Adaptation of Venezuelan equine encephalitis virus lacking 51-nt conserved sequence element to replication in mammalian and mosquito cells

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Abstract

Replication of alphaviruses strongly depends on the promoters located in the plus- and minus-strands of virus-specific RNAs. The most sophisticated promoter is encoded by the 5′ end of the viral genome. This RNA sequence is involved in the initiation of translation of viral nsPs, and synthesis of both minus- and plus-strands of the viral genome. Part of the promoter, the 51-nt conserved sequence element (CSE), is located in the nsP1-coding sequence, and this limits the spectrum of possible mutations that can be performed. We designed a recombinant Venezuelan equine encephalitis virus genome, in which the promoter and nsP1-coding sequences are separated. This modification has allowed us to perform a wide variety of genetic manipulations, without affecting the amino acid sequence of the nsPs, and to further investigate 51-nt CSE functioning. The results of this study suggest a direct interaction of the amino terminal domain of nsP2 with the 5′ end of the viral genome.
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Introduction

The alphavirus genus of the Togaviridae family contains almost 30 members, some of which are important human and animal pathogens (Griffin, 2001; Strauss and Strauss, 1994). Alphaviruses are widely distributed on all continents except in the Arctic and Antarctic areas. They efficiently replicate both in mosquito vectors and vertebrate hosts. However, in mosquitoes they cause a persistent, lifelong infection that does not noticeably affect biological functions of the vectors, while in vertebrate hosts, the infection is acute and characterized by high-titer viremia, rash and fever, until the virus is cleared by the immune system. The New World alphaviruses can cause severe encephalitis in humans and animals that can result in death or neurological disorders (Dal Canto and Rabinowitz, 1981; Griffin, 2001; Johnston and Peters, 1996; Leon, 1975). These encephalitogenic alphaviruses include Venezuelan (VEEV), eastern (EEEV) and western equine encephalitis (WEEV) viruses and represent a serious public health threat in the US (Rico-Hesse et al., 1995; Weaver and Barrett, 2004; Weaver et al., 1994, 1996). They continue to circulate in the Central, South and North Americas and cause severe, and sometimes fatal disease in humans and horses. During VEEV epizootics, equine mortality due to encephalitis can reach 83%, and in humans, while the overall mortality rate is below 1%, neurological disease including disorientation, ataxia, mental depression and convulsions, can be detected in up to 14% of all infected individuals, especially children (Johnson and Martin, 1974). Sequelae of VEEV-related clinical encephalitis in humans and rats are also described (Garcia-Tamayo et al., 1979; Leon, 1975). In spite of the continuous threat of VEEV epidemics, the biology of this virus and the mechanism of its replication are insufficiently understood.

The VEEV genome is represented by a ca. 11.5 kb-long, single-stranded RNA of positive polarity (Strauss et al., 1984), which mimics the structure of the cellular mRNAs, in that it contains a 5′ cap and poly(A)-tail at the 5′ and 3′ ends, respectively. The genome contains two polyprotein-coding sequences. The first, a 7500 nt-long, 5′-terminal open reading frame (ORF) is translated into viral nonstructural proteins (nsP1–4) that form, together with the cellular proteins, the enzyme complex required for genome replication and transcription of the subgenomic RNA. The latter RNA encodes the

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second polyprotein that is co- and post-translationally processed into viral structural proteins (capsid, E2 and E1) that form infectious viral particles.

The replication of the alphavirus genome is a multi-step process that begins with synthesis of a full-length, minus-strand RNA intermediate that, in turn, serves as a template for synthesis of the plus-strand viral genomes and transcription of the subgenomic RNA. Synthesis of these RNA species is a highly synchronized process regulated by differential processing of the viral nsPs (Lemm and Rice, 1993; Lemm et al., 1994; Shirako and Strauss, 1994). At the early stages of viral replication, the ns polyprotein is partially processed by nsP2-associated protease into P123 and nsP4, an enzyme complex capable of minus-, but not plus-strand RNA synthesis. Then, after further processing of the polyproteins into nsP1+P23+nsP4, the intermediate polymerase functions in synthesis of both plus and minus genome-sized RNA, but appears to be unable to efficiently use the internal promoter for 26S mRNA synthesis. Finally, after complete P1234 processing to individual nsP1−4, the replication complex (RC) efficiently (Wang et al., 1994) synthesizes the plus-strand viral genome and subgenomic RNA, but is no longer capable of minus-strand synthesis (Lemm et al., 1994, 1998; Sawicki and Sawicki, 1987). Thus, the early and mature RCs most likely utilize different RNA promoters located in the plus- and minus-strands of virus-specific RNAs.

The critical element of the promoter for minus-strand RNA synthesis is a 19-nt-long, conserved sequence element (CSE) adjacent to the poly(A) and located at the 3′ end of the viral genome (Hardy, 2006; Hardy and Rice, 2005; Kuhn et al., 1990). The mutations in this sequence have a deleterious effect on alphavirus replication. However, it can be replaced by an artificial AU-rich sequence that might efficiently function in RNA synthesis (Raju et al., 1999). It was also demonstrated that the presence of poly(A) at the 3′-terminus and cap at the 5′ terminus strongly stimulates the RNA replication (Hardy and Rice, 2005). Moreover, the sequence of the 5′UTR determines the minus-strand RNA synthesis as well (Frolov et al., 2001; Gorchakov et al., 2004), and these facts strongly suggest the importance of the 5′–3′ end interaction in the RNA replication, which most likely proceeds through formation of the translation initiation complex. The subgenomic promoter required for transcription of the subgenomic RNA is well defined (Levis et al., 1990; Ou et al., 1983; Wielgosz et al., 2001) and represented by a 24-nt CSE located in the minus-strand genome intermediate. The latter CSE covers not only the nucleotides adjacent to the start of the subgenomic RNA, but the first nucleotides of the subgenomic RNA as well. This promoter is recognized in vitro by the protein complex containing all of the viral nsPs (Li and Stollar, 2004).

The most sophisticated promoter of the RNA synthesis is encoded by the 5′ end of the alphavirus genome. Its functioning was intensively studied in the context of Sindbis virus (SINV) (Fayzulin and Frolov, 2004; Gorchakov et al., 2004; Niesters and Strauss, 1990a, 1990b; Strauss and Strauss, 1994), but is still poorly understood. The 5′ terminus appears to contain two elements: the 5′-terminal sequence, encoded by the 5′UTR (that appears to represent a core promoter) and a 51-nt CSE, found in the nsP1-coding sequence that might be a replication enhancer, functioning in a virus- and cell-dependent manner (Fayzulin and Frolov, 2004; Niesters and Strauss, 1990b). The problem with investigation of the 5′ promoter lies in the involvement of its sequence in initiation of translation of viral nsPs, and synthesis of both minus- and plus-strands of the viral genome. In addition, part of the promoter, the 51-nt CSE, is located in the nsP1-coding sequence, and this strongly limits a spectrum of mutations that can be done without affecting nsP1 functioning.

We considered these difficulties when we undertook the VEEV promoter investigation and designed a VEEV genome, in which the promoter sequence and the nsP1-coding sequence are separated. This modification allowed us to both perform a wide variety of genetic manipulations without affecting the amino acid sequence of the nsPs, and study the function of 51-nt CSE in virus replication. The results of this study suggest a direct interaction of the amino terminal domain of VEEV nsP2 with the 5′ end of the viral RNA and a possibility of applying a similar approach in studying the 5′-terminal promoter functioning in replication of other RNA-positive viruses.

Results

Recombinant viruses with the 5′ promoter sequences located outside of the nsPs' ORF

In our initial experiments, we attempted to develop an experimental system to study the effect of extended genetic manipulations in the 5′-terminal alphavirus genome elements without affecting the amino acid (a.a.) sequence of the nsPs. Thus, we separated the 5′ end-specific promoter and the nsP-coding sequence in the context of the VEEV TC-83 genome. To achieve this, we introduced 95 mutations into the first 300 nt of the nsP1-coding gene of VEEVmut (Fig. 1A), and these mutations did not change the encoded protein sequence. However, based on computer predictions, they destroyed the original secondary structure of the fragment, including the secondary structure of the 51-nt CSE. After transfection of the in vitro-synthesized RNA into BHK-21 cells, no cytopathic effect (CPE) was detected and no infectious virus was recovered even after following blind passages of the harvested media on the naive BHK-21 cells (data not shown). This indicated that the introduced mutations and, most likely, the destabilization of the secondary structure made the genome RNA incapable of replication, and neither the reverting nor pseudoreverting mutations accumulated in the VEEVmut genome.

In the next step, we cloned a 347-nt-long 5′-terminal fragment of the VEEV TC-83 genome into the VEEVmut genome upstream from the initiating AUG (see Figs. 1A and B for details). In the VEEVubi, fusion of the cloned sequence and the nsP1 was performed through the ubiquitin (Ubi) gene to preserve synthesis of the following nsP1 protein in its natural form, including the first methionine. Thus, the ORF started from the AUG located downstream from the 5′UTR and continued through the cloned fragment of nsP1 and Ubi into the P1234 polyprotein.