Functional Role of Conserved RNA Sequences in the DENV2 Genome

Background

• Dengue virus (DENV) is a mosquito-borne, positive-sense, single-stranded RNA virus with four genetically distinct serotypes (DENV1–4). DENV1-4 cause an estimated 100–400 million infections annually, setting a record of 14.1 million cases worldwide in 2024 (CDC 2019; Guzman et al. 2016; Haider et al. 2025).
• RNA sequence and structural elements within both coding and non-coding regions in the DENV genome regulate essential processes in the viral life cycle, including genome cyclization, translation initiation, and virion assembly (Alvarez et al. 2005; Clyde and Harris 2006; Groat-Carmona et al. 2012).
• Conserved RNA sequences within protein-coding regions in the DENV genome therefore carry two types of information: protein-coding information and RNA-level regulatory signals, as shown with the capsid-coding hairpin (cHP) and capsid-coding region 1 (CCR1) elements (Clyde and Harris 2006; Groat-Carmona et al. 2012).
• EnvCR-1 and NS1CR-1 were selected as candidate RNA regulatory elements because these regions showed greater nucleotide conservation across DENV1-4 when compared to the surrounding background sequence (Table 1). Here, nucleotide identity ≥70% used as the homology threshold for identifying such candidate region of interest. NS1CR-1 was also found to be conserved in the tick-borne encephalitis virus serogroup(Table 1).These observations are consistent with other conserved coding-region RNA elements that were previously identified in DENV1-4. (Clyde et al. 2006, Groat-Carmona et al. 2012; Boerneke et al. 2023).
NS1CR-1 was also found to be conserved in the tick-borne encephalitis virus serogroup(Table 1).

Table 1. Sequence conservation for candidate RNA elements, EnvCR-1 and NS1CR-1, among DENV1–4 and related flavivirus serogroups, Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). Percent nucleotide identity within each region of interest was calculated using genomic sequence alignments (Clustal Omega). Values represent nucleotide conservation within each region of interest as well as within the surrounding genomic background sequence (shown in parentheses).

Viral Serogroup	EnvCR-1 (Background %)	NS1CR-1 (Background %)
DENV1-4	83.3% (52.8%) *	81.5% (48.1%) *
JEV	55.6% (38.9%)	37% (29.6%)
TBEV	61.1% (61.1%)	74.1% (44.4%) *

Research Question:

Do EnvCR-1 and NS1CR-1 perform regulatory RNA-level functions beyond encoding viral proteins?

Study Objective:

Introduce silent mutations into the candidate RNA elements, EnvCR-1 and NS1CR-1, to test their proposed regulatory function in DENV2 replication.

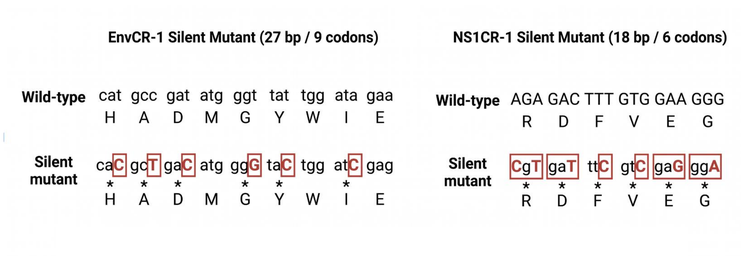

Figure 1. Silent mutations in conserved coding-region RNA elements candidates, EnvCR-1 and NS1CR-1. Nucleotide substitutions were designed to alter the RNA sequence while preserving the encoded amino acid sequence. Asterisks (*) and red boxes mark nucleotide/amino acid positions that differ between the wild-type and silent mutant. WT, wild-type; MUT, silent mutant.

Experimental Plan

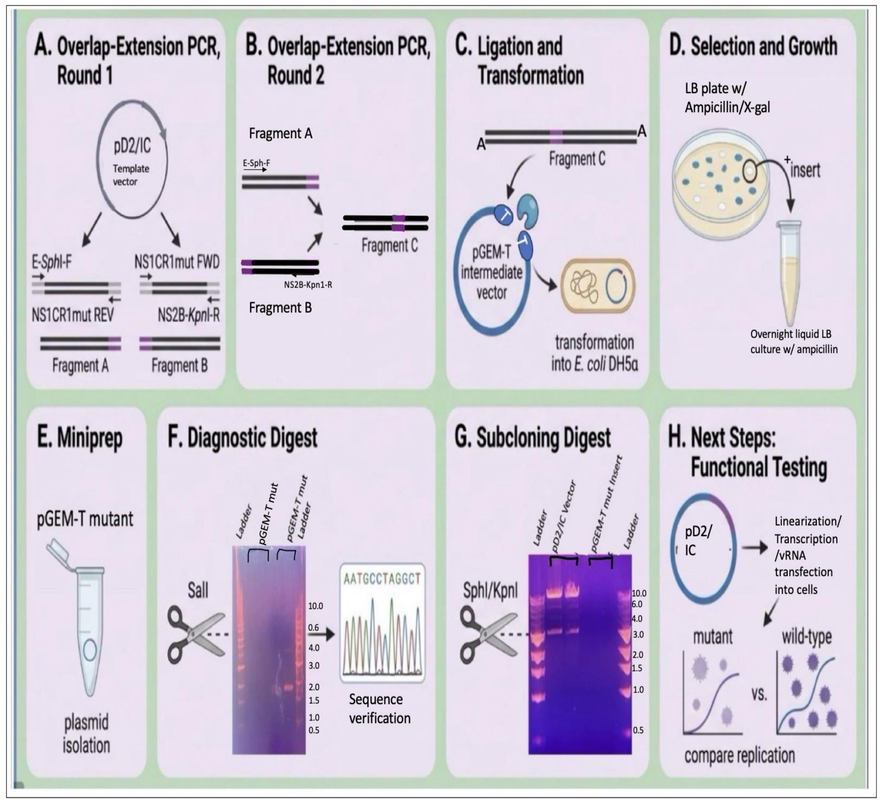

Figure 2. Experimental workflow for construction and molecular verification of the EnvCR-1 and NS1CR-1 mutants. (A) Two overlapping fragments were generated during the first round of overlap extension PCR using the pD2/IC template plasmid. Fragment A for NS1CR-1 mut requires primers E-SphI-F and NS1CR1mut REV, and Fragment B requires primers NS1CR1mut FWD and NS2B-KpnI-R. Each fragment (A & B) introduces half of the silent mutations with an overlap region at the fragment junction. (B) Fragments A and B were joined during the second round of overlap-extension PCR to generate the full-length mutant product (fragment C). (C) Fragment C was ligated into the pGEM-T intermediate vector and transformed into chemically competent *Escherichia coli* DH5α. (D) Transformants were selected on LB plates containing ampicillin/X-gal, and white colonies were picked to create overnight liquid cultures. (E) Plasmid DNA was recovered by miniprep (pGEM-T mutant). (F) SalI digestion of the pGEM-T mutant plasmids produced two bands of 3.9 kb and 2.1 kb (lanes 4-5), with the NS1CR-1 insert being present in reverse orientation. 1 kb DNA ladder shown in lanes 1 and 6. (G) SphI/KpnI double digestion of the pD2/IC vector (lanes 2–3) released the expected 10.6 kb and 3.1 kb fragments, confirming correct vector digestion. The corresponding digest of the pGEM-T mutant plasmid containing the NS1CR-1 mut inserts (lanes 4–5) did not yield visible insert bands, indicating the fragments could not be recovered for subcloning into pD2/IC. 1 kb DNA ladder shown in lanes 1 and 6. (H) Sequence-verified pD2/IC mutant plasmids will be linearized, so that vRNA can be transcribed and used to transfect mammalian cells for functional testing. Mutant DENV2 replication will be compared to wild-type virus to assess the potential regulatory role of EnvCR-1 and NS1CR-1 in the viral life cycle.

Sequence Conservation

Across DENV1–4 serogroup, both EnvCR-1 (83.3%) and NS1CR-1 (81.5%) were shown to be conserved compared to the surrounding genomic sequences (52.8% and 48.1%, respectively; Table 1), supporting their selection as candidate RNA regulatory elements. Conservation of EnvCR-1 and NS1CR-1 was more variable among related flaviviruses, ranging from ~37–83% depending on serogroup (Table 1). However, NS1CR-1 (74.1%) was also shown to be conserved in the TBEV serogroup (44.4%; Table 1).

Cloning Problems

• Diagnostic digests frequently resulted in no bands or a single inconclusive band, indicating supercoiled plasmid DNA. Issue was consistent for both mutant pGEM-T constructs.
• Several rounds of plasmid DNA isolation (pGEM-T backbone) produced no bacterial pellet during miniprep, indicating presence of satellite colonies on transformation plates.

Troubleshooting

• Switched to a new batch of chemically competent *E. coli* cells and used fresh T4 DNA ligase to improve transformation and ligation efficiency.
• Adopted the Promega miniprep kit with an added dry-spin step, improving plasmid DNA yield and purity.
• Re-screened additional colonies after inconclusive diagnostic digests.

Future Directions

• Screen additional mutant pGEM-T colonies to identify clones with the proposed mutations in NS1CR-1 and EnvCR-1 inserts and confirm mutant sequence by Sanger sequencing.
• Use cloning digests (SphI/KpnI or SacI/SphI) to recover mutant fragments and complete subcloning into pD2/IC for both EnvCR-1 and NS1CR-1.
• Generate recombinant mutant DENV2 plasmids (pD2/IC backbone) and compare viral replication to wild type.

Significance

EnvCR-1 and NS1CR-1 are conserved regions when compared to the surrounding sequence across the DENV1–4 serogroup, supporting their identification as candidate RNA regulatory elements. Efforts to introduce silent mutants into these regions is ongoing. Understanding whether these RNA elements regulate the DENV replication cycle independent of their protein-coding function could reveal new mechanisms controlling the viral life cycle. Dengue is a growing global concern, and the disease burden underscores the need for new therapeutic strategies (Guzman et al. 2016; Haider et al. 2025). Identifying novel RNA elements could reveal RNA-level targets for future antiviral drug development or new vaccines (Groat-Carmona et al. 2012; Boerneke et al. 2023; Korrapati et al. 2012).

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References