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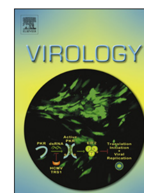

A single adaptive point mutation in Japanese encephalitis virus capsid is sufficient to render the virus as a stable vector for gene delivery

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ABSTRACT

Japanese encephalitis virus (JEV) is a neurotropic flavivirus that has broad range of hosts. Stable JEV vector has not been reported yet. Here, we constructed a JEV-EGFP by inserting a fragment of C38 (the N-terminal 38 amino acids of capsid)-EGFP-FMDV2A into the junction between 5′UTR and the N-terminus of capsid gene. An adaptive nucleotide mutation T45G (location at the N-terminus of capsid gene), resulting in an amino acid change from asparagine to lysine (N15K), was identified by genome sequencing. It stabilized the vector and enlarged the virion. The stabilizing effect might be general because it is also stable when EGFP was replaced with another marker, SNAP. A model was proposed for this stabilization effect based on previously published and our data. This finding may be used to construct various JEV-based stable delivery systems for virological studies and neural circuit tracing.
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Introduction

Neurotropic virus is able to transport in neuron and spread between neurons. The infection of animal can result in serious disease and some fatalities. However, viruses are double-edged swords. They are harmful for hosts, but could be used to deliver critical genes into organ for curing diseases. Many neurotropic viruses have been used in mapping neural circuit (Beier et al., 2013; Cannon et al., 2011; Ghosh et al., 2011; Lo and Anderson, 2011; Smith et al., 2000; Soudais et al., 2004; Wickersham et al., 2013; Zampieri et al., 2014), analyzing the function of interesting genes, and performing gene therapy (Benkhelifa-Ziyyat et al., 2013; Beutler, 2010).

Japanese encephalitis virus (JEV), which belongs to the genus Flavivirus in the family Flaviviridae, is the causative agent of Japanese encephalitis mainly in Asia. JEV genome, approximately 11 kb in length, has one open reading frame which encodes a single poly-protein. The poly-protein is cleaved into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by viral and host enzymes. The non-structural proteins play important roles in viral replication (Murray et al., 2008; Youn et al., 2013), the structural proteins involve in viral particle assembly and egress (Li et al., 2008; Roby et al., 2015). The E protein is an important antigen that is responsible for interaction with the receptor on cell surface. In central nervous system, the JEV can infect neuron and astrocyte in mouse, monkey and human (Myint et al., 2014), indicating its potential to deliver interesting genes into brain. Several groups have constructed different reporter flaviviruses by engineering the IRES (internal ribosomal entry site)-reporter gene at the 3′UTR of viral genome (Pierson et al., 2005; Yun et al., 2003). However, these reporter viruses have common drawbacks that the reporter genes are easily lost after a couple of rounds of viral infection cycles. Although new strategy has been used by fusing reporter gene into the N terminus of capsid gene (McGee et al., 2010; Schoggins et al., 2012; Shustov et al., 2007; Zou et al., 2011), none of these manipulations has generated a stable reporter system.

The aim of this study is to generate a recombinant competent JEV for stable expression of heterologous genes *in vitro* and *in vivo*. Here, we have identified a single adaptive mutation that enables the JEV to stably deliver EGFP and SNAP genes into cells, yielding a system that might be used for studies in virology, biology and neuroscience.

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Results

Construction of JEV-EGFP

In previous study, a version of JEV-GFP has been developed by engineering the IRES-GFP gene at the 3′UTR of viral genome (Yun et al., 2003), the reporter virus was unstable since the reporter gene was easily deleted after several rounds of viral infection. In order to construct a stable JEV-EGFP, we took a strategy as shown in Fig. 1. A fragment covering the sequence of the N-terminal 38 amino acids of capsid protein (C38), an EGFP gene and a FMDV2A (F2A) sequence was inserted into the junction between the 5′UTR and the N-terminus of capsid gene. The nucleotide sequence corresponding to the first 38 amino acids of capsid protein is required for maintaining genome cyclized. The F2A sequence is engineered to ensure that the EGFP protein was properly processed.

To test the ability of virus production of the construct, the 5 μg of genome RNA was transfected into BHK-21 cells and the medium was collected at different time points. The EGFP signals were detected at 2 days post-transfection (dpt), and enhanced with time (Fig. 5A). These results indicate that the JEV-EGFP is successfully produced.

JEV-EGFP cannot stably deliver EGFP gene into cells in vitro

The stability of JEV-EGFP is prerequisite for its application. We tested the stability of the system *in vitro* by monitoring the EGFP signals and plaque morphology. By continuous passaging of culture mediums from the early round on BHK-21 cells (3–4 days per passage), it was found that the sizes of P0 virus plaques were significantly different (Fig. 2A), suggesting the existence of different viruses. This is consistent with the fact that continuously and blindly passaging of P0 virus in BHK-21 cells for three rounds (termed as P1, P2 and P3) yielded virus with larger plaque size consistent with wild type (Fig. 2A). To confirm the genotype of these viruses, the viral RNA from each passage was extracted and the RT-PCR was performed using specific primers for targeting the fragment comprising the 5′UTR and capsid gene. The P0, P1, P2 and P3 viral RNA indeed produced multi-bands (Fig. 2B), indicating that the EGFP was deleted in some viruses during replication (Fig. 2C). These results suggest that the JEV-EGFP does not stably express EGFP *in vitro*.

Generation of stable JEV-EGFP

Although we adopted a new strategy, the produced JEV-EGFP was still unstable. Since virus usually evolves during life according to different conditions, we speculated that the larger plaques represent wild type JEV, and the smaller plaques, JEV-EGFP (Fig. 2A). To prove our speculation, we picked the small plaque from P0 virus into BHK-21 cells for three rounds (termed as P1′, P2′ and P3′), each round picks the small plaque for the next infection (Fig. 3A). The P3′ virus showed uniform and small plaque size (Fig. 3B), and could infect BHK-21 cells, leading to expression of the EGFP *in vitro* (Fig. 3C). The genome sequencing shows that the P3′ virus is not mixture (Fig. 3D). These results indicate that the P3′ virus is stable *in vitro*.

To test the stability of P3′ virus *in vivo*, the virus was injected into the VPM region of mouse brain. We found that the EGFP signals were detected at the VPM and part of the cortexes at 5 days post-injection (dpi, data not shown), and that the signals were distributed the whole slice at 7.5 dpi (Fig. 3E).

To rule out the possibility that the recombinant JEV also lost the marker gene *in vivo*, we carried out the staining with anti-envelope antibody, and found that the distribution of EGFP and JEV envelope is highly overlap (Fig. 3E). These results indicate that a stable JEV-EGFP is generated.

N15K adaptive mutation is sufficient for stable JEV-EGFP

The RNA virus genome changes frequently during replication. To answer the question that whether the stable system contains only one type of virus, we sequenced the genome of P3′ virus from three independent experiments. We found that a nucleotide change from T (location at the 45 position of N-terminus of capsid gene) to G, which resulted in an amino acid change from asparagine (Asn, N) to lysine (Lys, K) located at the 15 position of N-terminus of capsid (Fig. 4A). Sequence alignment shows that the N is conserved among the members of Japanese encephalitis serological group (Fig. 4B).

In order to confirm the role of the adaptive mutation in maintaining the stable JEV-EGFP, we reconstituted the N15K mutation into JEV-EGFP infectious clone and analyzed mutant virus. The green signals were detected at 1 dpt, indicating that JEV-EGFP-N15K can infect BHK-21 cells and enhanced the expression of marker gene compared with the original version (data not shown). The mutated virus (P0 virus) was blindly passaged for three rounds (termed as P1, P2, and P3), same as the procedures for the original construct in 3.2. Plaque size of each passage was uniform and small (Fig. 4C), and each passage viral RNA produced a single band with the expected size (Fig. 4D). The P3 virus can infect BHK-21 cells and express EGFP (Fig. 4E). The stability of the N15K mutation was confirmed by genome

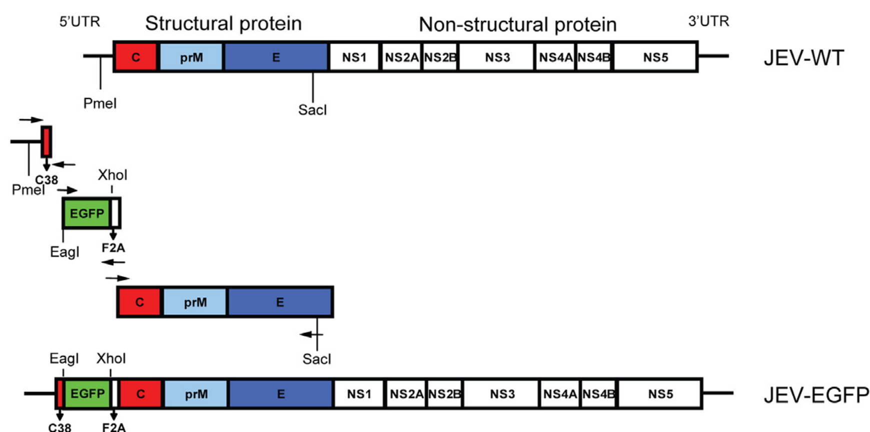

Fig. 1. Cloning diagram of recombinant JEV-EGFP. A fragment containing the sequence of the N-terminal 38 amino acids of capsid protein (C38), an EGFP gene, and a FMDV2A (F2A) sequence, which was inserted into the junction between 5′UTR and the N-terminus of capsid gene.

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