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Developing New Mammalian Gene Expression Systems Using the Infectious cDNA Molecular Clone of the Japanese Encephalitis Virus

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Abstract

Major advances in positive-sense RNA virus research have been facilitated by the development of reverse genetics systems. These systems consist of an infectious cDNA clone that encompasses the genome of the virus in question. This clone is then used as a template for the subsequent synthesis of infectious RNA for the generation of synthetic viruses. However, the construction of infectious cDNA for the Japanese encephalitis virus (JEV) has been repeatedly thwarted by the instability of its cDNA. As JEV is an important human pathogen that causes permanent neuropsychiatric sequelae and even fatal disease, a reliable reverse genetics system for this virus is highly desirable. The availability of this tool would greatly aid the development of effective vaccines as well as facilitate studies into the basic biology of the virus, including the molecular mechanisms of viral replication, neurovirulence, and pathogenesis. We have successfully constructed a genetically stable infectious JEV cDNA containing full-length viral RNA genome. Synthetic RNA transcripts generated *in vitro* from the cDNA were highly infectious upon transfection into susceptible cells, and the cDNA remained stable after it had been propagated in *E. coli* for 180 generations. Using this infectious JEV cDNA, we have successfully expressed a variety of reporter genes from the full-length genomic and various subgenomic RNAs *in vitro* transcribed from functional JEV cDNAs. In summary, we have developed a reverse genetics system for JEV that will greatly facilitate the research on this virus in a variety of different fields. It will also be useful as a heterologous gene expression vector and aid the development of a vaccine against JEV.

Introduction And Results

Research investigating positive-sense RNA viruses has been considerably advanced by the development of the reverse genetics system. Here, infectious cDNA clones of the viral genome in question are constructed and these then become the templates for infectious RNA synthesis that generates synthetic viruses. In the classical 'RNA-launched' approach, cells are transfected with RNA transcripts made from the infectious cDNA clones and the synthetic viruses are then recovered from these cells. However, an alternative 'DNA-launched' approach also exists. This approach was

first reported for poliovirus and has been adapted for alphaviruses. Here, synthetic viruses are generated by directly transfecting infectious cDNA clones into susceptible cells. Either of these two approaches has been used to construct infectious cDNA clones for many positive-sense RNA virus families. These clones have been invaluable in addressing many questions regarding the positive-sense RNA viruses. However, the construction of a full-length infectious cDNA clone for the Japanese encephalitis virus (JEV) has been hampered, largely because of the genetic instability of the cloned cDNA. Despite extensive efforts, a genetically stable full-length infectious cDNA molecular clone for JEV did not exist.

JEV is a member of the *Flaviviridae* family and is transmitted by mosquitoes. It is an important human pathogen that causes permanent neuropsychiatric sequelae and even fatal disease, especially in children. Transmission of the virus has recently been observed in the Southern Hemisphere, indicating this virus could become a worldwide public health threat. Mostly based on genome structure similar to other flaviviruses, JEV is a small-enveloped virus with a single-stranded, positive-sense RNA genome approximately 11 kb in length. The genome contains a single long open reading frame (ORF) flanked by 5' and 3' nontranslated regions (NTRs) that are important *cis*-acting elements for viral replication. The RNA genome has a type I cap structure at its 5'-terminus but lacks a poly (A) tail at its 3'-terminus. The ORF is translated into a large polyprotein that is co- or post-translationally processed into three structural and seven nonstructural proteins whose genes are arranged in the genome as follows: C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Further information, for example on the function of the majority of the JEV gene products and the molecular mechanisms involved in JEV replication, neurovirulence, and pathogenesis, is limited largely because of the lack of a reliable reverse genetics system. Here we report the JEV reverse genetics system that we have developed and its applications for heterologous gene expression system.

By sequentially linking the overlapping JEV cDNA fragments at natural restriction sites, we assembled six full-length cDNA clones of CNU/LP2. These cDNA clones all had the SP6 or T7 promoter transcription start at the beginning of the viral genome so that synthetic RNA transcripts with an authentic 5'-end would be generated. To ensure that the 3'-end of the viral genome after run-off transcription would be authentic or close to authentic, we placed a unique restriction endonuclease site, either *Xho* I or *Xba* I, at the end of the viral genome. At every cloning step during the assembly process, the structural integrity of the cloned cDNAs was assessed by extensive restriction and nucleotide sequence analyses. Structural instability of the inserts leading to deletions or rearrangements was never observed.

We first examined the specific infectivity of the synthetic RNAs transcribed from the three SP6-driven constructs. For run-off transcription, the constructs were linearized by digestion with *Xho* I or *Xba* I. SP6 polymerase run-off transcription of the two *Xho* I-linearized plasmids in the presence of the $m^7G(5')ppp(5')G$ cap structure analog yield capped synthetic RNAs containing three nucleotides (CGA) of virus-unrelated sequence at their 3'-ends. This is the result of copying the 5'-overhang left by the *Xho* I digestion. Similarly, SP6 polymerase run-off transcription of the *Xba* I-linearized JEV cDNA plasmid produces capped synthetic RNAs with four nucleotides (CTAG) of virus-unrelated sequence at their 3'-ends. When susceptible BHK-21 cells were transfected with the synthetic RNAs

from these constructs, all were highly infectious (>10^5 PFU/μg). It has been reported that for some flaviviruses, the presence of unrelated sequences at the 3'-end of synthetic RNAs transcribed from infectious cDNA diminishes or abrogates their specific infectivity. This motivated us to generate synthetic RNAs lacking the unrelated sequences by treating the *Xba* I-linearized cDNA plasmid with mung bean nuclease (MBN) prior to transcription reaction, which removed the four excess nucleotides of CTAG. RNA transcripts from *Xba* I-linearized and MBN-treated cDNA plasmid had increased the specific infectivity by about 10-fold compared to the untreated transcripts. Thus, the authentic 3'-end of the JEV genome should be present to ensure highly infectious synthetic JEV RNA transcripts are generated.

We confirmed that the specific infectivity requires the transcription of RNA from the full-length JEV cDNA template by adding DNase I or RNase A during or after the transcription reaction. A genetic marker, a silent single point mutation (A^{8171}→C) that had been introduced into the infectious cDNA, was observed in the genome of the recovered virus, indicating that the cDNA can be manipulated. Significantly, the infectious BAC remained genetically stable even after 180 generations of serial growth in *E. coli*.

JEV infect a variety of commonly used animal cell types and replicate to high levels. In our study, we demonstrated that recombinant JEV viruses and replicons have been useful as vectors for foreign gene expression. In this regard, foreign genes of interest as well as the dominant selective marker puromycin N-acetyltransferase, which confers resistance to the drug puromycin, were expressed as genomic and subgenomic transcripts of infectious JEV cDNA. We will discuss a variety of ways to express heterologous genes by engineering functional JEV cDNA plasmid

Conclusions

Here we report the development of a convenient and reliable reverse genetics system for JEV that can be used to generate synthetic viruses from genetically stable full-length infectious JEV cDNAs. Previous attempts to develop such a system, including our own, were thwarted by the instability of the cloned JEV cDNA. The system described in this paper will greatly facilitate the study of the molecular mechanisms of replication, virulence and pathogenesis employed by the virus. Furthermore, we showed that this system can be used as a JEV RNA vector that will rapidly express heterologous genes in a wide variety of eukaryotic cells.

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