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The flavivirus-conserved penta-nucleotide in the 3′ stem-loop of the West Nile virus genome requires a specific sequence and structure for RNA synthesis, but not for viral translation

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Abstract

A reporting replicon of West Nile virus (WN) was used to distinguish between the function of the 3′ untranslated region (UTR) in viral translation and RNA replication. Deletions of various regions of the 3′ UTR of the replicon did not significantly affect viral translation, but abolished RNA replication. A systematic mutagenesis showed that the flavivirus-conserved penta-nucleotide (5′-CACAG-3′ located at the top of the 3′ stem-loop of the genome) requires a specific sequence and structure for WN RNA synthesis, but not for viral translation. (i) Basepair structure and sequence at the 1st position of the penta-nucleotide are critical for RNA replication. (ii) The conserved nucleotides at the 2nd, 3rd, and 5th positions, but not at the 4th position of the penta-nucleotide, are essential for RNA synthesis. (iii) The nucleotide U (which is partially conserved in the genus *Flavivirus*) immediately downstream of the penta-nucleotide is not essential for viral replication. Published by Elsevier Inc.

Keywords: Flavivirus; RNA replication; Replicon; West Nile virus

Introduction

West Nile virus (WN) belongs to the genus *Flavivirus* within the family *Flaviviridae*. Viruses of this genus are usually transmitted to vertebrates by mosquitoes and ticks, and frequently cause significant morbidity and mortality in humans (Burke and Monath, 2001). Since its introduction into the United States in 1999, WN has spread to nearly every state in the continental US, and it has exhibited a dramatic increase in both the rate and severity of disease in humans. During 2003, more than 8567 human cases of WN infection were reported in the US (CDC, 2003). Besides WN, other human pathogens in the genus *Flavivirus* include yellow fever virus (YF), dengue virus (DEN), Japanese encephalitis virus (JE), Murray Valley encephalitis virus (MVE), St. Louis encephalitis virus (SLE), and tick-borne encephalitis virus (TBE). It is a public health priority to develop effective means of prevention and treatment of flavivirus infection.

WN virus is an enveloped, positive-sense RNA virus (Mukhopadhyay et al., 2003). The WN genome is approximately 11 kb in length and contains a type 1 cap at its 5′ terminus but lacks a poly(A) tail at its 3′ end (Brinton and Dispoto, 1988; Brinton et al., 1986; Mandl et al., 1993; Rice et al., 1985, 1989; Wengler, 1981). The genome encodes a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs) (Brinton, 2002). The 5′ and 3′ UTRs of the flavivirus genome are approximately 100 and 500–700 nucleotides (nt) in length, respectively. Translation of the viral genome generates a long polyprotein, which is co- and post-translationally cleaved by a combination of host and virally encoded proteases into 10 viral proteins.

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The N-terminal one-third of the ORF encodes the viral structural proteins (core [C], membrane [prM/M], and envelope [E]), that are involved in viral particle formation. The remaining C-terminal two-thirds of the ORF encodes seven nonstructural (NS) proteins (the glycoprotein NS1, NS2a, the protease cofactor NS2b, the protease and helicase NS3, NS4a, NS4b, and the methyltransferase and RNA-dependent RNA polymerase [RdRp] NS5), which are primarily involved in viral RNA replication (Lindenbach and Rice, 2001). Recent studies suggest that NS2a (Kummerer and Rice, 2002; Liu et al., 2003), and possibly, NS4b (Pugachev et al., 2004) plays critical roles in assembly and release of flavivirus particles.

The flavivirus 3′ UTR was assumed to regulate minus-strand RNA synthesis, viral translation, or packaging. The terminal nucleotides of the flavivirus genome form a highly conserved RNA structure (Brinton and Dispoto, 1988; Brinton et al., 1986; Mandl et al., 1993; Proutski et al., 1997; Rice et al., 1985, 1989; Shi et al., 1996). Deletions and mutations of the 3′ terminal stem-loop suggest that this conserved RNA structure is critical for flavivirus replication (Cahour et al., 1995; Khromykh and Westaway, 1997; Mandl et al., 1998; Men et al., 1996; Shi et al., 2002a, 2002b; Zeng et al., 1998). However, the question of whether the 3′ UTR affects flavivirus translation remains enigmatic. A previous study using a non-replicative system indicated that the 3′ terminal stem-loop of the WN genome represses viral translation in vitro (Li and Brinton, 2001). Interestingly, a recent report, also using an in vitro assay, showed that nucleotide substitutions located upstream of the 3′ stem-loop of the 3′ UTR of DEN reduced viral translation (Edgil et al., 2003). These in vitro results prompted us to examine the function of the WN 3′ UTR in viral translation in an in vivo system. Furthermore, it is important to distinguish between the roles of the WN 3′ UTR in viral translation and in RNA synthesis.

Besides the conserved 3′ stem-loop structure, several sequence elements are universally conserved within the 3′ UTR of genus *Flavivirus*. These conserved sequence (CS) elements include the 3′ terminal CU_{OH}, the CS1 elements that are involved in genome cyclization, and a penta-nucleotide in the top of the 3′ terminal stem-loop (Hahn et al., 1987; Wengler, 1981; Wengler and Castle, 1986). Recent studies showed that the 3′ terminal CU_{OH} (Khromykh et al., 2003; Nomaguchi et al., 2003; Tilgner and Shi, 2004) and genome cyclization (Bredenbeek et al., 2003; Corver et al., 2003; Khromykh et al., 2001, 2003; Lo et al., 2003; Molenkamp et al., 2003; You and Padmanabhan, 1999) are both essential for flavivirus replication. However, the function of the flavivirus-conserved penta-nucleotide is currently unknown. Although one Kunjin replicon containing a 3-nt substitution within the conserved penta-nucleotide was recently reported to be replication-defective (Khromykh et al., 2003), the penta-nucleotide's specific sequence and structural requirements for flavivirus replication remain to be determined.

The experiments described in this paper were designed to address two issues: (i) the in vivo function of the 3′ UTR of the WN genome in viral translation, and (ii) the specific requirement of the flavivirus-conserved penta-nucleotide for WN replication. Using a reporting replicon of WN virus, we showed that deletions of various regions of the 3′ UTR of the replicon did not significantly affect viral translation, but abolished RNA replication. We also demonstrated that a specific sequence and structure of the penta-nucleotide are required for WN RNA synthesis, but not for viral translation.

Results

Experimental system

Two types of self-replicating reporting replicons of WN virus were used in this study. The type 1 replicon contains a *Renilla* luciferase (Rluc) gene fused in-frame with the ORF in the position where the viral structural region was deleted, and is termed RlucRep (Fig. 1A). The type 2 replicon contains an auto-cleaving HDVr immediately downstream of the 3′ end of the RlucRep to yield a precise 3′ end of the genome and is termed RlucRep-HDVr (Fig. 2A). Both replicons were previously characterized in detail (Lo et al., 2003; Tilgner and Shi, 2004). Transfection of BHK cells with RlucRep-HDVr RNA yields two distinctive Rluc peaks, one at 2–10 h and the second after 25 h post-transfection (p.t.). In contrast, transfection of BHK cells with a mutant RlucRep-HDVr that contains a defective RdRp (resulted from a frameshift upstream of the GDD motif of the polymerase active site) yields only the first peak, not the second peak. These results suggest that the first Rluc peak (at 2–10 h p.t.) represents translation of the input RNA, while the second peak (after 25 h p.t.) represents viral RNA replication (Tilgner and Shi, 2004). A similar Rluc expression pattern was obtained with the RlucRep (without 3′ HDVr) (Lo et al., 2003). We previously demonstrated that both types of replicons can be reliably used to quantify and differentiate viral translation and RNA replication and are ideal for use in large-scale mutagenesis (Lo et al., 2003; Tilgner and Shi, 2004).

Deletions of the 3′ UTR of WN replicon do not significantly affect viral translation, but abolish RNA replication

The 3′ UTR of the New York strain of WN is 634 nt in length (including the stop codon of the ORF, GenBank accession no.AF404756), and contains a 3′ terminal stem-loop and several mosquito-borne flavivirus CSs (Fig. 1B). To examine the global role of the 3′ UTR in viral translation, we prepared three RlucRep variants containing large deletions of the 3′ UTR (Fig. 1B). RlucRep (Fig. 1A), rather than RlucRep-HDVr (Fig. 2A), was chosen for

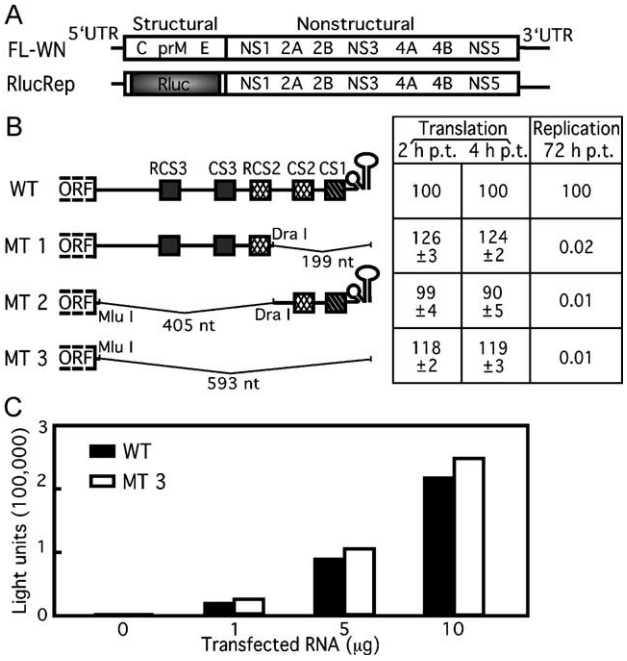

Fig. 1. Deletion analysis of WN 3′ UTR on viral translation and RNA replication. (A) Top panel: full-length (FL) WN genome. Bottom panel: a *Renilla* luciferase-reporting replicon (RlucRep) in which the Rluc reporter is fused in-frame with the ORF where the structural region was deleted (from nt 190 to 2379, GenBank accession no.AF404756). (B) Deletion analysis of WN 3′ UTR. The WN 3′ UTR contains a 3′ terminal stem-loop and the mosquito-borne flavivirus conserved sequences (CS). CS1, CS2, and repeated CS2 (RCS2), are retained among mosquito-borne flaviviruses. The JE subgroup and YF also have a distinct CS3 and repeated CS3 (RCS3). Mutant (MT) 1 contains a deletion of the 3′ terminal 199 nt of the genome. MT 2 contains a 405-nt deletion spanning the *Mlu*I and *Dra*I sites within the 3′ UTR. MT 3 contains a deletion of the 3′ terminal 593 nt of the genome. Equal amounts of replicon RNA (10 μg) were transfected into BHK cells and quantified for Rluc activity at 2 and 4 h, and 72 h p.t. to indicate viral translation and RNA replication, respectively. The relative efficiencies of translation and replication of the deletion mutants are represented as the replicons' percentage of the wild-type level of Rluc activity. The transfection efficiency of various replicons was normalized by co-transfection of a plasmid containing a firefly luciferase gene. A summary of four independent experiments is shown. (C) A linear correlation between input RNA amounts for transfection and initial level of RNA translation. BHK cells were transfected with various amounts (0, 1, 5, and 10 μg) of wild-type and mutant 3 replicon RNAs and were assayed for Rluc signals at 2 h p.t.

this type of study. This is because, if RlucRep-HDVr had been used for such analysis, 3′ terminal deletion mutants (in vitro transcribed from replicon template linearized at the *Dra*I and *Mlu*I sites for mutant 1 and 3, respectively) would not have the 3′ HDVr, whereas the wild type and 3′ internal deletion mutant 2 (in vitro synthesized from replicon DNA linearized with at the *Xba*I site located immediately downstream of the 3′ HDVr cDNA sequence) would have the 3′ HDVr. This heterogeneity among various replicon RNAs may complicate data interpretation.

Equal amounts (10 μg) of wild-type and mutant replicon RNA were transfected into BHK cells which were assayed for luciferase activities at 2 and 4 h, and at 48 and 72 h p.t., to determine viral translation and RNA replication, respectively (Lo et al., 2003). Mutant 1 contained a deletion of the 3′ terminal 199 nts (including the 3′ stem-loop, CS1, and CS2), and exhibited a translation level approximately 25% greater than the wild-type level. Mutant 2 contained a 405-nt internal deletion (including the RCS2, CS3, and RCS3), and displayed a translation efficiency of 90–99% of the wild-type level. Mutant 3 contained a 3′ terminal 593-nt deletion and showed a translation level about 19% higher than the wild-type level. None of the mutants was replication-competent, as indicated by background levels of luciferase signals collected at 48 h (data not shown) and at 72 h p.t. (Fig. 1B), and negative IFA results at 72 h p.t. (data not shown).

We were concerned that, in the experiments described above, the amount of transfected replicon RNA (10 μg) may have overloaded the host translation machinery and, therefore, possible differences between translation efficiency of the wild-type and mutant replicons may have been masked by a saturation effect. To exclude this possibility, we transfected BHK cells with decreasing amounts (10, 5, and 1 μg) of wild-type and mutant 3 replicon RNAs. Rluc signals at 2 h p.t. exhibited a linear correlation with the amount of transfected RNA for both wild-type and mutant 3 replicons (Fig. 1C). Moreover, the Rluc signals derived from the mutant 3 replicon were consistently higher (between 10% and 19%) than those of the wild type at each amount of transfected RNAs. A similar linear correlation was observed at 4 h p.t. between the Rluc activity and the amount of transfected RNA (data not shown). The results clearly suggest that our experimental conditions for translation analysis were within the linear range of the system, and should be appropriate for comparison of translation efficiency among various replicon RNAs.

We were also concerned that the marginal suppressive effect of the 3′ stem-loop on translation may be underestimated in our replicon system. This is because deletion of the 3′ stem-loop can potentially affect replicon stability, and consequently, reduce viral translation. To test this possibility, we transfected equal amounts of mutant 1 (containing the 3′ terminal 199-nt deletion) and wild-type replicon RNA into BHK cells. A real-time RT-PCR assay was performed to quantify replicon RNA extracted from the cells at 3, 5, 8, and 12 h p.t. The results showed that approximately 20% of RNA present at 3 h p.t. was retained at 12 h p.t. (data not shown). No dramatic difference in RNA copy number between mutant and wild-type replicons was detected (data not shown). The results indicate that the 3′ stem-loop deletion replicon has an RNA stability similar to that of the wild-type replicon; therefore, the slight suppressive effect of the 3′ stem-loop on translation was not underestimated in our system. Overall, the results indicate that deletions of the 3′ UTR of WN replicon do not significantly affect viral translation, but abolish RNA replication.

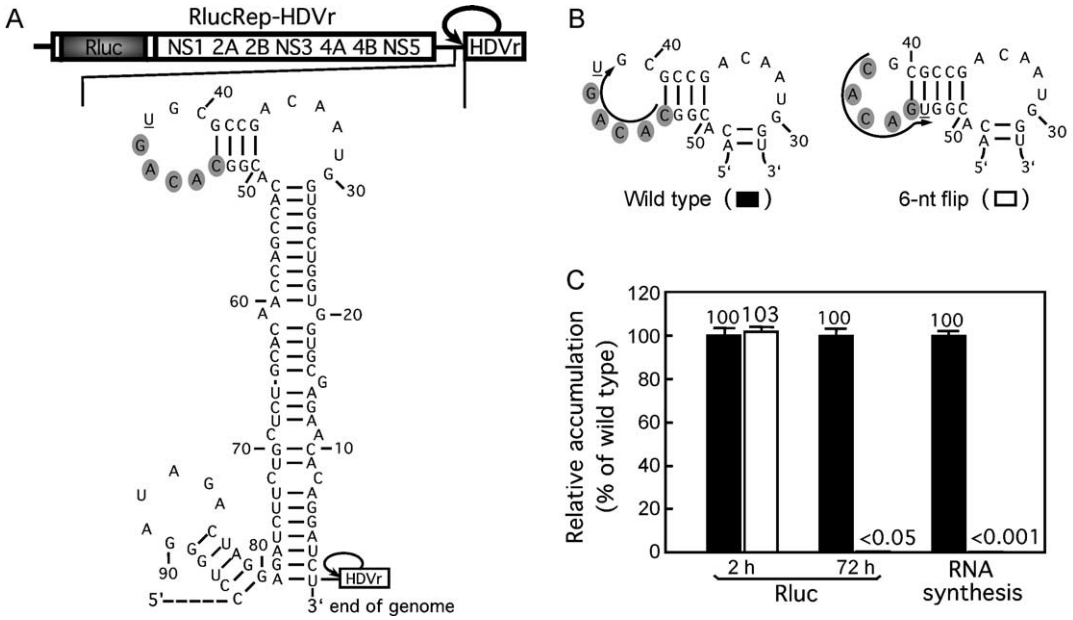

Fig. 2. Reversal of the orientation of the 6 nt containing the flavivirus-conserved penta-nucleotide abolishes WN RNA replication. (A) Top panel: a Rluc-reporting replicon containing an auto-cleaving HDVr immediately downstream of the 3′ end of the replicon RNA (RlucRep-HDVr). Bottom panel: a stem-loop structure formed by the 3′ terminal sequence of the WN genome. The flavivirus-conserved penta-nucleotide is shaded. The nucleotide U (underlined), immediately downstream of the penta-nucleotide, is partially conserved in the genus *Flavivirus* (Wengler and Castle, 1986). The RNA sequence is numbered from the 3′ end. (B) RNA folding of the 6-nt flip mutant, in which the orientation of the 6-nt containing the penta-nucleotide was reversed as indicated. Compared with the wild type, the 6-nt flip mutant has an extra basepair formed between the C at position 40 and the G at position 46. (C) Translation and replication of the 6-nt flip mutant replicon. Equal amounts of wild-type and mutant replicon RNA were transfected into BHK cells and assayed for Rluc activities at 2 and 72 h p.t. RNA synthesis at 72 h p.t. was directly quantified through real-time RT-PCR.

Reversal of the orientation of a 6-nt element containing the flavivirus-conserved penta-nucleotide abolishes WN RNA synthesis

The flavivirus-conserved penta-nucleotide 5′-CACAG-3′ (shaded in Fig. 2A) is located at the top of the 3′ terminal stem-loop of the WN genome. Among these nucleotides, the C at position 47 potentially forms a basepair with a G at position 39 (numbered from the 3′ end of the genome). Immediately downstream of the penta-nucleotide is a U or A that is partially conserved in the genus *Flavivirus* (Wengler and Castle, 1986). In the case of WN, this partially conserved nucleotide is a U (underlined in Fig. 2A). The conformation of the penta-nucleotide, as well as the rest of the predicted 3′ stem-loop structure, was previously demonstrated in WN genome through RNA structural probing (Brinton et al., 1986).

To test the requirement of the above 6 nts for viral replication, we initially reversed the orientation of the sequence from the wild-type 5′-CACAGU-3′ to 5′-UGACAC-3′ in RlucRep-HDVr, resulting in the 6-nt flip mutant (Fig. 2B). We chose RlucRep-HDVr, rather than RlucRep, for the penta-nucleotide analysis, because the RlucRep-HDVr replicates more efficiently than the RlucRep upon transfection (Tilgner and Shi, 2004). RNA structural analysis of the 3′ terminal 100 nts of the replicons using the M-Fold program indicated that, compared with the wild type, the 6-nt flip resulted in an extra basepair formed between the C and G at positions 40 and 46, respectively (Fig. 2B). Transfection of BHK cells with the 6-nt flip mutant yielded a luciferase activity of 103% of the wild-type level at 2 h p.t. However, at 72 h p.t., the 6 nt flip mutant exhibited a luciferase signal and a viral RNA synthesis level (measured by real-time RT-PCR) of less than 0.05% and 0.001% of the wild type, respectively (Fig. 2C). The 6-nt flip mutant-transfected cells also showed a negative IFA result (data not shown). These data demonstrate that reversal of the orientation of the 6-nt element is lethal for WN RNA synthesis, but not for viral translation.

Basepair structure and sequence of the 1st nt of the penta-nucleotide are important for RNA synthesis

To identify the elements of the conserved penta-nucleotide that are essential for viral replication, we systematically mutated the penta-nucleotide sequence in the RlucRep-HDVr. Each base of the penta-nucleotide was mutated to the three alternative bases. Substitution of the 1st nt C of the penta-nucleotide at position 47 with an G, A, or U resulted in mutant replicons C47→G, C47→A, and C47→U, respectively (Fig. 3A). Among them, mutants C47→G and C47→A abolished the potential basepair with the G at position 39. Neither C47→G nor C47→A was replication-competent, as indicated by the background levels of luciferase signals, RNA synthesis (quantified by real-time RT-PCR), and IFA staining at 72 h p.t. (Figs. 3B–C). In

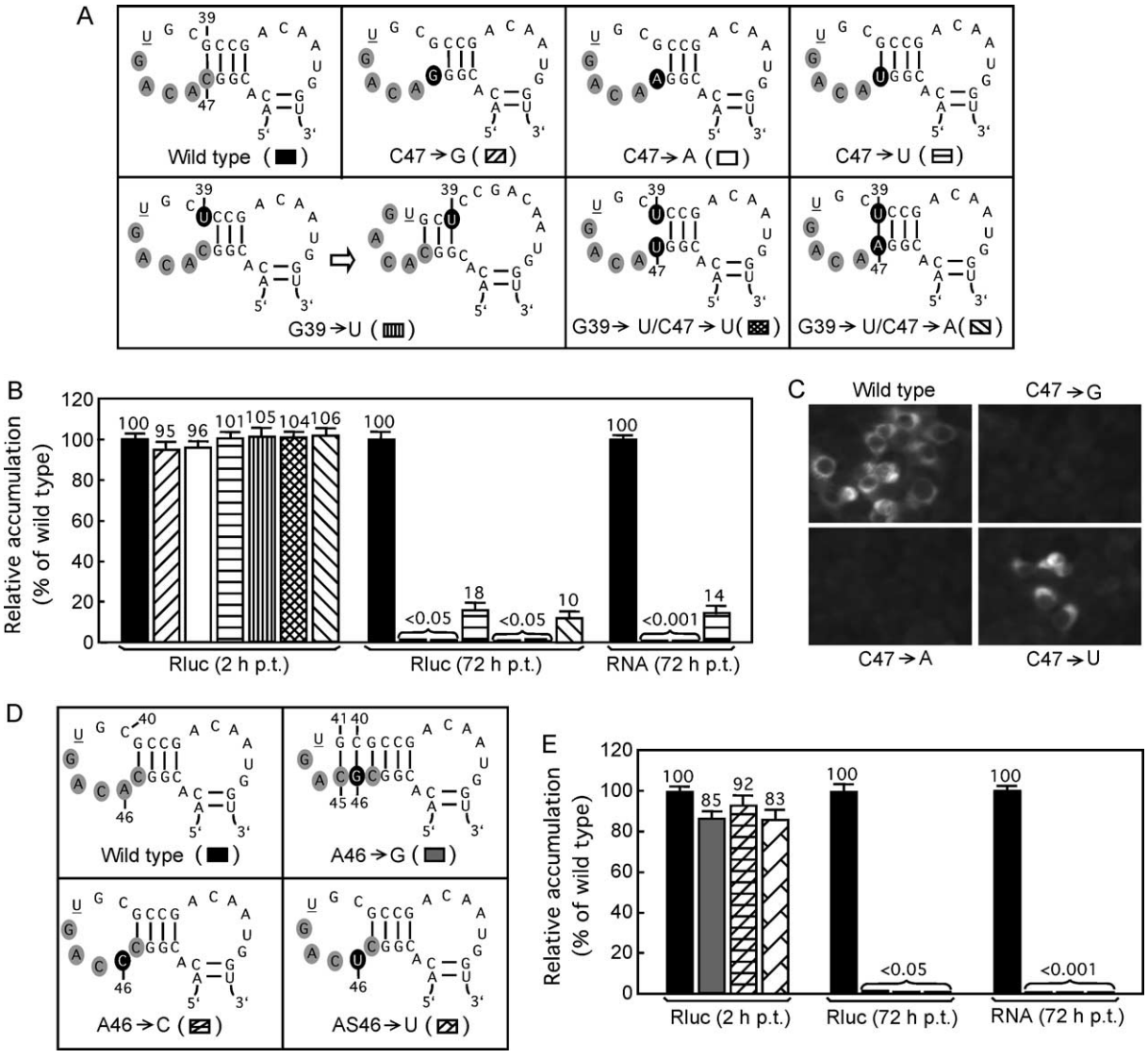

Fig. 3. Mutagenesis of the 1st nt (A–C) and 2nd nt (D–E) of the flavivirus-conserved penta-nucleotide. Each position of the penta-nucleotide was individually mutated to the three alternative nucleotides (indicated as filled ovals). Mutant replicons were examined for RNA folding, viral translation (measured by Rluc signals at 2 h p.t.), and RNA replication (monitored by Rluc signals, real-time RT-PCR, and IFA at 72 h p.t.). For some replicons, double mutations (G39→U/C47→U and G39→U/C47→A) or a point mutation outside the penta-nucleotide (G39→U) were engineered. For those replicons, only Rluc results (not the RNA synthesis data) were presented in panel B. A summary of three independent experiments is shown.

contrast, mutant C47→U, which maintained a potential basepair structure (between position 39 and position 47), exhibited an RNA synthesis level that was 14–18% of the wild-type level. It is noteworthy that the relative Rluc activity at 72 h p.t. agreed well with the real-time RT-PCR results, demonstrating that the Rluc signals reliably reflect the replication level of the replicon in the system. IFA of BHK cells at 72 h p.t. also correlated with the luciferase and real-time RT-PCR results (Fig. 3C). The results suggest that the basepair structure of the 1st nt of the penta-nucleotide is essential for WN RNA synthesis.

To further validate the importance of the basepair structure of the 1st nt of the penta-nucleotide in viral replication, we prepared three additional mutants. (i) Mutant G39→U was originally designed to retain the wild-type nucleotide C at the 1st position of the penta-nucleotide, but to abolish its potential basepair structure. However, based on M-Fold program, the G39→U mutation substantially changed the local folding of the RNA conformation (indicated by a hollow arrow in G39→U, Fig. 3A). Transfection experiments showed that mutant G39→U was replication-defective, as suggested by its Rluc activity of less than 0.05% of the wild-type level at 72 h p.t. (Fig. 3B). (ii) Double mutant G39→U/C47→U was constructed to abolish the basepair structure at the 1st position of the penta-nucleotide (Fig. 3A). Rluc activity at 72 h p.t. indicated that mutant G39→U/C47→U was completely replication-incompetent (Fig. 3B). (iii) Double mutant G39→U/C47→A was prepared to restore the basepair structure at the 1st position of the penta-nucleotide through

a non-viral U39-A47 interaction (Fig. 3A). Remarkably, mutant G39→U/C47→A was viable, with a replication efficiency of approximately 10% of the wild type at 72 h p.t. (Fig. 3B). These results support the conclusion that the basepair interaction of the 1st nt of the penta-nucleotide is essential for WN RNA synthesis. However, although mutants containing basepair structure at the 1st position (C47→U and G39→U/C47→A) were replication-competent, their replication efficiency was only 10–18% of the wild type (as indicated by the Rluc activity at 72 h p.t.). These results suggest that the sequence identity at these positions also contribute significantly to the RNA replication. Finally, all mutant replicons exhibited a translational efficiency similar to that of the wild type, as indicated by the luciferase activities at 2 h p.t. (Fig. 3B).

Wild-type sequence at the 2nd, 3rd, and 5th positions of the penta-nucleotide is absolutely required for RNA replication

We substituted the sequence at the 2nd (Figs. 3D, E), 3rd (Figs. 4A, B), and 5th (Figs. 5A, B) positions of the penta-nucleotide with each of the three alternative nucleotides in replicon RlucRep-HDVr. Analysis with the M-Fold program indicated that all mutants retained an RNA structure identical to the wild type, except for mutant A46→G (Fig. 3D), in which the base substitution resulted in two extra basepairs (between positions 40–41 and 45–46) and reduced the size of the loop to 3 nt. Transfection of BHK cells with equal amounts of replicon RNA showed that all mutant constructs were translated at levels 83–100% of the wild type at 2 h p.t. None of the mutants were replication-competent, as indicated by the background level of luciferase activity, RNA synthesis, and IFA (data not shown) at 72 h p.t. The results illustrate that the conserved nucleotides at the 2nd, 3rd, and 5th positions of the penta-nucleotide are essential for WN RNA replication.

The conserved 4th nt of the penta-nucleotide is not essential for WN RNA replication

The conserved nucleotide A at the 4th position of the penta-nucleotide was changed to a G, C, or U, resulting in mutant replicons A44→G, A44→C, and A44→U, respectively (Fig. 4C). As predicted by the M-Fold program,

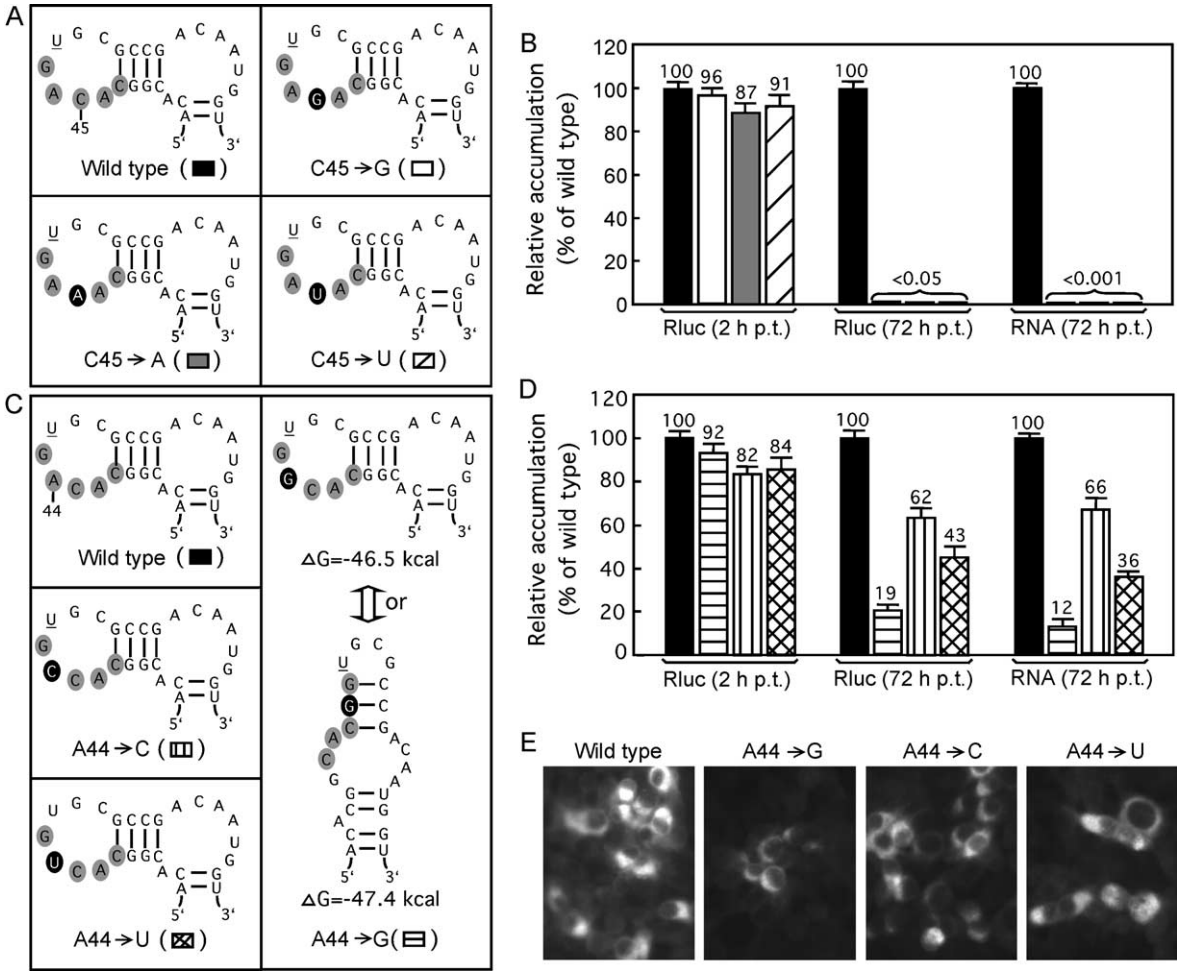

Fig. 4. Mutagenesis of the 3rd nt (A–B) and 4th nt (C–E) of the flavivirus-conserved penta-nucleotide. See details in Fig. 3 legend.

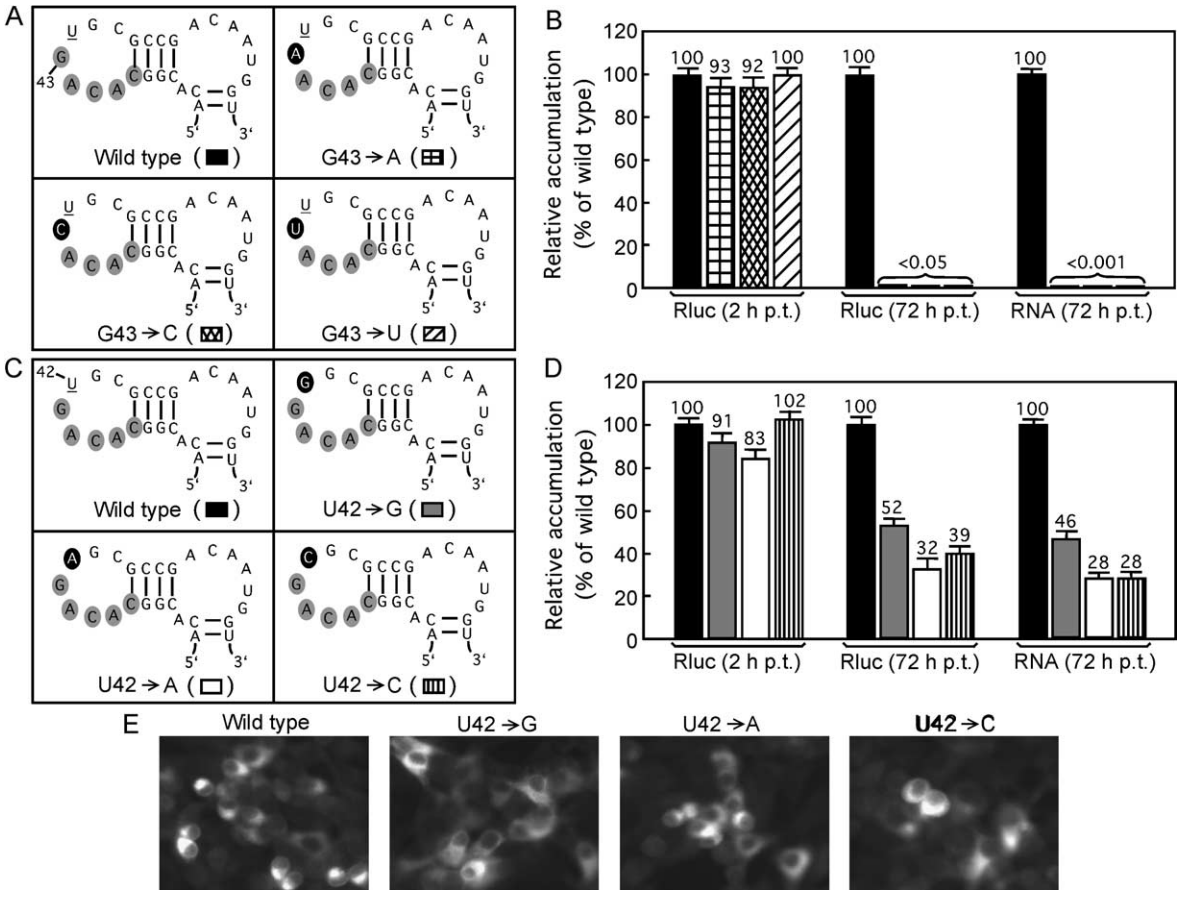

Fig. 5. Mutagenesis of the 5th nt (A–B) of the flavivirus-conserved penta-nucleotide and the nucleotide immediately downstream of the penta-nucleotide (C–E). See legend to Fig. 3 for details.

mutant A44→C and A44→U retained an RNA structure identical to that of the wild type. However, mutant A44→G was predicted to form two alternative structures having similar free energies (ΔG of −46.5 and −47.4 kcal); one of these structures was identical to the wild type (Fig. 4C). Transfection of BHK cells showed that translation efficiency of the mutant replicons was similar to that of the wild type, as indicated by the luciferase activity at 2 h p.t. (Fig. 4D). All three mutant replicons were replication-competent at 72 h p.t., exhibiting 12–19%, 62–66%, and 36–43% of the wild type replication level for A44→G, A44→C, and A44→U, respectively. IFA of BHK cells at 72 h p.t. (Fig. 4E) correlated well with the luciferase and real-time RT-PCR results.

It is interesting to note that, among the three mutants, replicon A44→G, which was predicted to form two alternative structures, displayed the lowest replication capability (12–19% vs. 62–66%, and 36–43% for mutant A44→C, and A44→U, respectively). It is possible that the two RNA conformations equilibrate and only one of them (most likely the one with the folding similar to the wild type) is replication-competent. Nevertheless, our results clearly demonstrate that the conserved nucleotide A at the 4th position of the penta-nucleotide is not essential for WN replication.

The identity of the partially conserved U immediately downstream of the penta-nucleotide is not essential for viral replication

The nucleotide immediately downstream of the penta-nucleotide is a partially conserved U or A in the genus *Flavivirus* (Wengler and Castle, 1986). To test the requirement of the partially conserved U for WN replication, we mutated the U to a G, A, or C, resulting in replicons U42→G, U42→A, and U42→C, respectively. Transfection experiments showed that mutant replicons exhibited translation levels similar to that of the wild type at the 2 h p.t., whereas replication efficiency at 72 h p.t. varied among the mutants. U42→G, U42→A, and U42→C exhibited 46–52%, 28–32%, and 28–39% of the wild type replication level, respectively (Fig. 5D). Complementary IFA results were observed in BHK cells at 72 h p.t. (Fig. 5E). These results suggest that the nucleotide U at position 42, which is partially conserved among flaviviruses, is not absolutely required for WN RNA replication.

Sequencing analysis of viable mutant replicons

To examine whether the mutated nucleotides were retained in replicons after replication, we recovered each

viable mutant replicon at 72 h p.t., and sequenced the 3′ terminal 250 nt (including the entire 3′ stem-loop structure) of the recovered RNA by 3′ RACE. The sequencing results showed that all viable replicons retained the engineered changes without any secondary mutation (Table 1). However, we could not exclude the possibility that compensatory mutation(s) exist outside the sequenced region of the recovered replicons.

Discussion

Cis-elements within the 3′ UTR of the flavivirus genome are postulated to modulate viral translation and RNA replication. The goal of this study was to distinguish between the function of the WN 3′ UTR in viral translation and RNA replication, and to define the specific requirement of the flavivirus-conserved penta-nucleotide (located at the top of the 3′ terminal stem-loop of the genome) for RNA replication. We used a luciferase-reporting replicon of WN that can differentiate between viral translation and RNA replication for the proposed study.

WN 3′ UTR does not significantly modulate viral translation

Deletions of various regions of the 3′ UTR of the replicon did not significantly affect viral translation, but

Table 1
Mutagenesis of the flavivirus-conserved penta-nucleotide within the 3′ stem-loop of the WN genome

Replicon	Translation[a] (%)	Replication[a] (%)	RNA synthesis[b] (%)	IFA[c]	Reversion[d]
Wild type	100	100	100	+++	–
6-nt flip (42–47)	103%	<0.05	<0.001	–	N.D.
Position 47					
C47→G	95	<0.05	<0.001	–	N.D.
C47→A	96	<0.05	<0.001	–	N.D.
C47→U	101	18	14	+	–
G39→U/C47→U	104	<0.05	N.D.	–	–
G39→U/C47→A	106	10	N.D.	+	–
Position 46					
A46→G	85	<0.05	<0.001	–	N.D.
A46→C	92	<0.05	<0.001	–	N.D.
A46→U	83	<0.05	<0.001	–	N.D.
Position 45					
C45→G	96	<0.05	<0.001	–	N.D.
C45→A	87	<0.05	<0.001	–	N.D.
C45→U	91	<0.05	<0.001	–	N.D.
Position 44					
A44→G	92	19	12	+	–
A44→C	82	62	66	++	–
A44→U	84	43	36	++	–
Position 43					
G43→A	93	<0.05	<0.001	–	N.D.
G43→C	92	<0.05	<0.001	–	N.D.
G43→U	100	<0.05	<0.001	–	N.D.
Position 42					
U42→G	91	52	46	++	–
U42→A	83	32	28	++	–
U42→C	102	39	28	++	–
Position 39					
G39→U	105	<0.05	N.D.	–	N.D.

[a] The translation and replication levels of each mutant replicon are expressed as that replicon percentage of the wild-type level of Rluc activity at 2 and 72 h p.t., respectively.
[b] Viral RNA synthesis is presented as a percentage of the wild-type level as quantified by real-time RT-PCR.
[c] Viral protein expression is monitored by an indirect immunofluorescence assay (IFA) at 72 h p.t., using WN immune mouse ascites fluid and Texas red-conjugated goat anti-mouse IgG antibody as primary and secondary antibodies, respectively (Shi et al., 2002). The IFA is arbitrarily scored by "+" to indicate the relative number of positive cells and their fluorescence intensities. Negative IFA is indicated as "–".
[d] Viable mutant replicons were extracted at 72 h p.t., polyadenylated, and subjected to 3′ RACE analysis (Tilgner and Shi, 2004). Sequences were not determined (N.D.) for non-replicative replicons.

abolished RNA replication (Fig. 1B). Deletions encompassing the 3′ terminal 199 nt of the 3′ UTR (mutant 1 and 3) marginally increased translation (approximately 19–25%), whereas truncation of the upstream portion of the 3′ UTR (mutant 2) did not seem to affect viral translation (90–99% of the wild-type level). These results suggest that deletion of the 3′ terminal 199 nt of the replicon, which includes the 3′ stem-loop, CS1, and CS2, causes a slight enhancement of translation. We previously showed that mutations or deletions of CS1 and CS2 did not affect viral translation (Lo et al., 2003). Therefore, deletion of the 3′ terminal stem-loop could be the major contributor to the marginal enhancement of translation observed in mutants 1 and 3. Our results are in line with the findings of a previous study that the 3′ stem-loop of WN suppresses viral translation (Li and Brinton, 2001). In that study, chimeric mRNAs composed of a CAT reporting gene flanked by WN 5′ and 3′ terminal sequences were used to examine the effect of the 3′ terminal stem-loop on viral translation. The 3′ sequences of the chimeric reporting mRNA included only the 3′ terminal stem-loop of the WN genome, not the upstream region of the 3′ UTR. Deletion of the 3′ stem-loop of the chimeric mRNAs enhanced translation by more than 2-fold in an in vitro assay, whereas the same construct increased translation by only 20% in vivo (Li and Brinton, 2001). It was speculated that the suppressive effect of the 3′ stem-loop on translation is due to its binding to host protein(s) that are essential component(s) of the translation machinery. Deletion of the WN 3′ stem-loop prevents such sequestration, and consequently, enhances translation efficiency. If the above assumption is true, then the discrepancy between the in vitro and in vivo results is likely due to a greater abundance of translation factors and ribosomes in the cytoplasm of transfected cells (in vivo) than in the reticulocyte extracts (in vitro) (Li and Brinton, 2001).

The marginal effect of the WN 3′ UTR on viral translation in vivo does not fit the current model for translation initiation of cellular mRNA. During the formation of the translation initiation complex, the 5′ and 3′ ends of an mRNA are brought into proximity, forming a closed-loop complex. The topology of the closed-loop complex increases the stability of the mRNA and promotes recruitment and recycling of ribosomes for translation (Gale et al., 2000). Since flavivirus genomic RNAs are not polyadenylated, the closed-loop complex cannot be formed via interactions between proteins that bind to the 3′ poly(A) tail and proteins that bind to the 5′ cap, as occurs with cellular mRNAs. However, flavivirus genome cyclization could be achieved by basepairing between the conserved 3′ CS1 and a complementary sequence (5′ CS) in the core coding region (Hahn et al., 1987). Recent results suggest that, although flavivirus genome cyclization is essential for RNA replication (Bredenbeek et al., 2003; Corver et al., 2003; Khromykh et al., 2001, 2003; Lo et al., 2003; Molenkamp et al., 2003; You and Padmanabhan, 1999), it does not substantially modulate viral translation (Lo et al., 2003). Overall, the in vivo results presented here and elsewhere (Li and Brinton, 2001) suggest that the 3′ UTR of WN does not significantly affect viral translation.

A specific sequence and structure of the penta-nucleotide are required for WN RNA synthesis, but not for viral translation

The results of mutagenesis on the flavivirus-conserved penta-nucleotide are summarized in Table 1. Our results agree with a recent report that a 3-nt substitution within the penta-nucleotide was lethal for Kunjin replication (Khromykh et al., 2003). We extended our study by defining the requirement of each individual nucleotide of the penta-nucleotide (as well as the partially conserved nucleotide downstream of the penta-nucleotide) for WN replication. The results showed that a specific sequence and structure of this region are critical for WN RNA synthesis, but not for viral translation. The basepairing structure at the 1st position of the penta-nucleotide is essential for viral replication; mutant replicons that maintained a potential basepairing structure at this position were replication-competent, whereas alternative replicons that blocked the basepairing structure were replication-defective (Figs. 3A–C). Point mutations at the 2nd, 3rd, and 5th positions, but not at the 4th position of the penta-nucleotide, completely abolished RNA synthesis. In addition, replicons containing substitutions of the nucleotide U (which is partially conserved in the genus *Flavivirus*) immediately downstream of the penta-nucleotide were replication-competent. It should be noted that, for all viable mutant replicons, the wild-type replicon replicated substantially better than did these mutant replicons (Table 1), suggesting that nucleotide identity at those positions also contribute to the replication efficiency.

Recent results from several laboratories have led to a working model for the initiation of WN minus-strand RNA synthesis. During the initiation of minus-strand RNA synthesis, *cis*-elements within the 5′ and 3′ UTRs are juxtaposed through genome cyclization to interact with the replicase complex. Since the flavivirus replicase complex contains multiple viral proteins (Khromykh et al., 1999; Lindenbach and Rice, 1999, 2001; Mackenzie et al., 1998; Westaway et al., 1997) and possibly host protein(s) (Blackwell and Brinton, 1997; Li et al., 2002; Ta and Vrati, 2000), the flavivirus-conserved penta-nucleotide and other *cis*-elements may function as binding sites for replicase components (viral or host proteins) to assemble the initiation complex for minus-strand RNA synthesis (Khromykh et al., 2003). This working model is supported by the following lines of evidence. First, structural and functional analysis of the 3′ terminal six nucleotides of the WN genome showed specific sequence and structural requirements of this region for viral replication (Tilgner and Shi, 2004). Additional support comes from an earlier report that the 3′ terminal two nucleotides CU_{OH} (conserved within the genus *Flavivirus*) play a critical role in Kunjin RNA replication (Khromykh et

al., 2003). The results from Kunjin and WN agree well with a mechanism for initiating RNA-dependent RNA polymerization derived from a crystal structure of the initiation complex of the bacteriophage Φ6 RdRp (Butcher et al., 2001). Second, genome cyclization through the 5′CS/CS1 interaction is essential for WN RNA replication (Lo et al., 2003). Similar results were previously reported for a DEN in vitro replication assay (You and Padmanabhan, 1999), a Kunjin replicon (Khromykh et al., 2001), a YF replicon (Corver et al., 2003; Molenkamp et al., 2003), and a YF infectious clone (Bredenbeek et al., 2003). Furthermore, blockage of genome cyclization was recently shown to affect minus-strand RNA synthesis, but not plus-strand RNA synthesis, in a WN RdRp assay (Nomaguchi et al., 2004). Third, host and viral proteins (including NS3 and NS5) were found to interact specifically with the 3′ UTR of the flavivirus genome (Blackwell and Brinton, 1997; Chen et al., 1997; De Nova-Ocampo et al., 2002; Li et al., 2002; Ta and Vrati, 2000; Tan et al., 1996). Fourth, the 5′ cap of a WN subgenomic RNA was shown in an in vitro RdRp assay to suppress 3′ copy-back initiated RNA synthesis, but not de novo initiated RNA synthesis (Nomaguchi et al., 2004). Finally, as demonstrated in this study, replication of WN replicons requires a specific sequence and structure of the flavivirus-conserved penta-nucleotide in the 3′ terminal stem-loop.

The molecular details of how the flavivirus 5′ and 3′ UTRs regulate viral replication are currently not known. Although it is important to identify *cis*-elements essential for viral replication, it is more critical and challenging to identify specific viral and host proteins that interact with the *cis*-elements during replication. A combinatorial approach of genetics, biochemistry, and crystallography is required to reveal such information. These studies will provide greater insight into flavivirus replication and pathogenesis, and will facilitate the development of flavivirus vaccines and antiviral therapies.

Materials and methods

Plasmid constructions

DNA manipulation and cloning were performed by standard protocols with modifications (Shi et al., 2002a, 2002b). RlucRep was used to examine the role of the 3′ UTR in WN viral translation (Figs. 1A, B). Mutant 1 contained a deletion of the 3′ terminal 199 nt of the genome, and was transcribed from the RlucRep plasmid linearized at the unique *Dra*I site. Mutant 2 contains a 405-nt deletion within the 3′ UTR. The mutant 2 plasmid was constructed by digestion of the wild-type RlucRep plasmid at the unique *Mlu*I (Lo et al., 2003) and *Dra*I sites, followed by filling in the 5′ overhang with dNTPs and by removal of the 3′ overhang with T4 DNA polymerase. The resulting plasmid was then blunt-end ligated using T4 DNA ligase. Mutant 2 and wild-type RlucRep were transcribed from their respective plasmids linearized with the *Xba*I as previously described (Lo et al., 2003). Mutant 3 contained a deletion of the 3′ terminal 593 nt of the genome, and was transcribed from the RlucRep plasmid linearized at the *Mlu*I site.

RlucRep-HDVr was used to perform mutagenesis of the flavivirus-conserved penta-nucleotide (Figs. 2–5). All mutant RlucRep-HDVr variants were constructed through a PCR-mediated mutagenesis. DNA fragments containing targeted mutations were prepared through fusion PCR (Sambrook and Russel, 2001), digested with *Mlu*I (at nt position 10436 of the genome) and *Xba*I (at the 3′ end of the HDVr sequence), ligated into the wild type RlucRep-HDVr cDNA plasmid (digested with *Mlu*I and *Xba*I), and verified by DNA sequencing.

RNA transcription, transfection, immunofluorescence assay, luciferase assay, 5′ nuclease real-time RT-PCR, and sequencing of recovered replicon RNAs

In vitro transcription of replicon cDNAs of all constructs was driven by a T7 promoter. A mMESSAGE mMACHINE kit (Ambion, Austin, TX) was used to synthesize replicon RNAs as previously described (Lo et al., 2003). RNA was precipitated using lithium chloride, washed with 70% ethanol, resuspended in RNase-free water, and quantified by spectrophotometry. We found that accurate quantification of RNA transcript is critical to minimize experimental variations. To ensure that RNA quantification is within the linear range of the instrument, we routinely use one tenth of the total RNA (derived from a 20-μl transcription reaction) for spectrophotometry. Transfection, immunofluorescence assay, luciferase assay, and 5′ nuclease real-time RT-PCR were performed as previously described (Lo et al., 2003). For viable replicons, we recovered the replicon RNA at 72 h p.t., and sequenced the 3′ terminal 250 nt (including the entire 3′ stem-loop structure) of the recovered RNA by 3′ RACE as described elsewhere (Tilgner and Shi, 2004). The RT-PCR products derived from the 3′ RACE were directly subjected to DNA sequencing.

Thermodynamic folding of RNA stem-loop structure

The 3′ terminal stem-loop structure of each mutant replicon was analyzed through minimization of free energy using the M-Fold program in the GCG package (Genetics Computer Group, Madison, WI).

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