Four Single-Base Mismatches within the 3'-Terminal Stem-Loop Are Critical for Replication of the Genomic RNA of Japanese Encephalitis Virus

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Abstract

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that causes a spectrum of neurological disease in humans. The virus contains an ~11-kb, single-stranded positive-sense RNA genome that has a flavivirus-conserved 84-nucleotide stem-loop (SL) at its 3'-end, with the imperfectly base-paired stem harboring four single-base mismatches of unknown function. To elucidate the role of each of the four mismatched base pairs (A²⁴₈:A²⁷₁₁, A²⁷₁₁:A²⁷₁₂, C³⁶₍:A¹¹, and C⁷⁷⁻C⁸) present within the 3'SL in JEV genome replication, we performed comprehensive site-directed mutagenesis using an infectious cDNA clone to replace each of the eight unpaired nucleotides with all three alternative nucleotides. We demonstrated that the competence and efficiency of viral genome replication was influenced by the position and type of nucleotide mismatched, with A²⁷₁₁:A²⁷₁₂ being the most tolerant and C⁷⁷⁻C⁸ being the least tolerant. In particular, both cytosines in the C⁷⁷⁻C⁸ mismatch were required for efficient JEV genome replication. Characterization of a large pool of pseudorevertants derived from three replication-incompetent C⁷⁷⁻C⁸ mutants provided compelling genetic evidence for the functional importance of creating a mismatch or bulge at the base of the 3'SL in JEV genome replication. In all cases, acquisition of novel sequences at or near the C⁷⁷⁻C⁸ mismatch decreased the stability of 3'SL, thereby restoring replicability. Our results indicate that four single-base mismatches within the 3'SL of JEV genomic RNA are critical for viral RNA replication.

Introduction

The regions of the world where Japanese encephalitis virus (JEV) is endemic place over 4 billion people at risk of infection. Most of Asia and more recently Indonesia and Australia are regions where JEV has been reported (Fig. 1). The emergence of JEV and closely related West Nile virus (WNV) and their recently discovered tendency to colonize new regions necessitate the study of their molecular structure, replication, and pathogenesis. Previous data from extensive genetic mapping and pathogenesis studies suggest that the 3' stem-loop (SL) structure of the JEV genome is essential for viral replication [1,2,3]. This is a genetic study of the highly conserved flavivirus 3' stem-loop and this is the first time examination of the functional importance of the four mismatched base-pairs in the 3' stem-loop have been extensively studied.

Replication and Transmission Cycles of JEV

![JEV replication cycle](image)

![JEV transmission cycle](image)

Figure 1. Global distribution of the four members of the JE serogroup: Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV).

Results

- Using our infectious JEV cDNA technology, combined with large-scale site-directed mutagenesis, we have demonstrated the role of each of the four mismatched base pairs in regulating the competence and efficiency of viral genomic RNA replication.
- Cloning of a large number of pseudorevertants derived from three replication-incompetent mutants revealed the functional importance of opening up the base of the 3'SL duplex stem and thus reducing its stability, thereby restoring the replication competence.
- Currently, we are reconstructing several second-site mutations to examine their functional importance in JEV RNA replication.

Materials & Methods

- Performed a full-scale site-directed mutagenesis by replacing each of the eight unpaired nucleotides with all three alternative nucleotides (24 mutants total).
- Examined the functional phenotypes of the 24 mutants: RNA infectivity, plaque morphology, JEV RNA production, and JEV NS1 protein accumulation.
- Recovered a large pool of replication-competent pseudorevertants.
- Determined the 5' and 3'-terminal sequences of the recovered pseudorevertants.
- Identified a large number of novel sequences capable of restoring JEV RNA replication.
- Currently reconstructing the newly discovered compensatory mutations in our infectious JEV cDNA molecular clone.

Conclusions & Discussion

Figure 2. JEV replication cycle.

Figure 3. JEV transmission cycle. The principal vector of JEV is Culex tritaeniorhyncus

Figure 4. The highly conserved flavivirus 3' stem-loop (SL) mismatched base pairs are required for replication of JEV genomic RNA. The nucleotides in yellow are members of the four mismatched base pairs within the stem of the 3'-SL. Functional analysis of 24 JEV 3'-SL mutants. The mutants are grouped in accordance with the unpaired nucleotide that was mutated. Green represents the alternative nucleotide that replaced the original unpaired nucleotide. Specific infectivities were determined by infectious center assays and are given as PFU/microgram of RNA with their representative plaque morphology. Mutants L1, L2, and L1 were not replication-competent exhibiting a 3- or 4-log reduction in RNA infectivity. JEV protein accumulations are shown at the indicated time points.

Figure 5. Accumulation of JEV genomic RNA of 24 mutants at 20 h post-transfection (hpt) compared to wild-type (WT).

References


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