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Essential Role of Cyclization Sequences in Flavivirus RNA Replication†

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A possible role in RNA replication for interactions between conserved complementary (cyclization) sequences in the 5′- and 3′-terminal regions of *Flavivirus* RNA was previously suggested but never tested in vivo. Using the M-fold program for RNA secondary-structure predictions, we examined for the first time the base-pairing interactions between the covalently linked 5′ genomic region (first ~160 nucleotides) and the 3′ untranslated region (last ~115 nucleotides) for a range of mosquito-borne *Flavivirus* species. Base-pairing occurred as predicted for the previously proposed conserved cyclization sequences. In order to obtain experimental evidence of the predicted interactions, the putative cyclization sequences (5′ or 3′) in the replicon RNA of the mosquito-borne Kunjin virus were mutated either separately, to destroy base-pairing, or simultaneously, to restore the complementarity. None of the RNAs with separate mutations in only the 5′ or only the 3′ cyclization sequences was able to replicate after transfection into BHK cells, while replicon RNA with simultaneous compensatory mutations in both cyclization sequences was replication competent. This was detected by immunofluorescence for expression of the major nonstructural protein NS3 and by Northern blot analysis for amplification and accumulation of replicon RNA. We then used the M-fold program to analyze RNA secondary structure of the covalently linked 5′- and 3′-terminal regions of three tick-borne virus species and identified a previously undescribed additional pair of conserved complementary sequences in locations similar to those of the mosquito-borne species. They base-paired with ΔG values of approximately −20 kcal, equivalent or greater in stability than those calculated for the originally proposed cyclization sequences. The results show that the base-pairing between 5′ and 3′ complementary sequences, rather than the nucleotide sequence per se, is essential for the replication of mosquito-borne Kunjin virus RNA and that more than one pair of cyclization sequences might be involved in the replication of the tick-borne *Flavivirus* species.

Despite its essential role in the virus replication cycle as a template for the synthesis of minus-strand RNA, the conformation of the genomic RNA of *Flavivirus* species has not been defined. Particularly important is the mode of its presentation to the RNA-dependent RNA polymerase NS5, and possibly other components of the replicase complex (RC) (28, 51), in order for copying to commence correctly from the 3′ end. The size of the *Flavivirus* genome is about 11 kb, and the complete nucleotide sequence is available for a range of species, (7, 12, 14–16, 18, 25, 27, 29, 33, 40, 45, 54). All sequences share a common gene order (5′-C prM E NS1 NS2A NS2B NS4A NS4B NS5-3′), i.e., structural (C, prM, and E) followed by nonstructural (NS) genes, and are flanked by 5′ and 3′ untranslated regions (UTR) of about 100 and 600 nucleotides, respectively (39). Conserved complementary cyclization sequences (CS) of 8 nucleotides in the 5′ region of the core, or capsid, gene, and in the 3′ UTR, were noted in genomic RNA for several mosquito-borne species (20). Hahn et al. (20) suggested that cyclization of *Flavivirus* genomic RNA could "help ensure that virus RNA molecules that are replicated are full-length RNA, if a viral replicase were required to bind to both 5′ and 3′ regions simultaneously in order to initiate RNA replication." For Kunjin virus (KUN) RNA, these CS are located at nucleotides 137 to 144 in the 5′ region and at nucleotides 97 to 104 from the 3′ terminus (see Fig. 1A). Another important sequence in replication may be the conserved pentanucleotide loop 5′-CACAG(A/U)-3′ (49) in the upper half of the 3′-terminal stem-loop, also described as the 3′ long stable hairpin structure (37).

During the initial copying of KUN genomic RNA, double-stranded RNA was formed, and on completion, this replicative form (RF) was shown to function as a recycling template from which progeny RNA plus strands were copied in an asymmetric and semiconservative manner via a replicative intermediate with an average of only one nascent RNA strand (9, 11, 50). At least during replication of KUN replicon RNA, no free minus-strand RNA appears to be present in the cells (26). In order to analyze minus-strand RNA synthesis in vitro, the putative RNA polymerase (NS5) of dengue virus type 1 (DEN-1), West Nile virus, and KUN has been expressed either in *Escherichia coli* or from recombinant baculoviruses (19, 43, 47). These purified NS5 proteins exhibited relatively weak RNA-dependent RNA polymerase activity on RNA templates representing the homologous RNA templates and also on nonspecific RNA templates (19, 43, 47). Using the endogenous DEN-2 RC in infected cell lysates and exogenous truncated viral RNA tem-

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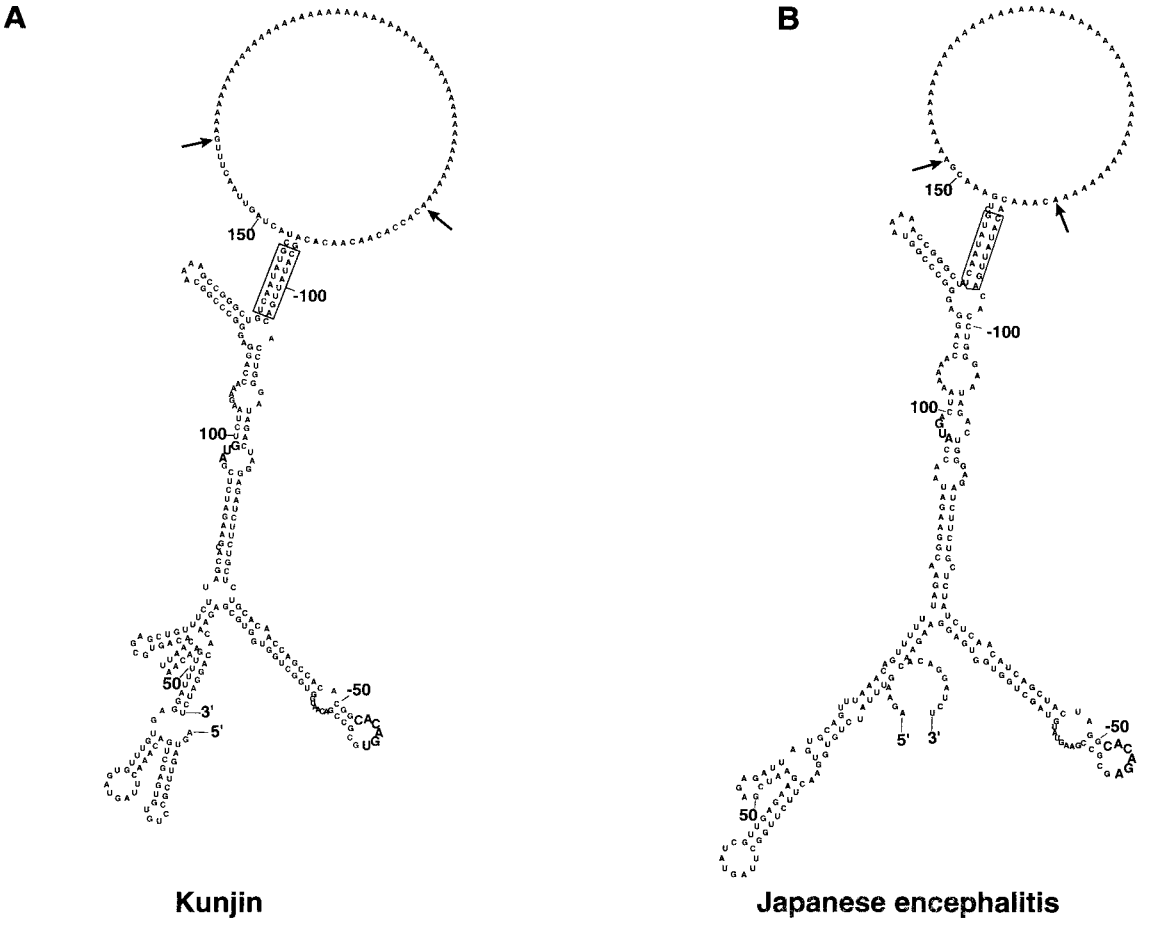
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plates, You and Padmanabhan (52) showed that self-primed RNA synthesis occurred by elongation of the authentic 3′ end of the template RNA. Interestingly, the RNA synthesis required both 5′- and 3′-terminal regions containing the conserved cyclization motifs, either connected in one molecule or with the isolated (not linked) 5′-terminal region added in *trans* to the 3′-terminal region. Mutation of the conserved CS in either the 5′ or 3′ region blocked this elongation, but elongation ability was restored when both regions contained compensatory mutations that allowed interaction between them. Because these experiments measured only 3′-terminal elongation and copy back of the 3′-terminal plus-strand RNA to yield predominantly a double-stranded hairpin molecule, no evidence was obtained of de novo minus-strand RNA synthesis, the first and essential prerequisite for enabling subsequent synthesis of progeny plus-strand RNA during the normal replication cycle.

The most convincing evidence for an essential role of the CS must come from analyses of *Flavivirus* RNA replication in cells. For this purpose we used the KUN replicon RNA, which has a deletion of most of the structural gene region but is still able to replicate autonomously after transfection into cells (26). The results obtained using RNAs with wild-type and mutated CS, or RNAs with compensatory mutations in these 5′ and 3′ sequences, showed that complementary cyclization motifs are essential for viral RNA replication in vivo.

Computer-generated conformation of the linked 5′ and 3′ regions of KUN RNA. We first verified that the covalently linked CS, each flanked by longer sequences representing the 5′ region (first 150 to 170 nucleotides) and part of the 3′ UTR (last 110 to 120 nucleotides) of genomic RNA from four mosquito-borne *Flavivirus* species and separated from each other by a stuffer poly(A) sequence, could hybridize to form a proposed panhandle RNA structure using the M-fold program (31, 55) (Fig. 1). Base-pairing of the proposed CS clearly occurred for all four species of RNA. Because of base-pairing elsewhere between the 5′ and 3′ sequences, i.e., upstream and downstream of the CS, respectively, the conformations differ from those obtained previously from comparable regions analyzed only as either the 5′ or 3′ sequence of several other mosquito-borne species. The previous analyses of secondary structures for isolated 5′- or 3′-terminal regions, obtained either by computer predictions or by biochemical analyses, uniformly showed the presence of terminal stem-loops, each of about 100 nucleotides. The 3′ stem-loop was reported to be particularly well conserved in structure (although variants were noted) in all publications reviewed (3, 4, 17, 18, 20, 29, 30, 35–38, 40–42, 46, 49, 53). Only the upper half of the 3′-terminal stem-loop (nucleotides −15 to −66 for KUN) in the conserved secondary structure of the isolated 3′ UTR was retained (Fig. 1). This upper half comprises the previously reported secondary structure shown to be important for the replication of DEN-2 (53). Pertinently, NS3 and NS5 of Japanese encephalitis virus were shown to cooperatively bind to the 3′-terminal stem-loop by Chen et al. (8), who suggested that this binding may facilitate the process for minus-strand RNA synthesis. Furthermore, in gel shift assays the 3′-terminal stem-loop of DEN-1 RNA containing the pentanucleotide loop bound to NS3 (13). The binding of NS3 to the N-terminal regions of NS5 during their translation was suggested by complementation experiments involving deletion analyses of the KUN genome (24). Such binding, possibly involving other components of the assembling RC, such as NS2A (23) and/or cellular proteins (1, 2), to the upper portion of the 3′ stem-loop, e.g., the conserved pentanucleotide loop, may facilitate the cyclization process. There appears to be no opportunity in our M-fold-program-predicted models of 5′ and 3′ interactions (Fig. 1) for formation of the pseudoknot involving the lower half of the 3′-terminal stem-loop, as was proposed in the computer-predicted secondary structures of the isolated 3′ UTR of West Nile virus, yellow fever virus, and DEN-3 RNAs (41).

Computer-generated secondary structures of the 5′-terminal region of RNA of several *Flavivirus* species contain a terminal stem-loop incorporating a nonconserved loop and bulges (4). Cahour et al. (5) showed that most of several small (5- or 6-nucleotide) deletions between nucleotides 55 and 98 in the 5′ UTR of DEN-4 RNA were lethal. This region varied in secondary structure for the four viruses shown in Fig. 1. In the present analyses, two small stem-loops appeared in the first 70 nucleotides of the 5′ region, and thereafter, base-pairing occurred with 3′-terminal sequences, interspersed with an additional one or two stem-loops involving only the 5′ nucleotide sequence. To obtain viable KUN replicon RNA, it was essential to include the first 60 nucleotides of the core gene, which incorporates the 5′ CS (26). Overall, the patterns of structures produced by the M-fold program and shown in Fig. 1 have only limited similarity apart from the base-pairing of the CS. Notably, all stem-loops are formed by base-pairing only within an individual sequence of a 5′ or 3′ region; none involve direct interaction between the 5′ and 3′ regions. The secondary structures produced by the M-fold program and shown in Fig. 1 require confirmation using analyses of the whole genome, as employed for topological organization of picornaviral genomes (34). However, the large size of the *Flavivirus* RNA (11 kb) precludes such analyses at present.

Having established in principle that base-pairing of the CS is feasible in the KUN replicon RNA sequence, we mutated them in order to observe the effects on conformation (using the M-fold program) and replication in transfected cells. Figure 2A shows the relevant mutations and the designations of plasmids containing the cDNA for transcription. The wild-type sequence copied from pC17 includes both the conserved 5′ and 3′ CS (20) with flanking sequences as shown in Fig. 1A. In pC13, the 5′ cyclization motif has been deleted. In pC17-5′mut

FIG. 1. Computer-generated secondary-structure analysis of the interaction between the genomic plus-strand RNA at the 5′ and 3′ ends for four mosquito-borne flaviviruses. The predicted secondary structures of the proposed CS and some flanking sequences connected by a poly(A) insert were produced using version 3.0 of the M-fold program (31, 55). The conserved putative CS are boxed. The arrows indicate insertion points for the stuffer poly(A) sequence. The AUG initiation codon and the conserved pentanucleotide loop [5′-CACAG(A/U)-3′] in the 3′-terminal stem-loop are shown in bold. Nucleotides are numbered from the 5′ and 3′ termini. (A) KUN (12, 25); (B) Japanese encephalitis virus (45); (C) yellow fever virus vaccine strain 17D (40); (D) DEN-2 (15). The relevant GenBank accession numbers are shown in Table 1.

and pC17-3′mut, five mutations were introduced that left only three of the original eight base-pairings in the CS. Both sets of mutations were combined in pC17-5′&3′mut as compensatory mutations so as to restore the original number of base-pairings in the CS. When the secondary-structure analyses of the interactions between the wild-type and mutated 5′ and 3′ ends were compared by the M-fold program as shown in Fig. 1 and 2B, it was found that only the sequence with combined compensatory mutations (pC17-5′&3′mut) was able to achieve the base-pairing conformation and structure comparable to those of the wild-type sequence pC17. The structures of the mutants pC17-5′mut and pC17-3′mut showed drastic changes in the M-fold pattern (Fig. 2B); the deletion mutant pC13 also obviously differed substantially in structure from the wild type (result not shown).

Effects of mutations in CS on RNA replication in vivo. In order to ascertain the effect of the mutations on replication, KUN replicons incorporating the mutations shown in Fig. 2 into the wild-type replicon sequence of the plasmid pC17 (derivative of C20DXrep) were constructed (22). Briefly, cDNA fragments containing mutated KUN 5′ and/or 3′ regions were obtained by PCR amplification, using the appropriate primers with incorporated mutations and restriction sites, and cloned into pC17, replacing the wild-type sequences. (Further details of plasmid construction can be obtained from the corresponding author upon request.) Replicon RNAs were transcribed in vitro and subsequently transfected by electroporation into BHK cells as previously described (26). Amplification and expression of the replicon RNAs were initially monitored by immunofluorescence (IF) using antibodies to KUN NS3 at various time intervals as described previously (51). Most of the cells were strongly positive by IF at 24 h after transfection with the wild-type KUN replicon pC17, compared with only a small number of cells for the compensatory mutant pC17-5′&3′mut (Fig. 3). However, the number of strongly positive cells increased severalfold later in transfection with pC17-5′&3′mut RNA, and nearly all cells were strongly positive by 36 and 48 h after transfection. No positive cells were observed at 48 or 72 h after transfection for any of the other mutants.

We next examined the accumulation of replicating RNA by Northern blot assay. RNA was extracted from the transfected BHK cell cultures at 24, 36, and 48 h and assayed for viral RNA content by Northern blot analysis (Fig. 4) as described previously (26). No viral RNA was detected in cells transfected with the mutated RNAs designated pC13, pC17-5′mut, and pC17-3′mut, in accord with the IF results showing the lack of expression of NS3. A positive signal was obtained at 24 h with the compensatory mutant pC17-5′&3′mut, although the signal was very weak. However, the signal shown by Northern blotting from the compensatory mutant increased dramatically by 36 and 48 h; this increase correlated well with the increase in the number of positive cells and the intensity of IF staining. The low signal shown by Northern blotting and the small number of IF-positive cells at 24 h for the compensatory mutant indicate inefficient early RNA replication leading to an extended delay in expression and attainment of the threshold of protein (NS3) required for detection by IF. The retention of introduced mutations in both the 5′ and 3′ ends of pC17-5′&3′mut RNA during its amplification in transfected cells was confirmed by restriction digest analysis with *Sda*I restrictase of the DNA fragments obtained by reverse transcription-PCR amplification with appropriate primers of a total cellular RNA isolated 48 h after transfection (data not shown).

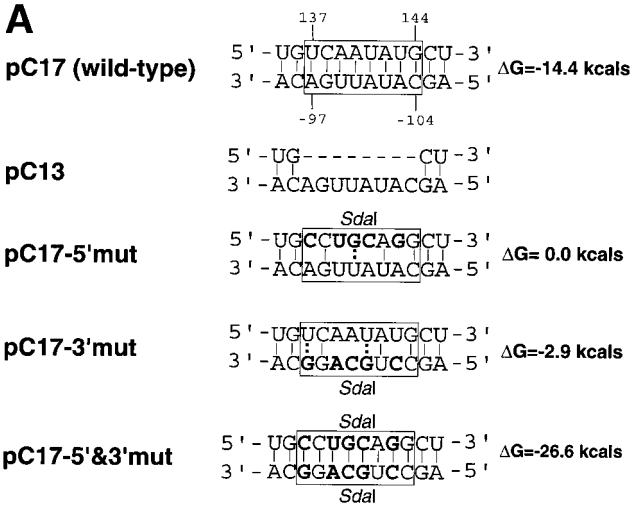

FIG. 2. Nucleotide sequence and secondary-structure analysis of wild-type and mutant KUN replicon RNAs. (A) Interaction between 5′ and 3′ ends of the putative cyclization motif (shown in boxes). Mutated nucleotides are shown in bold. Dashes indicate deleted nucleotides. The Δ*G* values shown are for base-pairing of the boxed CS. (B) Computer-generated secondary-structure analysis of the interaction between the genomic plus-strand RNA at the 5′ and 3′ ends of wild-type and mutant KUN replicon RNAs. The predicted secondary structures of the proposed CS and some flanking sequences connected by a poly(A) insert were produced using the M-fold program as for Fig. 1. The boxes enclose either the conserved cyclization motifs or the relevant mutated sequences. The arrows indicate insertion points for the stuffer poly(A) sequence. The AUG initiation codon and the conserved pentanucleotide loop [5′-CACAC(A/U)-3′] in the 3′-terminal stem-loop are shown in bold. Nucleotides are numbered from the 5′ and 3′ termini.

In earlier in vivo experiments, deletions of as many as 352 nucleotides after the stop codon in the KUN replicon, which left the 3′-terminal 272 nucleotides (including the 3′ CS) intact, partially inhibited RNA replication but were not lethal (26). Deletions in the DEN-4 3′ UTR of 30 to 262 nucleotides, including all but the 113 3′-terminal nucleotides, still permitted the recovery of progeny virus (32). However, a deletion of an additional 30 nucleotides was lethal; this region included the DEN-4 3′ CS (3′-terminal nucleotides −92 to −99) equivalent to those shown in Fig. 1 for other mosquito-borne viruses.

Comparisons with possible cyclization motifs in RNA of *Flavivirus* species not transmitted by mosquitoes. The genus *Flavivirus* includes a total of about 60 species (21). In addition to the numerous mosquito-borne *Flavivirus* species, there are a number of tick-borne viruses and some other species with no known vector. The conserved cyclization motifs first reported by Hahn et al. (20) for mosquito-borne viruses are absent in the other reported sequences. Putative CS were proposed in tick-borne encephalitis (TBE) virus RNA located at nucleotides 114 to 124 (5′ region) and at nucleotides 11061 to 11071 in the 3′ UTR (29). As successive deletions in the 3′ UTR progressed downstream from the variable region (nucleotides 10376 to 10795) into the core element, a deletion terminating at nucleotide 10919 severely impaired replication and a further

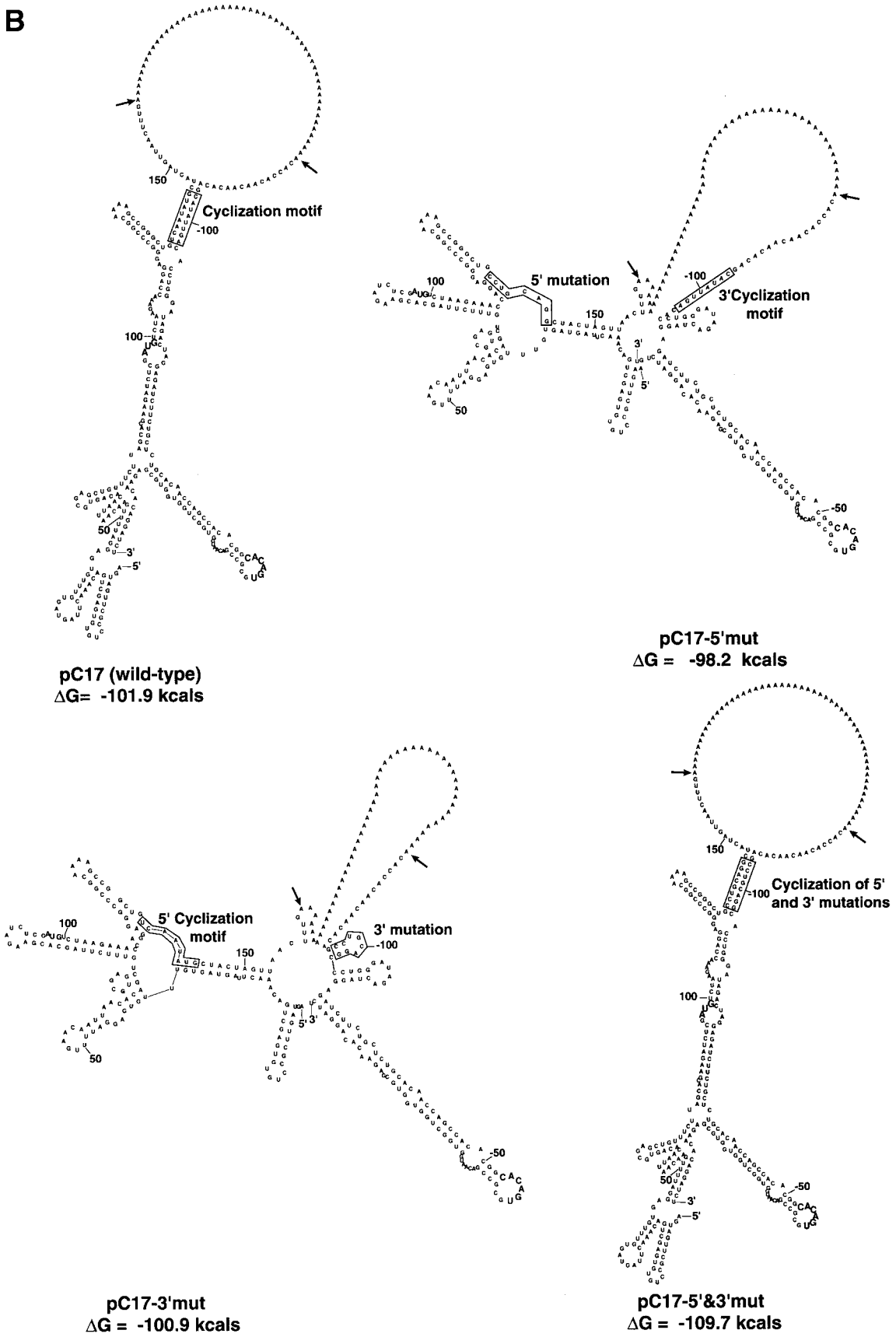

FIG. 2—*Continued.*

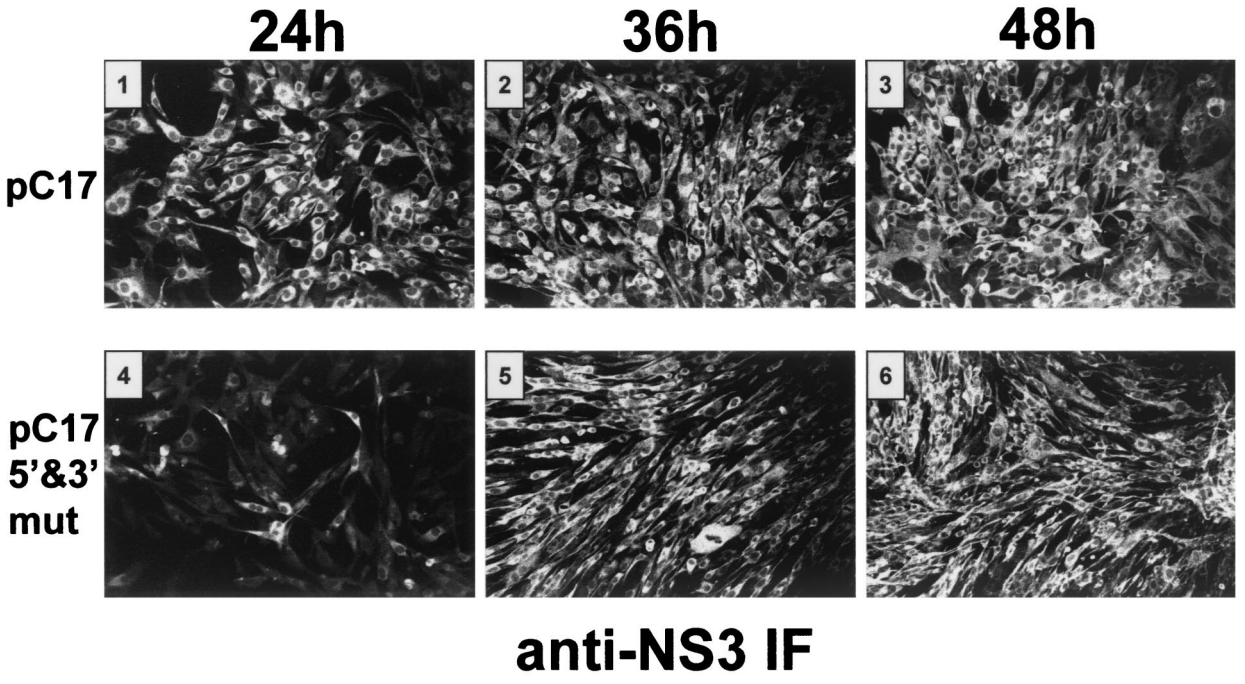

FIG. 3. Detection of replication and expression of the wild-type and mutated KUN replicon RNAs by IF analysis. BHK cells were electroporated with ~5 to 10 μg of in vitro-transcribed wild-type and mutated replicon RNAs as described previously (26) and assayed for expression of the NS3 protein by IF analysis with anti-NS3 antibodies (51) at 24, 36, and 48 h after electroporation. Panels 1 to 3 show the results of IF analysis of the wild-type (pC17) RNA, and panels 4 to 6 show the corresponding results for cells transfected with RNA containing simultaneous compensatory mutations in the 5′ and 3′ CS (pC17-5′&3′mut). Transfection with RNAs containing mutations only in the 5′- and 3′-terminal regions (pC17-5′mut and pC17-3′mut, respectively) (Fig. 2A) did not result in the detection of NS3-positive cells.

deletion extending to nucleotide 10994 was lethal (29). In this last deletion mutant the proposed 3′ CS was still retained in the terminal sequence from nucleotides 10995 to 11141. We therefore scanned the TBE virus RNA sequence between nucleotides 10795 and 10994 and continued to the 3′-terminal nucleotide 11141 using the M-fold program as used for Fig. 1 for any base-pairing with the 5′ region (Fig. 5). In addition to base-pairing in the CS, proposed by Mandl et al. (30) and shown here as CS "A," interactions were also observed between nucleotides 164 to 174 (5′ region) and nucleotides 10949 to 10958 (3′ UTR), shown as CS "B" in Fig. 5. These complementary sequences were conserved at corresponding locations in several TBE virus strains as well as in Powassan (POW) and louping ill viruses (Table 1) (18, 29, 48).

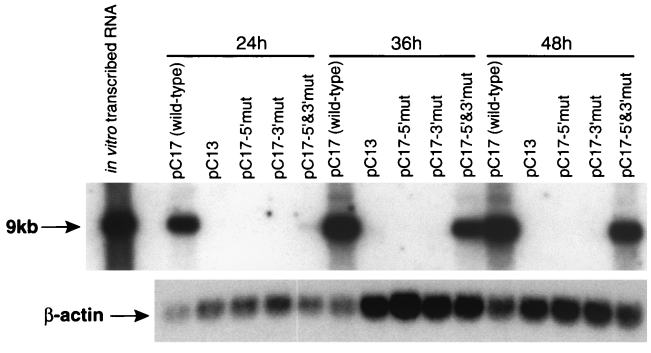

FIG. 4. Northern blot showing effects of mutations in the cyclization motifs on replication of KUN replicon RNA. BHK cells were electroporated with ~5 to 10 μg of in vitro-transcribed wild-type and mutated replicon RNAs as described previously (26), and total cellular RNA was harvested with Trizol (Gibco BRL) at 24, 36, and 48 h postelectroporation. Fifteen micrograms of total cellular RNA was separated electrophoretically in a 1% agarose gel under fully denaturing conditions and transferred to nylon (Hybond-N; Amersham), and the blot was probed simultaneously with two ^{32}P-labeled cDNA fragments encompassing either the entire KUN 3′ UTR or 291 nucleotides of human β-actin sequence. The upper panel was exposed to X-ray film for 22 h; the arrow indicates the position of RNA of ca. 9 kb. The lower panel was exposed for 2 h, and it indicates the relative abundance of the β-actin transcript in each RNA sample.

Cell fusing agent (CFA) was isolated from a mosquito cell line (44) and is classified as a tentative *Flavivirus* species (21). In the CFA genome, three sequences of 6, 7, and 12 nucleotides (designated A, B, and C, respectively) in the 5′ region, which are complementary to three sequences in the 3′ UTR, were noted by Cammisa-Parks et al. (6). Of these putative CS, two (CS "A" and CS "B") were base-paired in the 5′ region within the first 45 nucleotides, unlike all other CS examined; the 3′ component of the third (CS "C") commenced at nucleotide −471, nearly 200 bases upstream of all those described above (Table 1). However, other possible CS (now described as CS "D") were base-paired at nucleotides 169 to 179 (5′) and 10563 to 10572 (−134 to −125 in 3′ end) as shown in Fig. 5, in locations similar to those in the CS of the mosquito-borne viruses, especially in the 3′ UTR (commencing in the region from −99 to −112 [Table 1]).

In all secondary structures of *Flavivirus* RNA generated by the M-fold program which showed base-pairing of the proposed CS, the upper half of the 3′-terminal stem-loop and the

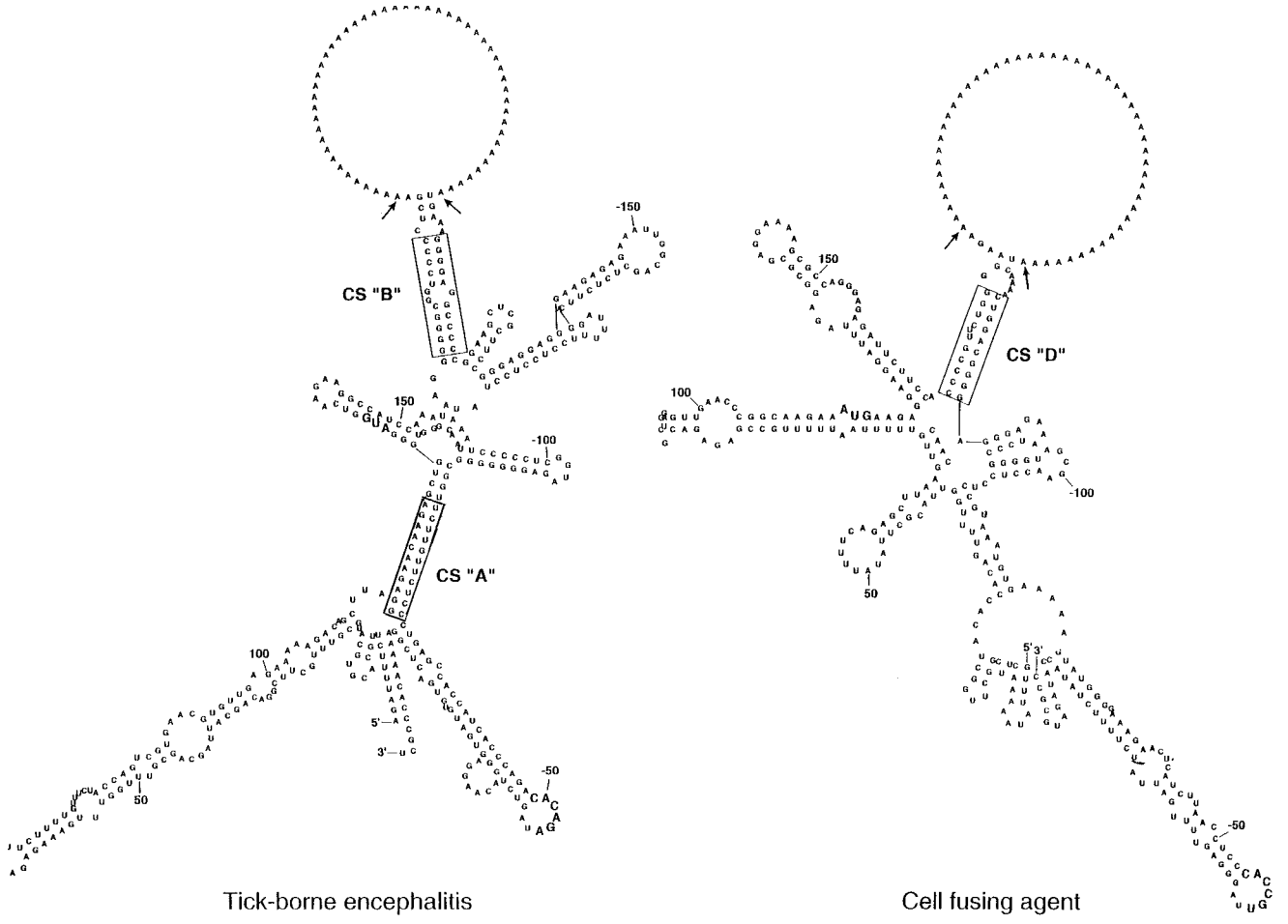

FIG. 5. Computer-generated secondary structures of the interaction between the RNA at the 5′ and 3′ ends of TBE virus (29) and of CFA (6). The predicted secondary structures of the proposed CS and some flanking sequences connected by a poly(A) insert were produced using the M-fold program as for Fig. 1. The putative CS are boxed. The arrows indicate insertion points for the stuffer poly(A) sequence. The AUG initiation codon and the conserved pentanucleotide loop [5′-CACAG(A/U)-3′] in the 3′-terminal stem-loop are shown in bold. Nucleotides are numbered from the 5′ and 3′ termini.

conserved pentanucleotide loop were retained. The latter is uniformly located for all species, viz., at −46 to −48 (mosquito borne), −49 (tick borne), or −45 (CFA) nucleotides from the 3′ terminus. However, the CFA pentanucleotide loop is changed at the fourth base (C instead of A). Table 1 summarizes the proposed CS and their relative locations within the nucleotide sequences of genomic RNA for the range of *Flavivirus* species for which data are available. There is remarkable uniformity among the nine mosquito-borne viruses. The conserved 5′ CS always commence 37 to 40 nucleotides downstream from the start of the initiation codon. The 3′ CS always commence 99 to 112 nucleotides before the 3′ terminus. The corresponding locations in CFA virus RNA that appear to best match the mosquito-borne viruses according to the M-fold program (Fig. 5) are newly defined CS "D" rather than CS "C" (6). However, the CS show no resemblance to the order of nucleotides in the mosquito-borne species. For the 5′ CS of the tick-borne species, the reference locations for CS "A," as proposed by Mandl et al. (29), for both the TBE and POW viruses commence 18 to 20 nucleotides before the initiation codon, whereas those of the mosquito-borne viruses commence about 40 nucleotides downstream of this codon, as noted above. The location of the proposed 3′ CS of the tick-borne viruses appears to be anomalous; each of these is located within the lower half of the 3′-terminal conserved stem-loop predicted when the 3′ UTR is analyzed in isolation from the remainder of the genome (29, 37). In contrast, the newly proposed 3′ CS "B" for both viruses is located further upstream, as for the mosquito-borne viruses. For louping ill virus, the CS and their locations conform to those described above for the TBE and POW viruses (Table 1). Whether or not our proposed CS in RNA of the tick-borne viruses and the CFA virus (Fig. 5; Table 1) are essential for replication remains to be tested experimentally.

The ΔG values for the conserved base paired CS for the mosquito-borne viruses, as cited by Hahn et al. (20), increase to as much as −33 kcal when four to six base pairs upstream are included, but those for DEN-2 remain at −12 kcal. The ΔG values of CS for the other viruses in Table 1 are −22.9 and −24.2 kcal for CS "C" and CS "D," respectively, of the CFA

TABLE 1. Relative locations of proposed CS in genomic RNA of *Flavivirus* species

Virus	Translation initiation[a]	Proposed CS and location[b]	GenBank accession no.	Reference for CS
Mosquito-borne		5′-**U**CAAUAUG 3′-AGUUAUA**C**		20
KUN	97	5′**(137)** **(−104)**3′	D00246, L24511, L24512	Fig. 1
Japanese encephalitis virus	96	5′**(136)** **(−111)**3′	M10370	Fig. 1
Murray Valley encephalitis virus	96	5′**(136)** **(−111)**3′	NC000943	20
West Nile virus	97	5′**(137)** **(−105)**3′	M12294	20
DEN-1	81	5′**(118)** **(−103)**3′	M87512	Compare Fig. 1
DEN-2	97	5′**(134)** **(−103)**3′	M20558	Fig. 1
DEN-3	94	5′**(132)** **(−103)**3′	M93130	Compare Fig. 1
DEN-4	102	5′**(136)** **(−99)** 3′	M14931	Compare Fig. 1
Yellow fever virus	119	5′**(156)** **(−112)**3′	NC002031	Fig. 1
CFA	114	5′**(169)****C**CCCGUUCUGG 3′ GGGGCA-GGU**C(−134)**	M91671	Fig. 5, CS “D”
	114	5′**(149)****G**CCAGGG 3′ CGGUCC**C(−471)**	M91671	6; CS “C”
Tick-borne				
TBE virus	133	5′**(164)****G**GGGC^{GG}UCCC 3′ CCCCG$_{G}$AGG**G(−193)**	U27495	Fig. 5, CS “B”
POW virus	112	5′**(136)****G**GGGGC^{GG}UCC 3′ CCCCCG−−AG**G(−189)**	L06436	Fig. 5, CS “B”
Louping ill virus	130	5′**(160)****G**GGGGC^{GG}UCC 3′ CCCCCG$_{G}$AG**G(−193)**	Y07863	Fig. 5, CS “B”
TBE virus	133	5′**(115)****G**GAGAACAAGA 3′ CCUCUUGUUC**U(−81)**	U27495	29; Fig. 5, CS “A”
POW virus	112	5′ **(88)****G**GAGAACAAGA 3′ CCUCUUGUUC**U(−81)**	L06436	29
Louping ill virus	130	5′**(112)****G**GAGAACAAGA 3′ CCUCUUGUUC**U(−81)**	Y07863	18

[a] Numbered position of first nucleotide in the AUG codon.

[b] The 5′ and 3′ CS shown are base-paired. The first nucleotide of each of the CS is shown in bold. Additional but nonconserved base pairs flanking the CS of the mosquito-borne viruses are not shown. Positions of the first nucleotide of each CS in relation to the 5′ terminus or the 3′ terminus of each genome are shown in bold within parentheses.

virus and −19.8 and −30.9 kcal for CS “A” and CS “B,” respectively, of tick-borne viruses. ΔG values for the five or six-base pairs in Table 1 preceding the mismatch or bulge region in CS “B” of tick-borne viruses are −19.2 or −24.0 kcal, respectively. These values compare favorably with those of the mosquito-borne viruses for stability of the CS.

Conclusions. All previous analyses of the conformation and possible role of 5′- and 3′-terminal nucleotide sequences of a range of *Flavivirus* species defined a variety of stem-loop structures (37), including a pseudoknot formed about 90 nucleotides from the 3′ terminus (41). However, all these analyses examined the structure of each 5′- or 3′-terminal region in isolation, and hence the possible interactions or base-pairing between the proposed CS when covalently linked with flanking 5′ and 3′ sequences have not hitherto been examined, either by the M-fold program or in infectivity testing of genomes with mutated CS. Our results with the KUN replicon establish the essential role of both the 5′ and 3′ CS in replication. Other functions of the CS in addition to the role in replication proposed by Hahn et al. (20) may be postulated. Cyclization of genomic RNA during or immediately after formation of the RC on, e.g., the 3′-terminal loop may allow ribosomes involved in translation to complete their traverse of the genomic RNA and their subsequent release but prevent their reattachment at the 5′ terminus for reinitiation. Assuming that a short delay occurs during cyclization and assembly of the RC before its commencement of copying, the risk of collision between the ribosome and RC moving along the template towards each other would be eliminated. It was proposed previously that during the initial assembly of the RC on the 3′ UTR of the plus-strand RNA template, it is transported to the membrane site of replication by the affinity of hydrophobic regions of components of the RC (24). Such binding may provide a sequestered environment that prevents the reattachment of ribosomes when the base-pairing of the CS is disrupted during the copying of the RNA template. In this scenario, cyclization of the RNA template is required only for minus-strand RNA synthesis, which is a relatively infrequent event throughout infection compared to synthesis of plus-strand RNA. There is a continuing major need for genomic RNA to function early and late as mRNA for synthesis of components of the RC and of the structural proteins, respectively. Hence, the M-fold-program-predicted structures shown in Fig. 1, 2, and 5 cannot be representative of the total viral RNA population, but they may be essential for providing the appropriate template conformation for minus-strand RNA synthesis. The presumably rigid structure of the double-stranded RNA in RF would not allow cyclization to occur when RF is being converted to the replicative intermediate during the initiation of synthesis of progeny RNA plus strands (9, 10). Proutski et al. (37) suggested that the essential role of cyclization of the *Flavivirus* genome may be in virion packaging rather than in RNA rep-

lication. However, the latter role is strongly supported by our results with the KUN replicon RNA which has a deletion in the structural genes, and hence, effects on replication do not involve packaging.

We believe that the results obtained by mutation analysis with the KUN replicon unequivocally establish the essential requirement of complementary CS in the 5′ region and 3′ UTR for replication in vivo. Because of the conservation of these motifs, the results can be extrapolated to other mosquito-borne flaviviruses. Clearly the results obtained with the inactive pC17-5′mut and the pC17-3′mut RNAs show that a single 5′ or 3′ cyclization motif is inadequate for replication. Results with the compensatory mutant pC17-5′&3′mut, show that base pairing of the CS provides the essential element rather than the nucleotide sequences per se. The slow initial amplification of the double mutant pC17-5′&3′mut, may indicate that the wild-type sequence confers some early advantage in replication, but this is only transient. Further definition of the role of CS in *Flavivirus* RNA replication will be possible when efficient systems are established for in vitro assays of *Flavivirus* RNA synthesis using specific RNA templates and the purified RC.

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