

# Identification of Mutated Cyclization Sequences That Permit Efficient Replication of West Nile Virus Genomes: Use in Safer Propagation of a Novel Vaccine Candidate<sup>∇</sup>

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**Existing live-attenuated flavivirus vaccines (LAV) could be improved by reducing their potential to recombine with naturally circulating viruses in the field. Since the highly conserved cyclization sequences (CS) found in the termini of flavivirus genomes must be complementary to each other to support genome replication, we set out to identify paired mutant CS that could support the efficient replication of LAV but would be unable to support replication in recombinant viruses harboring one wild-type (WT) CS. By systematic evaluation of paired mutated CS encoded in West Nile virus (WNV) replicons, we identified variants having single and double mutations in the 5'- and 3'-CS components that could support genome replication at WT levels. Replicons containing only the double-mutated CS in the 5' or the 3' ends of the genome were incapable of replication, indicating that mutated CS could be useful for constructing safer LAV. Despite the identity of the central portion of the CS in all mosquito-borne flaviviruses, viruses carrying complementary the double mutations in both the 5'- and the 3'-CS were indistinguishable from WT WNV in their replication in insect and mammalian cell lines. In addition to the utility of our novel CS pair in constructing safer LAV, we demonstrated that introduction of these mutated CS into one component of a recently described two-component genome system (A. V. Shustov, P. W. Mason, and I. Frolov, *J. Virol.* 81:11737-11748, 2007) enabled us to engineer a safer single-cycle WNV vaccine candidate with reduced potential for recombination during its propagation.**

West Nile virus (WNV) is a member of the genus *Flavivirus* of the family *Flaviviridae*, a genus that includes dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (12). The diseases caused by these viruses have a serious impact on public health, especially in developing countries. WNV has spread widely in the United States since its introduction in 1999 and become a major public health problem, producing significant morbidity and mortality (42).

WNV, like other flaviviruses, is an enveloped, positive-strand RNA virus with a genome of about 11 kb. A single open reading frame encodes the viral polyprotein, which is processed co- and posttranslationally into the structural (C, prM/M, and E) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. This open reading frame is flanked by 5' and 3' untranslated regions (UTRs). The viral structural proteins form infectious viral particles. The nonstructural proteins responsible for various enzymatic activities, including those of the RNA-dependent RNA polymerase, helicase, and protease, are all essential for viral genome replication (24). Furthermore, the NS proteins also are capable of interfering with some aspects of the infected cell's innate immune responses to infection (6, 23, 26, 31, 32), and some NS

proteins have also been shown to be required for efficient virion assembly (22, 25).

Early studies on mosquito-borne flaviviruses identified highly conserved short complementary sequences near the 5' and 3' ends of flavivirus genomes that included an eight-nucleotide core sequence that is conserved among all mosquito-borne flaviviruses. These sequences were predicted to circularize the genome (17) and have therefore been termed cyclization sequences (CS). The 5' CS is located within the C coding region, and the 3' CS is present in the 3' UTR, just upstream of a 3'-terminal stem-loop structure that is also highly conserved among all flaviviruses (11, 43) and is thought to function as a critical element of the promoter for minus-strand-RNA synthesis (10). Recently it was reported that another RNA interaction between a region upstream of the viral AUG (defined as the upstream AUG region, or 5' UAR) and a complementary 3' UAR found at the end of the viral genome are required for efficient replication of DENV2 (3) and WNV (45) genomes.

Several studies on CS have shown that their complementarity, rather than nucleotide sequence, is a prerequisite for replication of the genomes of WNV, YFV, and DENV (1, 2, 13, 19, 27). However, the precise nucleotide sequence of CS also appear to be important for efficient viral RNA replication, since genomes with unnatural CS, although viable, usually replicate more poorly than wild-type (WT) genomes (2, 19, 27).

Live-attenuated vaccines (LAV) for yellow fever and Japanese encephalitis are in widespread use, and LAV for other

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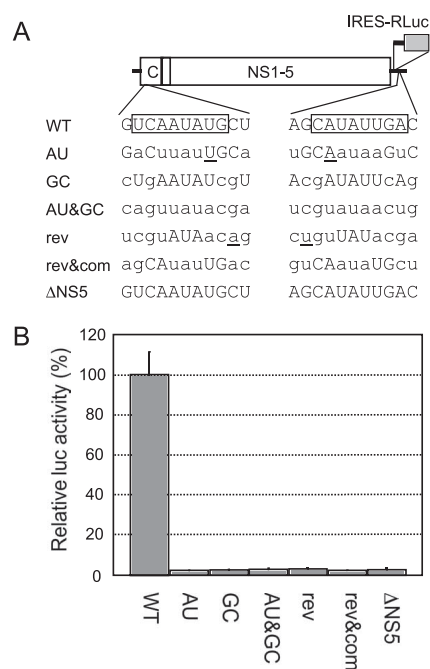
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flavivirus diseases, including dengue and West Nile encephalitis, are in different stages of development (7, 8, 16, 18, 21, 29, 34, 35, 40). LAV offer considerable advantage over inactivated viral vaccines or subunit vaccines in terms of efficacy and cost. However, there is a concern that recombination between these LAV and naturally circulated flaviviruses could result in generation of unnatural viruses with increased virulence over either natural or vaccine strains (38). This potential problem can be addressed by engineering unnatural CS in the LAV genomes such that their 5' and 3' CS are not complementary to the corresponding CS elements of circulating WT viruses. Thus, recombinant genomes generated by a single round of recombination between these LAV genomes and WT genetic material would not be viable. Recently, we showed that flaviviruses can replicate as two-component genome viruses, a system that opens up the opportunity of producing a new class of LAV (39). However, large-scale production of these viruses in vitro requires coinfection of two defective genomes in the same cell, providing a situation that could result in intergenomic recombination leading to the production of replication-competent viruses that could be pathogenic. Thus, the safety of this two-component system would be enhanced by the introduction of mutated CS into one of the two defective genomes. However, to be efficiently utilized for both enhancing LAV safety and improving our two-component genome system, there is a need for mutated CS that support highly efficient genome replication.

In this study, a large number of nucleotide substitutions were systematically introduced into the CS in both the 5' and 3' regions of a WNV subgenomic replicon (WNR), and the mutated genomes were tested for their replication efficiency. Using this strategy, we selected mutations in the 5' and 3' CS elements that supported efficient replication of WNV genomes and allowed them to be packaged into infectious particles. Two such mutations were combined (to increase their genetic stability) and further investigated to demonstrate that genomes with these mutations only in the 5' CS or only in the 3' CS were unable to replicate. These results suggest that the mutated CS could be useful for development of safer LAV with reduced probability of generating recombinant viruses with naturally circulating flaviviruses. Surprisingly, the replication of viruses engineered to contain these double-mutant CS in both mammalian and insect cells was indistinguishable from that of WT virus. Furthermore, we inserted these mutations into one component of our previously described two-component genome vaccine (39) and showed that a genome containing matched WT CS could efficiently complement a genome containing the matched mutant CS, producing high titers of infectious particles. These data demonstrate the utility of CS mutations in generating two-component genome vaccines with a reduced capacity to generate viable virus in the event of single-round recombination with only one crossover.

#### MATERIALS AND METHODS

**Cells, plasmids, and viruses.** BHK cells were maintained at 37°C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Vero cells were maintained at 37°C in MEM containing 6% FBS and antibiotics. BHK(VEErep/C<sup>s</sup>-E/Pac) packaging cells expressing the WNV C-prM-E proteins were propagated at 37°C in Dulbecco's MEM supplemented with 10% FBS and 10 µg/ml puromycin as previously described (14, 44). C7/10



**FIG. 1.** Effects of nucleotide sequence of CS on RNA replication. (A) Schematic representation of WNV replicon RNA structure (WNR-CNS1-5Rluc) and position of the Rluc-encoding cistron. The nucleotide sequences of CS are indicated next to their names (see the text). Boxes indicate the eight nucleotides that are 100% conserved among all mosquito-borne flaviviruses. Nucleotide substitutions in the 5' CS and 3' CS are in lower case. Underlined nucleotides were exceptions to the rules used to swap codons (see the text) that were implemented to avoid generation of stop codons in 5' CS that reside in the C coding region. ΔNS5 contains WT CS and a frameshift mutation upstream of the active-site GDD motif of the RNA-dependent RNA polymerase of NS5. (B) RNA replication of WT and mutant replicons in BHK cells. BHK cells were transfected with identical amounts of in vitro-transcribed Rluc-expressing WNR RNAs. Rluc activities were measured at 5 h and 2 days posttransfection. The Rluc activity obtained at 2 days is shown normalized to activity obtained at 5 h to account for slight differences in transfection efficiency (see the text). Data for each condition are averages of triplicate values with error bars showing standard deviations; the WT control value was set to 100%. The experiment was repeated at least twice, with consistent results.

mosquito cells were maintained at 30°C in Leibovitz's L15 medium supplemented with 10% FBS, 10% tryptose phosphate broth, and antibiotics.

A WNR genome containing the entire WNV C gene (WNR-CNS1-5) was described previously (9). WNR-CNS1-5Rluc was constructed from WNR-CNS1-5 by inserting an IRES (internal ribosome entry site)-driven humanized *Renilla* luciferase (Rluc) gene into an NsiI site located 39 bases downstream of the termination codon (see Fig. 1).

For studies requiring a full-length WNV cDNA, a previously described WNV cDNA derived from a human WNV isolate obtained in Texas in 2002 (36) was introduced into a bacterial artificial chromosome (BAC) plasmid to enhance its stability (41). A BAC-propagated cDNA derivative of our previously described single-cycle WNV lacking a functional C gene (28), designated RepliVAX WN.2, has been described (44). For the construction of the helper genome for our two-component genome system, we inserted a cassette consisting of the gene for the foot-and-mouth disease virus (FMDV)-2A autoprotease and a codon-optimized WNV C gene between the truncated C and NS1 coding sequences in WNR-CNS1-5.

CS mutations were introduced into the cDNAs encoding the WNV (and WNR) genomes by using PCR and confirmed by sequencing. The genome sequences used in this study are available from the authors upon request.

**RNA synthesis using in vitro transcription reactions.** Plasmids were prepared for runoff transcription by digestion with SmaI, and the resulting template DNAs

were *in vitro* transcribed using a MegaScript T7 synthesis kit (Ambion) supplemented with a 7mG(ppp)G cap analogue (New England Biolabs). The yield and integrity of transcripts were analyzed by gel electrophoresis under nondenaturing conditions, and aliquots of transcription reaction products were used for transfection without additional purification.

**RNA transfection.** RNA was transfected into BHK monolayers using Lipofectin (Invitrogen) by a slight modification of the manufacturer's suggested protocol. Three hours after transfection, the media containing the RNA and Lipofectin mixture were removed from the cell layer, and the cells were refed with growth medium and incubated at 37°C until assayed as described below. To produce infectious WNV genome-containing particles, BHK cells and BHK packaging cells expressing WNV structural protein, BHK(VEErep/C<sup>+</sup>-E/Pac) (see above), were electroporated by T7 transcription reactions (36), and virus or virus-like particles (VLPs) containing the WNR were collected as previously described (14).

**Analysis of VLPs, RepliVAX, and virus replication.** Cell monolayers prepared in multiwell plates were incubated with dilutions of virus, VLPs, or RepliVAX and helper particles (two-component system) and then overlaid with medium containing 1% FBS (in some cases containing 0.8% carboxymethyl cellulose). Following incubation for the appropriate time at the appropriate temperature (see the figure legends), the monolayers were fixed and immunostained as described previously (36), and foci (or individual cells) were counted and used to calculate a titer of focus-forming units/ml for spreading infections or infectious units/ml for nonspreading infections. For most studies, infected cells were immunostained with a polyclonal anti-WNV hyperimmune ascitic fluid, but in cases where the packaging cells (which expressed WNV structural proteins) were utilized, cells containing replicating WNV genomes were stained with a monoclonal antibody to NS1 (20) (provided by E. Konishi, Kobe University). In other cases, where assay cells infected with E-expressing RepliVAX genomes needed to be distinguished from cells infected with VLPs expressing "helper" genomes (see Results), a monoclonal antibody to E (7H2; Bioreliance) was utilized. For growth curves, BHK, Vero, or C7/10 cell monolayers were infected at defined multiplicities of infection (MOIs) (see the figure legends) and then incubated at the appropriate temperature. Media were removed (and replaced with fresh media) at the indicated time points and stored at -80°C for subsequent titration as described above.

**Renilla luciferase assay.** Monolayers of BHK cells transfected with Rluc-expressing RNAs by using Lipofectin were lysed by the addition of reporter lysis buffer (Promega), and the lysates were stored at -20°C for subsequent assays. Prior to the assays, the extracts were thawed and clarified to remove insoluble debris, and a portion of each extract was mixed with 5 volumes of an Rluc reaction buffer (100 mM EDTA, 50 mM Tris [pH 8.0] containing 5 µg/ml coelenterazine; Nanolight Technology) in black-walled 96-well microtiter plates. Following a 1-min incubation period, luminescence was determined with a Microplate luminometer (Applied Biosystems).

**RNA secondary-structure prediction.** RNA secondary structure and the value of  $\Delta G$  were predicted by using Mfold version 3.2 (46). In cases where multiple structures were predicted, the structure with lowest free energy of initial  $\Delta G$  was chosen for presentation.

## RESULTS

**Drastic alteration of CS abolishes RNA replication.** Previous studies demonstrated that CS need to be complementary to support genome replication of flaviviruses and that their complementarity, rather than nucleotide sequence, is a prerequisite for flavivirus RNA replication (2, 19, 27). However, those studies also showed that viral genome with complementary but "unnatural" CS replicated more poorly than genomes with WT CS. In those studies, introduction of unnatural CS resulted in changes in the overall free energy of the expected interactions between the 5' CS and 3' CS of the tested genomes. Therefore, these altered free energies of the interaction of CS as well as the alteration of specific nucleotides could have affected RNA replication efficiency and thus could have been responsible for the observed defects in replication of the mutant CS-bearing genomes.

In our initial attempts to generate CS mutants that could not productively recombine with WT sequences, we created a se-

ries of CS mutant WNRs designed to contain substantially altered CS with multiple base changes but predicted free energies of interaction identical to the those of WT CS (Fig. 1A). To this end, all CS mutations were designed to conserve the base pairing of the 11-nucleotide 5'- and 3'-CS regions as well as the overall number of A-U and G-C base pairs to ensure that mutant genomes maintained the free energy of WT WNV. These mutant constructions include a switch of all A-U pairs to U-A, a switch of all G-C pairs to C-G, a switch of both A-U and G-C pairs, reverse sequences, and reverse and complementary sequences. Certain changes required by these "swap" algorithms were not implemented, since they would have resulted in nonviable-stop-codon generation in the 5' CS that resides in the C coding region. In these cases, either WT nucleotides or other suitable substitutions were placed at these positions (Fig. 1A).

These mutations were incorporated into plasmids carrying Rluc-expressing WNR cDNAs (WNR-CNS1-5Rluc), and RNAs derived from these plasmids were generated *in vitro* and transfected into BHK cells. The Rluc activities were measured at 5 h posttransfection, providing a measurement of translation of transfected RNA, and at 48 h posttransfection, providing a measurement of RNA replication (27). The values obtained 5 h after transfection were nearly identical for all replicons, including  $\Delta$ NS5, which contains a frameshift mutation upstream of the GDD motif of NS5 for a negative control with no replication activity, indicating that the CS mutations had no effect on the initial translational activity of the reporter gene encoded by the transfected replicon RNA. To facilitate analyses of replication levels, these 5-h data were used to normalize for slight differences in transfection efficiency (5-h Rluc data were usually within 50% of each other within experiments, and these 5-h values were always less than 7% of the 48 h value in the case of WT CS replicons [WNR-CNS1-5Rluc]). As shown in Fig. 1B, all five replicons harboring the CS mutations showed levels of normalized Rluc activity indistinguishable from the Rluc activity of the replication-deficient control RNA ( $\Delta$ NS5). These data indicated that many possible complementary, unnatural CS with an overall free energy of hybridization similar to that of WT sequences were incapable of supporting RNA replication, demonstrating that the precise sequences of these regions are as important for replicational activity as complementarity per se.

**Effects of single-nucleotide substitution within the CS on RNA replication.** Following this demonstration that the large changes in CS examined in Fig. 1 were nonviable, despite their maintenance of WT free energies, we undertook a more systematic analysis of the CS. To this end, complementary single-nucleotide substitutions were introduced into CS in both 5'- and 3'-CS regions of WNR, as shown in Fig. 2A.

The Rluc activity from mutated replicons was compared with that of WT replicon RNA (WNR-CNS1-5Rluc). As shown in Fig. 2B, m1 and m2, which contain 1-bp substitutions at the terminal end of the paired CS, showed a drastic reduction of RNA replication. However, m4 and m7 displayed no detectable reduction in RNA replication. Other mutants showed moderately reduced replication efficiency (20 to 80% of WT efficiency). These data suggest that there are both critical and noncritical nucleotides in the CS. Using Mfold to produce structures from an artificial sequence created by fus-



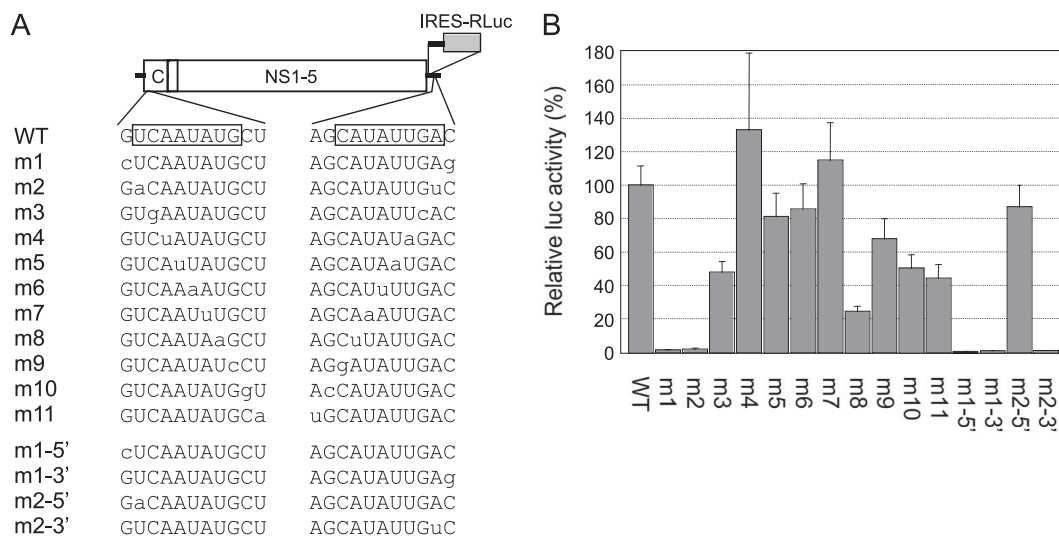


FIG. 2. Effect of single nucleotide mutations in CS on WNV RNA replication. (A) Schematic representation of WNR RNA structure. The nucleotide sequences of mutant CS are indicated next to their respective names. Boxes indicate the eight nucleotides that are 100% conserved among all mosquito-borne flaviviruses. Nucleotide substitutions in the 5' CS and 3' CS are in lower case. (B) RNA replication of WT and mutant replicons in BHK cells. Averages of triplicate relative luciferase activities at 2 days posttransfection were normalized to that at 5 h, and the WT control value was set to 100%; error bars show standard deviations. The experiment was repeated at least twice, producing similar results.

ing 52 bases of the C coding region, 31 bases of the 3' UTR, and a 38-nucleotide A stuffer, we were able to show that the predicted structures of all mutants were indistinguishable from those of the parental CS (data not shown). Thus, it appears that these apparent site-specific nucleotide requirements were likely not due to a large alteration of secondary structures predicted for this region.

To help determine if these site-specific requirements reflected sequence-specific needs in only one CS or the other, we examined the replication-incompetent m1 and m2 mutants in more detail. To this end, we constructed reporter RNA replicons containing the m1 or m2 mutation in only the 5' UTR or 3' UTR (Fig. 2A). As shown in Fig. 2B, m1-5', m1-3', and m2-3' showed drastically reduced replication of RNA, whereas m2-5' showed only slight reduction of RNA replication compared to the WT (WNR-CNS1-5Rluc). Surprisingly, m2-5' could replicate much better than m2, which altered both the 5' CS position (U to A) and 3' CS position (A to U), suggesting that A at position 2 in 3' CS, rather than base pairing, is critical for efficient RNA replication. In contrast, the nucleotide at position 1 in CS appeared to be critical in both the 5' UTR and 3' UTR, although we could not exclude the possibility that base pairing per se was critical at this residue.

**Effect of combined CS mutations on RNA replication and viral genome packaging.** To help produce an altered CS that would be unlikely to be able to support replication when combined with a WT sequence, we decided to create a CS with two separate mutations. To this end, we combined the two mutations (m4 and m7) that appeared to support WT levels of replication with each other, creating CS with a combination of these substitutions (m4/7; Fig. 3A). Transcribed RNA encoding this double mutant was introduced into BHK, and Rluc activity was determined. As shown in Fig. 3B, m4/7 replicated as efficiently as a WT replicon.

To further investigate the efficiency of m4/7 in supporting

replication, these mutations were inserted into a replicon without a reporter gene (identical to the constructs shown in Fig. 3A, except for the lack of a reporter gene) to examine their effect in a multicycle replication system. To this end, the WT and CS mutant replicon RNAs without a Rluc reporter gene were electroporated into BHK packaging cells, BHK(VEErep/C\*-E/Pac), expressing the WNV structural proteins C, prM, and E to produce VLPs (14). The resultant culture supernatants were harvested, and side-by-side focus formation assays of WT and m4/7 VLPs on BHK(VEErep/C\*-E/Pac) cells were performed. As shown in Fig. 3C, these VLPs produced foci indistinguishable in size, indicating that the m4/7 RNAs were very similar to WT sequences in their ability to support the RNA replication needed for all steps of the viral life cycle. Coincidentally, these mutations demonstrated that the m4/7 mutation did not interfere with WNV genome packaging in this trans-packaging system for testing multicycle replication. Titers (focus-forming units) of VLPs recovered in supernatants from electroporated packaging cells were also indistinguishable between WT and m4/7 (data not shown), providing additional support for the similarity of WT and m4/7 in supporting RNA genome replication in this WNR.

**Introduction of CS mutation into virus.** In the VLP experiments described above, the CS mutant replicon m4/7 encoded a C protein with two amino acid substitutions at positions which are otherwise highly conserved among mosquito-borne flaviviruses. However, these VLP studies did not demonstrate whether these amino acid substitutions in C resulting from the m4/7 mutation affected viral replication, since authentic C protein was provided by the Venezuelan equine encephalitis virus replicon in BHK(VEErep/C\*-E/Pac) cells. To examine the effect of CS mutation as well as alteration of the C protein on the replication of WNV, we introduced the m4/7 CS mutations into a full-length WNV genome (Fig. 4A). Recombinant WNV and infectious virus containing the m4/7 mutation were pre-

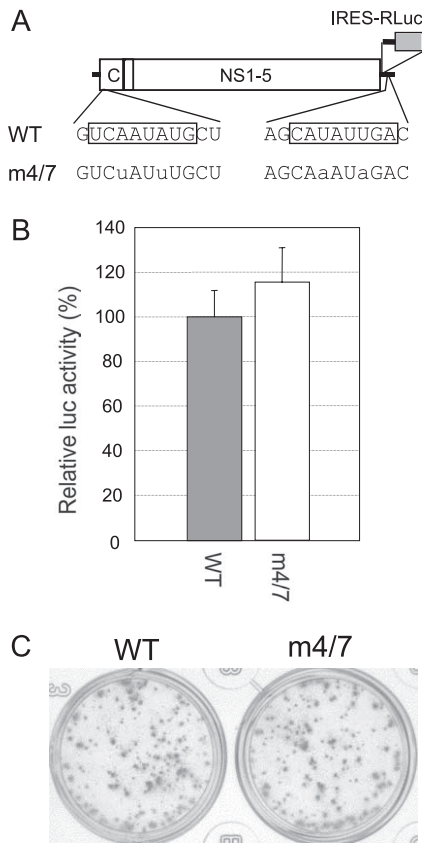


FIG. 3. Effect of multiple CS mutations on RNA replication and viral genome packaging. (A) Schematic representation of WNR RNA structure. The nucleotide sequences of CS are given next to their names. Boxes indicate the eight nucleotides that are 100% conserved among all mosquito-borne flaviviruses. Nucleotide substitutions in the 5' CS and 3' CS are in lower case. (B) RNA replication of WT and mutant replicon in BHK cells. Averages of triplicate relative luciferase activities at 2 days posttransfection were normalized to that at 5 h, and the WT control value was set to 100%; error bars show standard deviations. The experiment was repeated at least twice, producing similar results. (C) Photograph of monolayers of BHK packaging cells infected with WT or m4/7 mutant VLP, overlaid with semisolid medium, fixed, and immunostained with anti-NS1 antibody 48 h after incubation at 37°C.

pared, and side-by-side growth curves were generated with these viruses in BHK, Vero, and C7/10 cells (Fig. 4B).

Although mutated virus (m4/7) had two amino acid changes in the N-terminal C protein as well as both 5'-CS and 3'-CS mutations, we were unable to detect any dysfunction in its ability to replicate in either mammalian or mosquito cells (Fig. 4B). We also examined the focus phenotypes of these viruses on these cells, and both viruses formed similar-sized foci on monolayers of all three cell types (Fig. 4C), a finding that was particularly surprising in light of the absolute conservation of these sequences in all mosquito-borne flaviviruses sequenced to date.

These results, which show that m4/7 sequence was able to promote efficient replication of WNV, indicate that there is also no need to conserve this region of the C protein, since the m4/7 changes that altered the codons at these positions had no

apparent effect on C protein function in viral genome replication and packaging.

**Replicons containing two base substitutions only in 5' CS or only in 3' CS showed no RNA replication activity.** Although the predicted RNA base pairings between the 5' and 3' CS of m4/7 were similar to those produced by WT CS (Fig. 5A), the predicted base pairing in genomes containing position 4 and position 7 mutations in the 5' CS only or the 3' CS only were dramatically different from those of WT and m4/7. To examine the effect of these unmatched double mutations on RNA replication, transcribed replicon RNAs containing these unmatched mutations were introduced into BHK cells, and RLuc activities were determined. As shown in Fig. 5B, replicons containing two base substitutions only in the 5' CS (m4/7-5') or only in the 3' CS (m4/7-3') showed no detectable RNA replication activity at the 48-h time point.

Specific infectivities of WT and mutated replicon RNA without the reporter gene were also determined by serially diluting BHK packaging cells electroporated with transcribed replicon RNAs in naïve packaging cells, and incubating these mixtures for 48 h at 37°C under semisolid medium. After 48 h, cells were fixed and stained with anti-NS1 antibody. As shown in Fig. 5A, the genomes with unmatched CS were over 10,000 times less efficient at producing focus formation in BHK packaging cells, whereas replicon RNA with WT and m4/7 sequences of both 5' and 3' CS produced the same number of infectious foci.

Although replicons with unmatched CS (m4/7-5' and m4/7-3') showed no detectable foci at 48 h posttransfection, repeated passaging of the supernatant from transfected BHK(VEErep/C\*-E/Pac) cells with a longer incubation period (4 days) enabled us to rescue viable replicons from both WNR-CNS1-5 m4/7-5' and m4/7-3' RNA-transfected cells. To identify the nucleotide sequence in the CS region of these rescued replicons, supernatants from a second passage were used to infect BHK cells, and replicon RNAs were collected from the cells and reverse transcribed into cDNA. After PCR amplification, sequence analyses of the CS regions were performed. As shown in Fig. 5C, the rescued replicon population recovered from the m4/7-5' transfection appeared to be homogeneous and contained a 1-base reversion at position 4 (U to A) of the 5' CS, producing a CS with a single unmatched position equivalent to an m7 mutation in the 5' CS that was unable to pair with the WT 3' CS in the genomes found in this rescued population. The replicon population rescued from the m4/7-3' transfection contained reversions at position 4 (A to U) and position 7 (A to U). Both nucleotide positions appeared to contain mixtures of both original (transfected mutant RNA) and revertants to the WT nucleotide. It is likely that these represented mixtures of genomes with reversions at one position or the other; thus, the rescued population was likely to contain two different genomes, one with an m4 mutation in its 3' CS and the other with an m7 mutation in its 3' CS. It is interesting that replicons rescued from m4/7-5' or m4/7-3' showed nucleotide changes in mutated positions but not the WT portion of the CS. It should be noted that sequencing of similar blind-passaged WT and m4/7 WNRs did not reveal any nucleotide changes in their CS (Fig. 5C).

**Production of single-cycle WNV with two-component genomes.** Identification of mutated CS that permitted the efficient replication of WNV genome encouraged us to examine

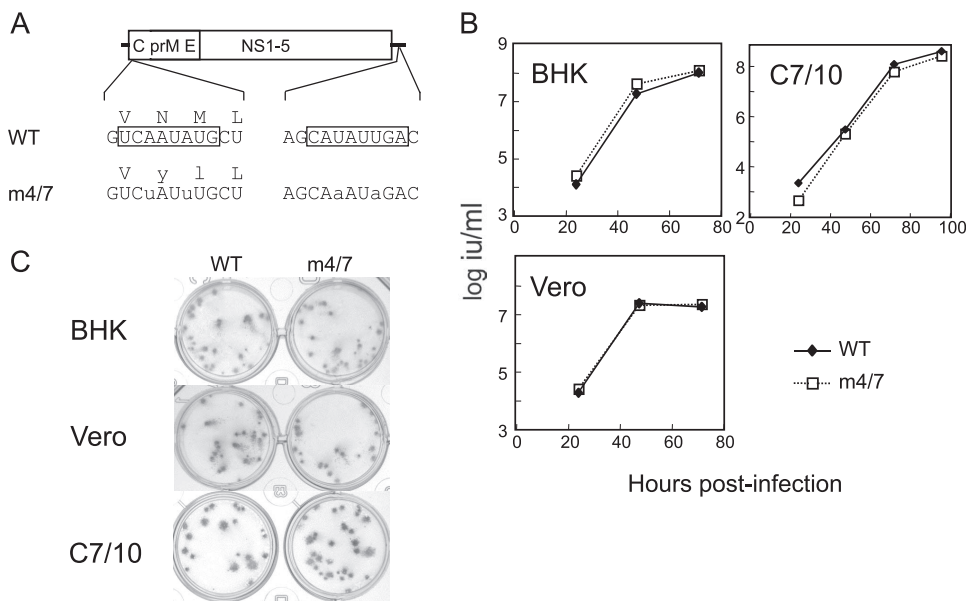


FIG. 4. Effect of multiple CS mutations on growth kinetics of recombinant virus. (A) Schematic representation of WNV RNA structure. The nucleotide sequences of CS and coding amino acid sequences are indicated next to their names. Nucleotide substitutions at 5' CS and 3' CS and substituted amino acid in the C-coding region are indicated in lower case. Boxes indicate the eight nucleotides that are 100% conserved among all mosquito-borne flaviviruses. (B) Growth curves of WT and mutant virus on BHK, Vero, and C7/10 cells. Monolayers of cells were infected with the indicated virus at an MOI of 0.05 for BHK and Vero cells and an MOI of 0.2 for C7/10 cells. At each time point, the media were removed and frozen for subsequent titration and fresh media were added. Virus titers in cell culture media were determined on Vero cells. (C) Photograph of monolayers BHK, Vero, and C7/10 cells infected with WT or m4/7 recombinant virus, overlaid with semisolid media, fixed at 40 h postinfection for BHK and Vero cells and 72 h postinfection for C7/10 cells, and immunostained with anti-WNV antibody.

their use in producing a two-component LAV based on our single-cycle WNV, named RepliVAX WN. The RepliVAX WN genome has a large deletion in the C-coding sequence so that it can be packaged only in C-expressing cell lines (28, 44). Recently we reported that a C-expressing flavivirus replicon can also complement RepliVAX genomes (39). However, there is a concern that intergenomic recombination between the two flavivirus genomes could produce a live infectious agent during LAV production or in LAV-vaccinated individuals. Although such an event is unlikely, this recombination potential could be significantly reduced by utilizing genomes with different pairs of CS, ensuring that recombinant genomes would not be replicationally active. To test if the m4/7 CS could be used for this purpose, we designed the C protein-expressing WNV helper genome (Fig. 6A) to contain these mutant CS. Based on work with the two-component YFV system indicating that helper genomes encoding the WT C gene did not efficiently complement RepliVAX genomes (39), we constructed our WNV helper genomes with a truncated C gene followed by the gene for the FMDV autoprotease 2A, a codon-optimized synthetic C gene, and the nonstructural protein genes (Fig. 6A). As expected, this genome was packaged into particles by cells expressing WNV prM/E from a Venezuelan equine encephalitis virus replicon, and helper genome packaging was dependent on cell line-expressed prM/E but did not require cell-expressed C (results not shown), demonstrating that the cassette comprising the FMDV autoprotease and the codon-optimized C gene was able to supply a functional C protein. When VLPs (referred to here as helper particles) containing this helper genome that were recovered from packaging cells

were mixed with RepliVAX WN.2 particles at an equal multiplicity, they were able to grow to high titers in Vero cells (Fig. 6B). The titer of these particles after repeated passage also remained high (Fig. 6C). These data indicate that the m4/7 CS mutations could be utilized for generating a two-component WNV LAV vaccine candidate with a reduced recombination potential.

**DISCUSSION**

In this study, we set out to identify unnatural CS that were capable of supporting efficient replication of the flavivirus genome so that these sequences could be utilized to enhance the safety of flavivirus LAV. Previous studies indicated that the complementarity of CS, rather than precise nucleotide sequences, was a prerequisite for flavivirus RNA replication (2, 13, 19, 27), but these studies also showed that viral genomes with complementary but unnatural CS replicated more poorly than WT genomes. Due to the nature of the mutant sequences reported in those works, it is unclear if the levels of replication achieved by genomes harboring these matched mutations were the result of the predicted free energy of the interactions between the mutated 5' CS and 3' CS of the genome or the precise nucleotide sequences selected for insertion in the reported mutant CS. To circumvent this problem, we undertook large-scale mutagenesis studies of the CS of WNV that preserved the predicted free energies of interaction between the 5' and 3' CS in order to find mutant CS that would support WT levels of genome replication.

To produce CS with an inability to revert to WT sequences

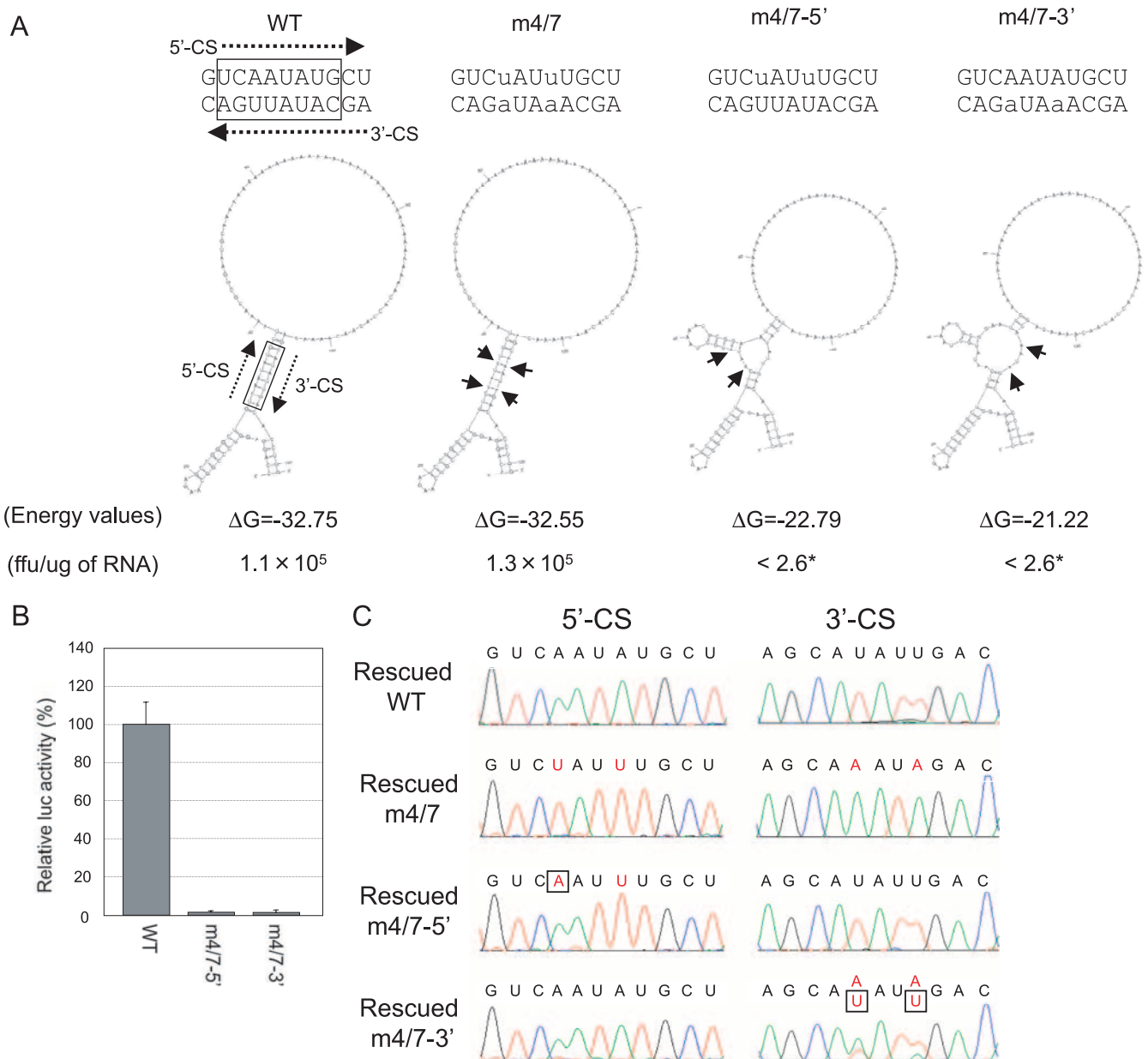


FIG. 5. Effect of unmatched CS mutation. (A) Nucleotide sequence and secondary structures of the CS predicted by Mfold. Nucleotide substitutions at 5' CS and 3' CS are in lower case. Dotted arrows show the 5'-to-3' direction of nucleotide in CS. Short arrows indicate nucleotide positions with substitutions. The  $\Delta G$  values and the structures were produced using an artificial sequence created by fusing 52 bases of C coding region, 31 bases of 3' UTR, and a 38-nucleotide A stuffer. The numbers of focus-forming units (ffu) per microgram of electroporated RNA are also indicated. Asterisks show no detection of foci. The limit of detection for this assay was 2.6 focus-forming units/ $\mu$ g. (B) RNA replication of unmatched CS mutant replicons in BHK cells. Average triplicate relative luciferase activities at 2 days posttransfection were normalized to those at 5 h, and the WT control value was set to 100%; error bars show standard deviations. The experiment was repeated at least twice, with consistent results. (C) Electropherograms showing the sequences of the blind-passed replicons derived from WT, m4/7, m4/7-5', and m4/7-3'. The nucleotide sequences of the CS are indicated above the electropherograms. Deliberately mutated CS nucleotides are shown in red; changes from these mutations detected in rescued populations are shown in black.

in the rare event of recombination with WT CS, we introduced a series of nonconservative multiple base changes within the CS, in such a manner that the mutations thus produced did not alter the overall structure or predicted free energy of the interaction between the 5' and 3' CS. Surprisingly, none of these mutant sequences supported detectable WNR genome replication levels, indicating that there were position-specific re-

quirements suggested by earlier studies (see the introduction). To systematically identify sites that could be mutated, we then undertook a site-by-site mutation strategy in which we independently switched each A-U pair to a U-A, each U-A to an A-U, each G-C to a C-G, and each C-G to a G-C of the 5' and 3' CS. As expected, this strategy indicated that swaps at certain positions (discussed in detail below) were highly deleterious,



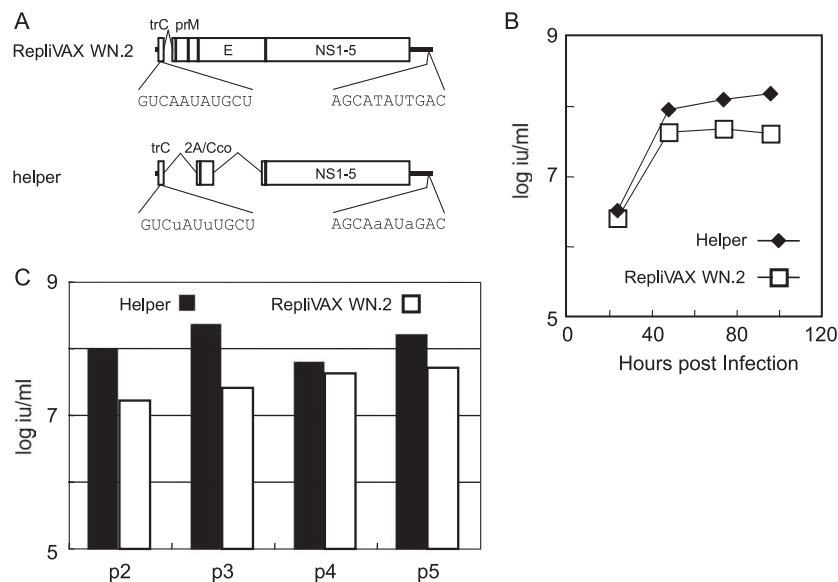


FIG. 6. Analysis of two-component genome replication. (A) Schematic representation of RepliVAX WN.2 and helper genome structure showing the position and composition of the WT (RepliVAX WN.2) and mutant (helper) CS. Nucleotide substitutions at 5' CS and 3' CS are in lower case. (B) Release of infectious particles containing the genome of RepliVAX WN.2 or helper from Vero cells infected with a mixture of particles at an MOI of 0.5. The titers of particles recovered at each time point were determined by an infectious-unit assay performed on Vero cells (see Materials and Methods). The titer of the RepliVAX WN.2 particles was determined by focus formation assays using an E protein-specific serum that detects only cells infected with the E-encoding RepliVAX WN.2 genome. The titer of helper particles was determined by subtraction of the focus formation assay titer of RepliVAX WN.2 from the combined titer of RepliVAX WN.2 and helper particles determined by using an anti-WNV serum (see Materials and Methods). (C) Titers of infectious particles containing the genome of RepliVAX WN.2 and helper after serial passage in Vero cells. Supernatants collected 3 days postinfection were used to inoculate fresh Vero cells. This procedure was repeated five times, and the titers of both helper and RepliVAX WN.2 in each passage were determined as described for panel B.

whereas some paired mutations produced WT levels of RNA replication.

Although the introduction of these singly mutated CS pairs into a flavivirus LAV would be expected to reduce its ability to produce a viable recombinant with WT virus, we sought to further reduce the possibility of generation of viable recombinants by combining mutations. Interestingly, the combination of two mutations into paired CS resulted in the production of WNR genomes that appeared to replicate at WT levels when introduced into BHK cells. To analyze these genomes in a multistep growth situation, we further compared their ability to replicate in a packaging cell system that should have reduced the effect of C-protein coding changes (that resulted from the CS mutations) on WNR packaging and spread. Using this system, we were unable to detect any differences between the replication (and packaging) activity of WT and the paired double-mutated-CS-bearing WNR.

Due to the highly conserved nature of the CS in all mosquito-borne flaviviruses, we were surprised by our ability to easily generate mutated CS with apparent WT levels of replication activity. To further test their ability to support WT levels of replication, the paired double CS mutations were introduced into an infectious WNV. When this virus was compared to its parental cDNA-derived WNV, it displayed indistinguishable growth properties in low-multiplicity single-cycle growth assays in two different mammalian cell lines, as well as in a mosquito cell culture. Since the CS changes we had selected based on replicational activity produced changes in the encoded C protein, our studies showing that viruses with mutated C proteins are

highly efficient in their replication also demonstrate that the high level of amino acid conservation in C in the region that overlaps the 5' CS is due not to a constraint on C protein structure but rather to a constraint on complementarity of the 5' and 3' CS. These data are consistent with reports from trans-packaging studies demonstrating that deletion of the C coding sequence corresponding to 5' CS did not prevent packaging of replicon genome of YFV (33) or WNV (37). Nevertheless, as discussed below, some of the activities of the C protein might be affected by mutations of the CS, complicating analyses of this busy portion of the flavivirus genome.

A direct analysis of WNR containing a combination of WT and either 5' or 3' double-mutant CS demonstrated that the intentional combination of these unmatched CS produced genomes that displayed undetectable levels of genome replication. However, blind passage of cells transfected with large amounts of these genomes resulted in rescue of replicationally active genomes that had undergone mutation. The nucleotide sequence analyses of these genomes showed that replicationally active genomes contained reversions in at least one of the mismatched positions. These data indicate that replicons with a single noncomplementary base pair at either the m4 or m7 position were viable, consistent with our findings that genomes with a single, noncomplementary mutation in position 2 (m2-5') were replicationally active. In contrast, WNR with our CS mutant m4/7 was stably maintained for multiple passages, indicating that the m4/7 mutation is not under strong selective pressure, consistent with its WT levels of replication. These



data suggest that the m4/7 mutation is suitable for use in the design of LAV.

As part of our studies to systematically identify mutations in the 5' and 3' CS that could support efficient replication, we were surprised to find two mutants (m1 and m2) for which a single pair of swaps of the nucleotides in the first or second positions, respectively, of the paired CS abolished replication. Studies with replicons containing mutations at these positions in only the 5' or only the 3' CS revealed that the precise nucleotides at the 3'-CS positions were more critical for replication than the corresponding 5'-CS nucleotides. These data suggest that these 3' CS play a critical role in RNA replication, either by themselves or as part of other local secondary structures.

Recently, it was reported that JEV C protein was processed by cathepsin L, which is associated with replication of JEV in neural and macrophage cells (30). Whether WNV C is also processed by cathepsin L and/or whether this processing is important for replication of WNV has not been determined, but the C protein sequences of these two viruses are nearly identical at the predicted cathepsin L cleavage site. The possibility that the C mutants created in our study had an effect on the processing of C by cathepsin L cannot be ruled out based on our studies. In the case of m1 and m2, both mutations generated amino acid changes at five codons upstream of the predicted cathepsin L cleavage site (V to L in m1 and V to D in m2). However, m2-5', which also contains the same amino acid substitutions as m2, showed only a slight reduction of RNA replication compared to that of WT genomes, indicating that these mutations in coding capacity, which could alter cathepsin L cleavage, do not have a serious effect on genome replication in our system. These results further support the possibility that the nucleotide sequence in CS is critical for efficient RNA replication, whereas the encoded protein sequences are not as critical (see above).

One stimulus for initiation of these studies was the concern that LAV for flaviviruses, especially those that carried foreign genes, could recombine with viruses in nature (38). To date, there is no evidence that this type of recombination has occurred; however, in the case of the distantly related poliovirus, recombinants between LAV and wild viruses have been identified in nature (4, 15). Recombination between a vaccine strain and persisting pestivirus, generating cytopathogenic virus and inducing lethal disease in cattle, has also been reported (5). We believe that the utilization of WNV LAV with the CS mutant that we have identified would provide an additional level of safety for LAV use, since these CS mutations would reduce the viability of recombinants with WT viruses. For similar reasons, our CS mutations also have a significant advantage in the production of a new class of vaccines recently described by Shustov et al. (39). These vaccines consist of a single-cycle virus (named RepliVAX) which encodes a C-gene-deleted genome that can be cocultivated with a helper genome encoding the C but not prM/E proteins (39). Although Shustov et al. did not detect any infectious full-length virus in cells forced to replicate both RepliVAX and its helper genome (39), the *in vitro* cocultivation of this type of vaccine could be rendered safer by utilizing CS mutations of the type we have identified here. Preliminary studies presented in this work demonstrate that repeated cocultivation of RepliVAX WN

with a helper encoding mutant CS can be accomplished, documenting the utility of CS mutants in a practical application.

In conclusion, we demonstrated the systematic evaluation of mutated CS on replication of WNV and identified mutant CS that permitted efficient replication of the WNV genome in both WNR and infectious virus. The WNR containing a combination of WT and either 5' or 3' mutant CS was very inefficient at producing a replicationally active genome. Therefore, recombinant genomes that arose from a single recombination event between LAV carrying these types of mutant CS and natural viruses would not be replicationally active. Thus, these studies demonstrated that the high level of replication of mutated CS genome could be used to enhance the safety of existing flavivirus LAV as well as enhance the safety of production and use of a recently described LAV based on two genomes with complementary packaging (39).

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