extracted twice with 400 μl of xylene and counted in a β counter to detect ^{14}C-acetylated chloramphenicol product (LSC assay). Chloramphenicol including any ^{14}C-labelled product (but not [^{14}C]-acetyl CoA) in the other half (200 μl) was precipitated with 1 ml of ethyl acetate, dried, and resuspended in 20 μl of ethyl acetate, and 10 μl was subjected to thin-layer chromatography (TLC assay) with a solvent of chloroform-methanol at 95:5 (vol/vol). TLC plates were air dried and autoradiographed.

RESULTS

Replicons with deletions in the structural region of the KUN genome. Initially we prepared two replicon constructs from the full-length KUN cDNA clone AKUN (21) by deleting the sequence coding either for all three structural proteins C, prM, and E (ΔCME, Fig. 1C) or for only prM and E (ΔME, Fig. 1B). In ΔME and ΔCME we retained the coding sequence for the last 22 (ΔME) or 30 (ΔCME) amino acids, respectively, of E protein (12), which includes the signal sequence for NS1 protein (Fig. 1A), in order to preserve correct processing and translocation of NS1 and of the remaining nonstructural polyprotein across the membrane of the endoplasmic reticulum. The sequence coding for mature core protein (first 107 amino acid) (12, 39) was included in the ΔME construct because of a postulated role of core protein in regulation of the synthesis of minus-strand RNA (41) as well as a possible role of the proposed RNA cyclization sequence in the C gene (Fig. 1E) (17) in flavivirus RNA replication. Initial evidence of replication of these RNAs after electroporation into BHK21 cells was sought by IF analysis with antibodies to KUN NS3 protein. No positive IF was observed in cells transfected with either ΔME or ΔCME at 2 and 8 h posttransfection, some very weak IF staining was detected at 12 h, and subsequently strong staining was observed in 10 to 20% of cells at 24 and 48 h after transfection only with ΔME RNA, with some cells remaining positive even at 15 days after transfection (data not shown). Note that there was also no apparent cytopathic effect observed in the great majority of the anti-NS3 positive cells at any time posttransfection. In contrast to ΔME-electroporated cells, all cells transfected with ΔCME RNA remained completely negative in IF analysis with anti-NS3 antibodies at any time after transfection. These results showing no IF staining at 2 or 8 h after transfection, and none at any time after transfection with ΔCME, indicate that detectable expression of NS3 required amplification of ΔME RNA, rather than expression only from unamplified input RNA.

Accumulation of KUN-specific RNA in cells transfected with ΔME and ΔCME RNA was initially studied by reverse transcription-PCR (RT-PCR) analysis of total cellular RNA with KUN-specific primers. No positive amplification was observed from RNA isolated from ΔCME-electroporated cells at any time posttransfection with RNA transcribed from three cDNA clones (data not shown). Although RT-PCR analysis showed virtually no KUN-specific RNA present at 6 h after transfection of ΔME RNA and full-length AKUN RNA (used as a positive control), further incubation of transfected cells produced a strong amplification of KUN-specific RNA at 24 (Fig. 2), 48, and 72 h posttransfection (data not shown). Accumulation of KUN-specific RNA of the expected size for ΔME at 24 h posttransfection was also confirmed by Northern

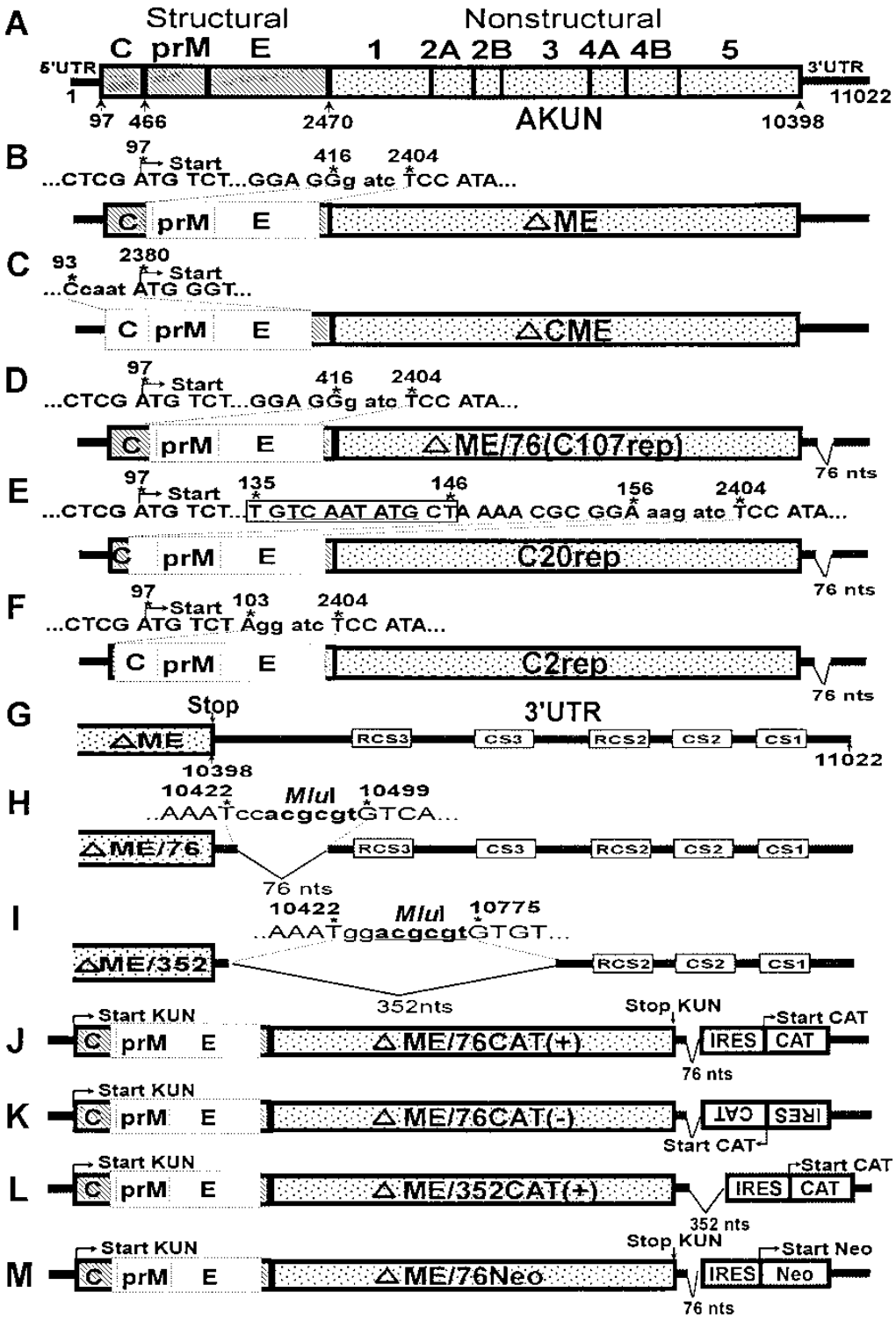


FIG. 1. Construction of KUN replicons. AKUN represents the complete genome (21), and the numbers represent nucleotide positions. Hatched boxes show the structural regions of the genome, stippled boxes indicate the nonstructural regions of the genome (12). Open boxes show parts of the KUN genome deleted in these replicons. Nucleotides shown as capital letters represent authentic KUN nucleotides, and lowercase letters represent additional nucleotides introduced during plasmid construction. Numbers above asterisks identify the nucleotides of the joined KUN cDNA sequence at the boundaries of the deletions. Underlined nucleotides in construct E represent a proposed cyclization sequence conserved in all flaviviruses and complementary to the conserved sequence in the CS1 region of the 3′UTR (see G; 17), while other nucleotides in the open box represent an extension of this complementary sequence for KUN RNA (21). Start and Stop, position of the first nucleotide of each initiating AUG codon or the last nucleotide of the termination codon for synthesis of KUN polyprotein, respectively. CS1 and CS2 in open boxes represent sequences conserved in all mosquito-borne flaviviruses, RCS2 shows the exact repeat of CS2 present in all mosquito-borne flaviviruses except YF, and CS3 and RCS3 indicate another conserved sequence and its repeat found only in flaviviruses from the Japanese encephalitis virus subgroup (17, 21). Bold underlined nucleotides in H and I represent the inserted unique recognition site for *Mlu*I restrictase. (J to L) Dicistronic KUN replicons with EMCV IRES-CAT insertions in the 3′UTR; Start CAT, initiation of translation of the CAT gene driven by the IRES of EMCV RNA. (+) and (−) in the names of the constructs indicate the orientation of the IRES-CAT insertion and therefore define the translation of CAT gene from either the plus (genomic sense) or minus (antigenomic sense) strands of dicistronic KUN replicon RNA, respectively. (M) The dicistronic KUN-Neo replicon, in which the CAT gene in ΔME/76CAT(+) is replaced by the Neo gene.

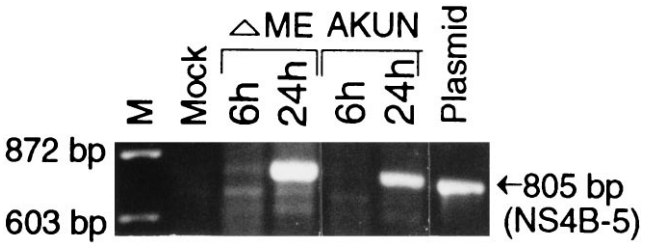
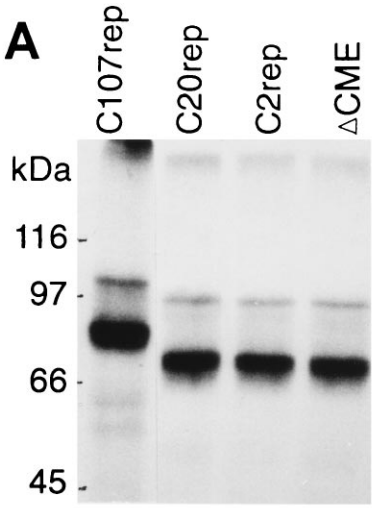


FIG. 2. Accumulation of ΔME RNA in transfected cells. One microgram of total cytoplasmic RNA isolated at 6 and 24 h after electroporation of ΔME or AKUN RNAs was subjected to RT-PCR analysis with KUN-specific primers. RT reaction was performed under standard conditions with avian myeloblastosis virus (AMV) reverse transcriptase (Promega), using a primer complementary to the minus strand of KUN RNA in the NS4B region; one-tenth of the RT reaction mixture was then used for PCR amplification with a pair of nested primers in the NS4B-5 region. Plasmid, PCR with ΔME plasmid DNA; M, DNA molecular weight standards; Mock, RT-PCR amplification of RNA from untransfected BHK cells. Arrow shows the correct size PCR fragment of 805 bp within the NS4B-5 sequence.



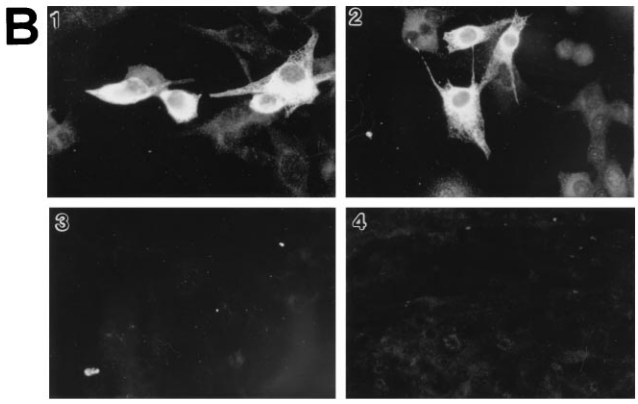


FIG. 3. In vitro translation and immunofluorescence analyses of replicon RNAs with deletions in the C coding sequence. (A) In vitro translation of deleted RNAs in rabbit reticulocyte lysates. RNA transcripts were prepared from the corresponding cDNAs digested with *Eco*RI at nucleotide 3924 of KUN cDNA (in codon 133 of the NS2A protein) and translated in rabbit reticulocyte lysates (Promega) supplemented with [^{35}S]methionine/cysteine for 60 min at 30°C. Radiolabelled translation products were identified by autoradiography after electrophoretic separation in a 10% polyacrylamide gel; positions of prestained molecular weight markers (Bio-Rad) are shown on the left. (B) Immunofluorescence with anti-NS3 antibody of BHK21 cells at 24 h after electroporation with ΔME/76(C107rep) and C20rep RNAs (panels 1 and 2, respectively) and at 48 h after electroporation with C2rep RNA (panel 3) or mock-electroporated BHK cells (panel 4). Transfected cells on coverslips were fixed with acetone at −20°C for 30 s, air dried, and incubated with rabbit anti-NS3 antibody at room temperature for 30 to 60 min and subsequently with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) for 30 min.

blot analysis with radiolabelled KUN cDNA probe (data not shown). Moreover, in vivo RNA labelling at 24, 48, and 72 h posttransfection with $^{32}P_i$ in the presence of actinomycin D and RNA gel analysis to identify products (8) showed that active replication of ΔME RNA occurred in transfected cells from 24 to 72 h posttransfection (data not shown). Thus, taken together, the results of RT-PCR detection, IF and Northern blot analysis, and in vivo RNA labelling strongly indicated continuous replication of ΔME but not of ΔCME RNA in transfected cells.

Following the results with ΔME (replicating) and ΔCME (nonreplicating) RNAs we attempted to define a minimal sequence in core protein required for RNA replication. Replicon constructs containing sequences coding for only the first 20 amino acids (C20rep; Fig. 1E) or only the first 2 amino acids (C2rep; Fig. 1F) of core protein were prepared from the ΔME/76 cDNA (C107rep; Fig. 1D), which will be described below. Note that ΔME/76(C107rep) and ΔME RNAs replicated with the same efficiency in transfected cells (see for example Fig. 5A). The C20rep construct includes a coding sequence for NMLKR (amino acids 15 to 19) conserved among mosquito-borne flaviviruses (except YF) (12) which also includes a perfectly conserved RNA sequence of eight nucleotides (KUN nucleotides 137 to 144; underlined in Fig. 1E) complementary to a proposed cyclization sequence in the flavivirus 3′UTR (KUN nucleotides 10918 to 10925) (17, 21). In KUN RNA this complementarity between the sequences in core and 3′UTR is extended to 12 nucleotides (open box in Fig. 1E) (21) and therefore this extended sequence was included in the C20rep construct. The C2rep construct was similar to the ΔCME construct, except that it contained the native KUN translation initiation codon with its flanking sequences as well as exactly the same sequence downstream of the deletion junction as in parental ΔME/76 (C107rep) or in ΔME and C20rep constructs (Fig. 1B, D, E, and F).

In order to monitor the effect of the deletions in the C coding sequence on the translation efficiency of the resulting RNA transcripts, we translated all these RNAs in rabbit reticulocyte lysates. The results showed that all RNAs produced products of a size expected by translation of their truncated transcripts (~75 to 80 kDa for C107rep RNA and ~66 to 70 kDa for C20rep, C2rep, and ΔCME RNAs) and that the efficiencies of translation were similar for all the RNAs (Fig. 3A). Thus, these deletions in C coding sequence including a complete deletion of C did not produce any noticeable effect on translation of the remaining polyprotein in vitro.

We next investigated the effect of these deletions in C on the replication activity in vivo of each replicon construct. IF analysis with anti-NS3 antibodies at 24 and 48 h after electroporation of the parental ΔME/76(C107rep) and the C2rep and C20rep RNAs into BHK21 cells showed that about 10 to 20% of cells transfected with either ΔME/76(C107rep) or C20rep RNAs were strongly positive at 24 h after transfection (Fig. 3B, panels 1 and 2), while no positive cells were detected by IF even at 48 h after transfection of C2rep RNA (Fig. 3B, panel 3), as observed previously with ΔCME-electroporated cells. We then performed a Northern blot analysis of total RNA

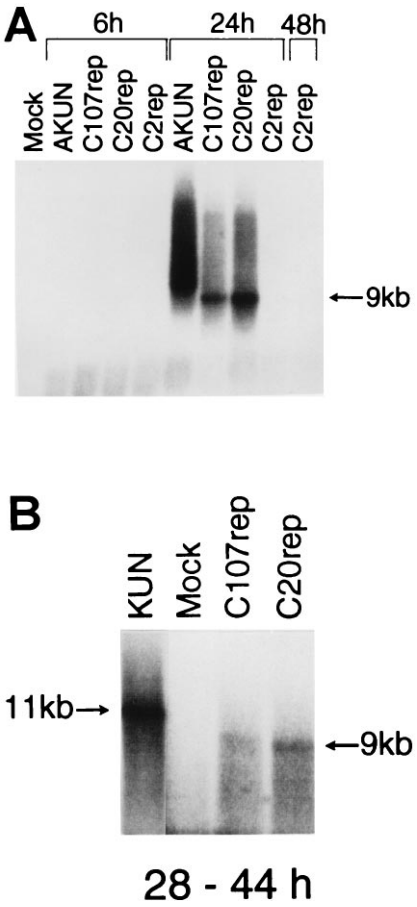


FIG. 4. Northern blot and in vivo RNA labelling analyses of replicon RNAs with deletions in the C coding sequence. (A) Autoradiogram of Northern blot hybridization of total RNA from electroporated BHK21 cells at the indicated times posttransfection, using a ^{32}P-labelled KUN cDNA probe representing the 3′UTR region. (B) In vivo RNA labelling of cells electroporated with C107rep and C20rep RNAs and incubated with $^{32}P_i$ from 28 to 44 h posttransfection in presence of actinomycin D. Note that for KUN-infected cells the labelling period was from 22 to 28 h. Labelled total RNA from cells was separated in a denaturing 1% agarose–2% formaldehyde gel. Arrows indicate the positions of RNAs of about 11 kb for KUN RNA and about 9 kb for other RNAs determined relative to migration in the same gels of ethidium bromide-stained λ DNA digested with *Bst*EII (New England Biolab).

from electroporated cells with a radiolabelled KUN cDNA probe to confirm an accumulation of KUN-specific RNA in transfected cells. No KUN specific RNA was detected in cells transfected with RNA of AKUN, C107rep, C20rep, or C2rep early (at 6 h) after transfection (Fig. 4A), reflecting the delayed onset (8 h) of detectable KUN RNA replication (8) as well as showing an absence of detectable surviving input RNA in the cellular RNA samples. Easily detectable labelled KUN-specific bands of the expected molecular sizes (~9 kb for replicon RNAs and ~11 kb for the full-length AKUN RNA) were observed in the total cellular RNA samples at 24 h after electroporation with AKUN, ΔME/76 (C107rep), and C20rep RNAs but not with C2rep RNA even at 48 h (Fig. 4A). The results of these Northern blot analysis are in complete agreement with the IF results, and together they suggest the importance of the first 60 nucleotides of the core protein coding sequence for RNA replication. Further confirmation of the continuous replication of replicon RNAs was obtained by in vivo RNA labelling of transfected cells after 28 h with $^{32}P_i$ in the presence of actinomycin D (Fig. 4B).

Effect of deletions in the 3′UTR on ΔME RNA replication. In order to study the role of the 3′UTR in flavivirus RNA replication, as well as to explore the possibility of insertion of reporter genes into the 3′UTR, we prepared two replicon constructs with deletions in the 3′UTR as shown in Fig. 1H and I. Both deletions commenced at nucleotide 10423, which is situated 26 nucleotides downstream of the termination codon, and were followed by insertion of a unique recognition sequence for *Mlu*I restrictase (Fig. 1H and I). Thus, the first construct (ΔME/76) has a deletion of 76 nucleotides including 21 nucleotides of a KUN unique insertion sequence (21), while the second construct (ΔME/352) has a deletion of 352 nucleotides including additionally the conserved sequences CS3 and RCS3 of viruses in the Japanese encephalitis flavivirus complex (Fig. 1G) (17).

IF analysis with anti-NS3 antibodies (results not shown) and Northern blot analysis with a radiolabelled KUN cDNA probe at 48 h after electroporation (Fig. 5A) showed that ΔME/76 RNA apparently replicated with the same efficiency as ΔME RNA, while the apparent efficiency of replication of ΔME/352 RNA was significantly impaired. The proportion of cells detectably expressing NS3 protein by IF was about 5 to 10 times less after electroporation of the smaller ΔME/352 RNA (data not shown) than after electroporation of the larger ΔME or ΔME/76 RNAs. Similarly, there was a difference of about 5- to 10-fold in the intensity of the radiolabelled replicon RNA bands detected in the Northern blot of the corresponding cellular RNA samples (Fig. 5A). The results were consistent in a number of experiments (data not shown). It is apparent from these results that the deleted sequence, representing 56% of the 3′UTR and including the RCS3-CS3 region, was important for establishment of efficient and easily detectable replication of KUN RNA. However, the sequence between nucleotides 10422 and 10499 could be removed without obvious deleterious effect on RNA replication.

Dicistronic KUN replicon RNAs expressing the CAT gene. The rationale behind the insertion of the CAT gene into KUN replicons was to provide a simple and quick assay for monitoring the effects of mutations/deletions on replication of KUN RNA as well as to distinguish replicon RNAs from helper KUN RNA in future experiments on complementation and packaging. The 3′UTR region 25 nucleotides downstream of the stop codon was chosen for insertion for a number of reasons. First, the insertion into this region should not interfere with upstream polyprotein processing. Second, a deletion of 76 nucleotides and insertion of 8 nucleotides in this region did not affect the efficiency of RNA replication (ΔME/76, Fig. 5A). Thus, an inserted unique recognition site for *Mlu*I restrictase in the construct ΔME/76 (Fig. 1H) was utilized to prepare dicistronic replicons by cloning the EMCV IRES-CAT sequence in both plus and minus sense orientations in order to monitor synthesis of plus and minus strands of replicon RNA during replication via expression of the CAT gene from the IRES-CAT cassette (Fig. 1J and K). The EMCV IRES sequence was inserted upstream of the CAT gene to ensure cap-independent internal initiation of translation of the CAT gene.

Initial IF screening with anti-NS3 antibodies showed approximately 2-fold and 10- to 20-fold reduction in the proportion of detectable positive cells after transfection with ΔME/76CAT(−) RNA and ΔME/76CAT(+) RNA, respectively, relative to ΔME/76-transfected cells (data not shown). Northern blot analysis at 48 h posttransfection showed the presence of easily detectable RNA bands of the expected size with both KUN- and CAT-specific radiolabelled cDNA probes in cells

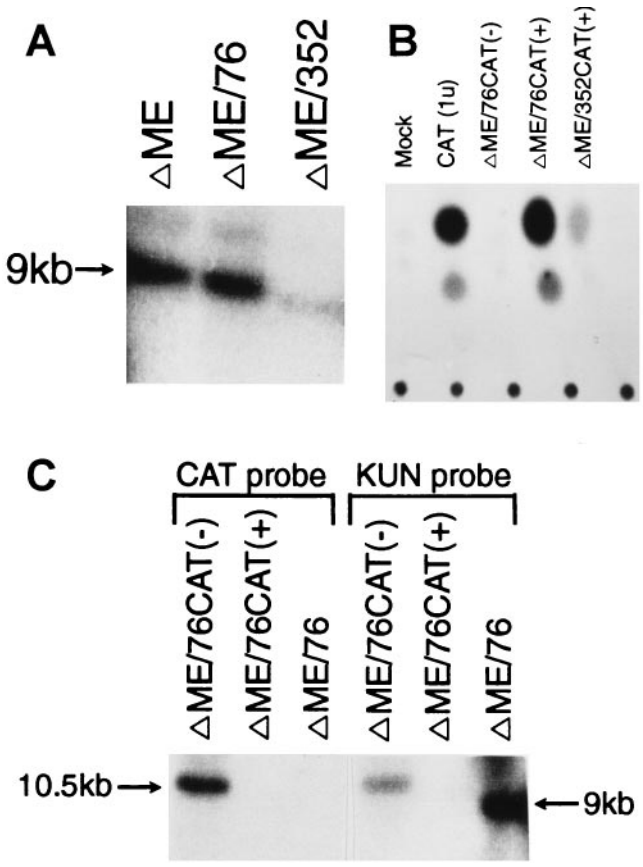


FIG. 5. Effects of deletions and insertions in the 3′UTR of ΔME replicons (Fig. 1G to L). (A) Autoradiogram of Northern blot hybridization of total RNA from cells at 48 h after electroporation with ΔME, ΔME/76, and ΔME/352 RNAs, using a ^{32}P-labelled KUN cDNA probe representing the NS4B region. (B) Autoradiogram of the CAT assay of the lysates from cells electroporated with dicistronic KUN-CAT RNAs at 48 h after electroporation. CAT assays with ^{14}C-acetyl CoA as donor substrate were performed as described in Materials and Methods. One unit of commercial CAT (CAT[1 u]; Promega) was included. Mock, assay for mock-transfected cells. (C) Autoradiogram of Northern blot hybridization of total RNA from cells electroporated with dicistronic ΔME/76CAT(+), ΔME/76CAT(−), or parental monocistronic ΔME/76 RNAs at 48 h after electroporation, using ^{32}P-labelled KUN and CAT cDNA probes representing the KUN NS4B region and IRES-CAT sequence, respectively. Arrows indicate the positions of RNAs with molecular sizes of about 9 kb (for ΔME/76 and ΔME/352 RNAs) and about 10.5 kb (for ΔME/76CAT RNAs) determined as described in the legend to Fig. 4.

transfected with ΔME/76CAT(−) RNA (Fig. 5C). No KUN- or CAT-specific RNA was detected in ΔME/76CAT(+)-transfected cells by Northern blots (Fig. 5C), consistent with the low proportion of cells expressing NS3 detected by IF. It should be noted that IF focuses on individual positive cells and therefore can easily detect a very low percentage of replicon-expressing cells which may not be detectable by Northern blot assay. In addition to positive detection by IF, a very sensitive CAT assay showed readily detectable expression of functional CAT in lysates from cells transfected with ΔME/76CAT(+) RNA (Fig. 5B). Significantly, no CAT activity at all was detected in the lysates from cells transfected with ΔME/76CAT(−) RNA (Fig. 5B), despite on apparently more efficient (about 10-fold) replication of this RNA than ΔME/76CAT(+) RNA (Fig. 5C).

To evaluate the ability of a CAT reporter assay to detect the inhibitory effect of the large deletion in the 3′UTR on replication of the replicon RNA we inserted the IRES-CAT cassette in a plus-sense orientation into ΔME/352 RNA [ΔME/352CAT(+), Fig. 1L]. Despite the very low proportion of detectable cells positive in IF analysis (severalfold less than for ΔME/352 RNA) with anti-NS3 antibodies at 48 h after transfection with ΔME/352CAT(+) RNA (data not shown), a functionally active CAT enzyme was detectable in the corresponding cell lysates but at a very low level relative to the signal for ΔME/76CAT(+) RNA (Fig. 5B). In a parallel LSC assay (see Materials and Methods), the radioactive counts in lysates from ΔME/76CAT(+)- and ΔME/352CAT(+)-transfected cells were 7,020 and 1,307 cpm, respectively, and these were 18.6- and 3.5-fold greater than the counts from mock-transfected cells, respectively. Similar results were obtained in two independent transfection experiments (data not shown). These easily detectable quantitative differences by CAT assay correlate well with the differences observed by IF and Northern blot analyses of the corresponding monocistronic replicon RNAs without IRES-CAT insertions [compare ΔME/76 and ΔME/352 Northern blots in Fig. 5A and CAT assay results with ΔME/76CAT(+) and ΔME/352CAT(+) in Fig. 5B].

Selection and recovery of BHK21 cells persistently expressing a dicistronic KUN-Neo replicon. Following our initial observations on detection of some BHK21 cells still expressing NS3 protein at 15 days posttransfection with the ΔME replicon (see above), we attempted to extend the period of expression and to increase the proportion of replicon-expressing cells by preparing a construct expressing the Neo gene. In this system, cells expressing the Neo gene can be selected from a mixed population with nonexpressing cells by maintenance in medium containing the antibiotic G418, also known as Geneticin, which is toxic for normal cells (38). To express the Neo gene in a KUN replicon we used the same approach as for expression of the CAT gene; an EMCV IRES-Neo cassette was inserted into the *Mlu*I site in the 3′UTR of ΔME/76 (Fig. 1H) to produce construct ΔME/76Neo (Fig. 1M). Similarly to the results with ΔME/76CAT(+) RNA, transfection of ΔME/76Neo RNA occurred at low efficiency, only about 0.5 to 1% of cells being positive with low intensity of staining in IF analysis with anti-NS3 antibodies at 48 h after transfection and prior to Geneticin selection (data not shown). After 8 days of incubation in medium containing 800 μg of G418 (Geneticin; Gibco BRL) per ml a number of growing cell colonies were observed, while all mock-transfected cells had died (data not shown). Continued passaging of surviving cells in medium containing 1 mg of Geneticin per ml resulted in recovery of a cell culture persistently expressing the ΔME/76Neo replicon, with the great majority of cells positive (with variable intensity) in IF analysis with anti-NS3 antibodies after as many as nine passages or 41 days after transfection (Fig. 6A). Although these cells grew more slowly than nontransfected BHK cells, most of the surviving cells appeared normal by high-power light microscopy, without obvious signs of cytopathic effect. Northern blot analysis of the total cellular RNA during nine passages using a radiolabelled Neo cDNA probe showed the presence of Neo-specific RNA of the expected molecular size (~10.5 kb) for a ΔME/76Neo replicon (Fig. 6B). To confirm continuous expression of the ΔME-Neo replicon we pulse-labelled cells from the second passage (at 16 days) for 5 h with [^{35}S]methionine/cysteine, followed by radioimmunoprecipitation of the cell lysates with anti-NS3 antibodies. The results showed active incorporation of the label into KUN NS3 protein (molecular size of ~70 kDa) during the pulse comparable to that observed under similar conditions in KUN-infected cells (Fig. 6C). This result also indicates that correct posttranslational processing of NS3 and probably of other KUN NS proteins occurred in ΔME/76Neo-expressing cells.

A long-term persistence of replicating and expressing KUN-

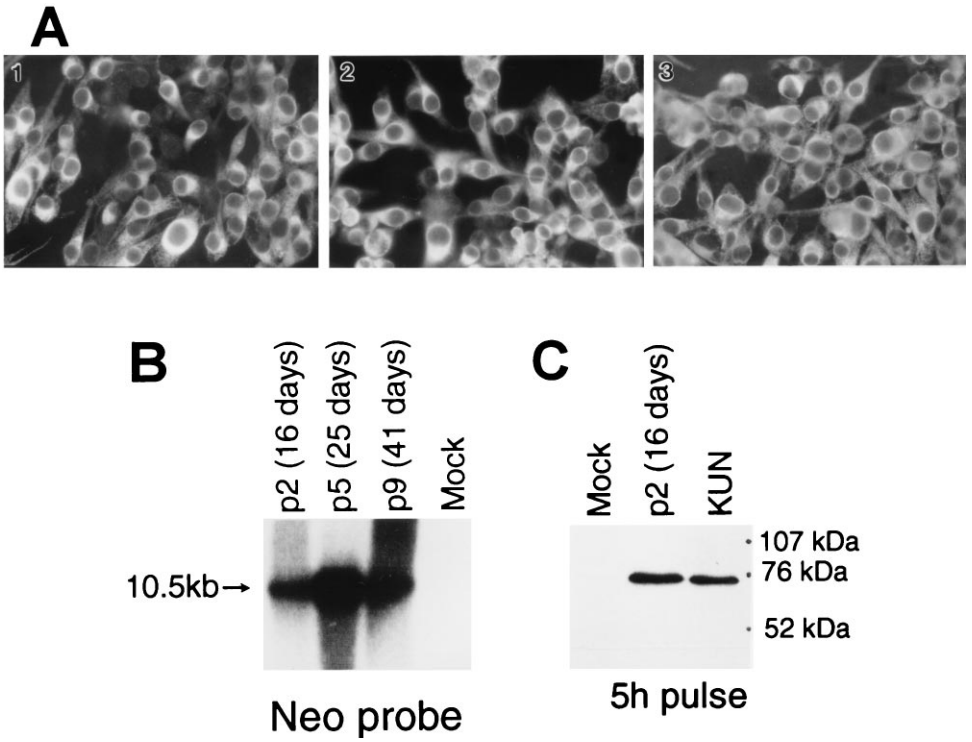


FIG. 6. Recovery and characterization of cells continuously expressing the dicistronic KUN-Neo replicon (Fig. 1M). (A) Immunofluorescence with anti-NS3 antibody of cells selected and maintained in medium with Geneticin for 16 days (passage 2, panel 1), 25 days (passage 5, panel 2), and 41 days (passage 9, panel 3) after initial transfection with ΔME/76Neo RNA. (B) Autoradiogram of Northern blot hybridization with radiolabelled Neo cDNA probe of total RNA isolated from ΔME/76Neo-expressing cells at passages two, five, and nine (p2, p5, and p9) during selection and maintenance in medium containing Geneticin. An arrow indicates the position of RNA of about 10.5 kb determined as described in the legend to Fig. 4. (C) Autoradiogram of the radioimmunoprecipitation with anti-NS3 antibody of lysates from cells in a 60-mm-diameter dish labelled for 5 h with [^{35}S]methionine/cysteine either at 16 days (p2) after selection and maintenance in medium containing Geneticin or at 22 h after KUN virus infection. Cells were lysed on ice with 0.5% Nonidet P-40 (NP-40) in 0.1 M NaCl, 20 mM Tris-HCl (pH 8.0), the nuclei were removed by centrifugation, and the supernatant was adjusted to 1% NP-40–0.5% deoxycholic acid sodium salt–0.1% sodium dodecyl sulfate. Approximately one-third of the KUN-infected cell immunoprecipitate relative to those of Mock or ΔME/76Neo-expressing cells were loaded on the gel.

Neo replicon RNA was thus observed in transfected BHK21 cells selected by Geneticin without causing any apparent changes in the morphology of surviving positive cells.

DISCUSSION

We have constructed KUN replicons able to replicate in vivo with large deletions in the structural region of the genome (ΔME [nucleotides 417 to 2403, inclusive] and C20rep [nucleotides 157 to 2403]) and in the 5′ region of the 3′UTR (ΔME/76 and ΔME/352). A range of assays was used to establish that amplification and expression of the transfected RNAs occurred and that the assays were not merely detecting unamplified RNA. These assays included comparisons with transfected infectious AKUN RNA and with RNA transcripts of the replicon constructs ΔCME and C2rep, in which the introduced deletions were found to be lethal for replication, even though both of these RNA transcripts were translated efficiently in vitro. Thus, the input (transfected) RNA or its expression was not detectable at 6 h after transfection by RT-PCR or IF for ΔCME, or by Northern blot assays for C2rep, similar to results with AKUN, ΔME, and C20rep RNAs. ΔCME and C2rep RNA remained undetectable later (24, 48, or 72 h after transfection) by IF, RT-PCR, and/or Northern blot assays. In contrast, the other replicon RNAs were clearly amplified and expressed as early as 24 h (and later), as judged by IF (ΔME, C20rep, ΔME/76, and ΔME/352), RT-PCR (AKUN and ΔME), and Northern blot assays (AKUN, ΔME, and C20rep) and by incorporation of a pulse of $^{32}P_i$ into KUN-specific RNA (ΔME and C20rep).

Deletions in the 5′ region of C gene resulting in the removal of 98% or all of the C coding sequence (C2rep or ΔCME, respectively) were lethal, indicating a possible role in replication of either the first 20 amino acids of core as in C20rep or of the RNA sequence itself which includes a proposed cyclization sequence (nucleotides 137 to 144). The lethal effect of these deletions in the 5′ region of C on replication was unrelated to translation, since the translation efficiency of all four (ΔME, C20rep, C2rep, and ΔCME) RNAs in rabbit reticulocyte lysates was similar. In further analyses, preliminary results indicated that initiation of translation at the next downstream AUG codon commencing at nucleotide 142 (effected by mutation of the native initiation codon in C20rep) did not abolish replication (20). Hence the RNA sequence itself including the cyclization sequence may be an essential requirement for replication. A direct proof of the importance of the cyclization sequence in RNA replication can be obtained only by further precise deletions in the C20rep RNA.

Deletions in the variable region of the flavivirus 3′UTR (32, 40) had no effect (ΔME/76) or a restrictive effect (ΔME/352) on replication, as indicated by a large reduction in the percentage of IF-positive cells and by Northern blot assay. These assays do not detect a possible subpopulation of transfected cells in which replicon ΔME/352 may be defective in initiating the establishment of productive replication, whereas in other transfected cells (positive by IF) the requisite threshold level of

detectable viral products to sustain replication is attained and maintained. The restrictive effect in ΔME/352 was associated with the deletion of two sequences, CS3 and RCS3, conserved amongst flaviviruses of the Japanese encephalitis virus complex. Recently published results with dengue 4 virus (DEN4) full-length RNA transcripts showed various effects of deletions in the 3′UTR on virus recovery (29). Although a direct comparison between our results and the DEN4 data is difficult because of the differences in size of both UTRs (624 nucleotides for KUN and only 384 nucleotides for DEN4) and an absence of sequences similar to KUN CS3 and RCS3 in DEN4 3′UTR, it is quite clear from the presented data that deletions in the 5′ half or in the CS2A region of the DEN4 3′UTR resulted in reduced efficiency of RNA replication as the size of the deletion increased, as judged by lower virus yield or by the delay of plaque formation in transfected cells and/or a smaller plaque size. Significantly, in view of the lethal deletion of the postulated cyclization sequence in the core gene of replicons C2rep and ΔCME, deletions of the CS1 sequence, or the sequence just upstream of the complementary postulated cyclization sequence in CS1 of DEN4, were also lethal.

Encouraged by the efficiency of replication of ΔME/76 containing a unique *Mlu*I restriction site 25 nucleotides downstream from the stop codon, we used that site for further insertion of IRES-foreign gene cassettes, initially the EMCV IRES-CAT sequence, in both plus and minus senses. Although ΔME/76CAT(+) replication was readily detected by CAT assay, a strong inhibitory effect of the inserted sequence was noted, presumably caused by competition on the same mRNA between the KUN initiation codon and the IRES-mediated initiation for the cellular translation machinery. This may cause some limitation on the utility of this strategy for expression of heterologous products. However, this strategy employing the use of dicistronic replicons could still be applied for monitoring the effects of deletions/mutations on replication with sensitive enzyme assays. For example, the CAT assay was sensitive enough to detect not only the replication of ΔME/76CAT(+) RNA but also the difference in the replication efficiency between two RNAs with different deletions in the 3′UTR [compare the results for ΔME/76CAT(+) and ΔME/352CAT(+) in Fig. 5B].

Importantly, while the ΔME/76CAT(−) replicon replicated efficiently (shown by IF and Northern blot analyses), the CAT assay was negative, indicating that free minus-strand RNA was not available for translation of the CAT gene, presumably because it remains bound in the replicative-form template after synthesis in accord with the postulated model of flavivirus RNA synthesis (8). As far as we are aware, this is the first demonstration that no free flavivirus-specific RNA of negative polarity is present during replication in cells.

In order to exploit the use of KUN replicons by pursuing biochemical and genetic studies on replication and to examine the ultrastructure of transfected cells, it became obvious that considerable improvement in efficiency of transfection or an enrichment procedure was necessary because the positive cells tended to be overgrown by normal cells. We chose the second option, encouraged by the apparent persistence of replicon replication for several days, the observation of some paired IF-positive cells indicating cell division (data not shown), and the demonstrated ability to make deletions and insert a reporter gene, as in ΔME/76CAT(+) RNA. Accordingly, the CAT gene was replaced by the Neo gene and the low percentage of replicon-expressing cells was strikingly enriched by selection during growth in the presence of the antibiotic G418. These selected cells were by far the dominant population throughout as many as nine passages (over 41 days posttransfection). It is noteworthy that no apparent cytopathic effect was observed in ΔME/76Neo-expressing cells; this distinguishes the KUN replicon from the replicons of other RNA viruses such as Sindbis virus which are associated with delayed but still profound cytopathic effect during replication in mammalian cells (14).

Our results provide valuable information on the construction and potential use of flavivirus replicons for studying effects of mutations/deletions on RNA replication as well as for the possible future development of a packaging system for flavivirus replicon-based expression of foreign genes similar to that of the alphaviruses Sindbis (5) and Semliki Forest (26). The persisting replication and absence of cell lysis shown here for KUN replicons could provide some advantages: for example, persistent antigen presentation without prematurely induced lysis of the antigen-presenting cells would provide adequate time for induction of the immune response, not available with replicons which cause rapid cell lysis. In addition, incorporation of an IRES-Neo cassette into the 3′UTR of flavivirus replicons overcomes the problem of low transfection efficiency by use of G418 selection. Since the minimal C coding sequence required for efficient replication of KUN replicon RNA is now defined to be within only the first 60 nucleotides, it should be possible to express foreign proteins as fusions with truncated C protein while still retaining the Neo gene in the 3′UTR for selection purposes. Taken together, the persistence of replication, an absence of cytopathic effect, and incorporation of an antibiotic resistance gene allowing simple and efficient selection of replicon-expressing cells make flavivirus replicons a unique and very useful addition to existing RNA virus expression systems.

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