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Virus Research 206 (2015) 99–107

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The 5′ and 3′ ends of alphavirus RNAs – Non-coding is not non-functional

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ARTICLE INFO

Article history:
Available online 25 January 2015

Keywords:
Translation regulation
Polyadenylation
mRNA capping
Viral pathogenesis
miRNAs
Protein–RNA interactions

ABSTRACT

The non-coding regions found at the 5′ and 3′ ends of alphavirus genomes regulate viral gene expression, replication, translation and virus–host interactions, which have significant implications for viral evolution, host range, and pathogenesis. The functions of these non-coding regions are mediated by a combination of linear sequence and structural elements. The capped 5′ untranslated region (UTR) contains promoter elements, translational regulatory sequences that modulate dependence on cellular translation factors, and structures that help to avoid innate immune defenses. The polyadenylated 3′ UTR contains highly conserved sequence elements for viral replication, binding sites for cellular miRNAs that determine cell tropism, host range, and pathogenesis, and conserved binding regions for a cellular protein that influences viral RNA stability. Nonetheless, there are additional conserved elements in non-coding regions of the virus (*e.g.*, the repeated sequence elements in the 3′ UTR) whose function remains obscure. Thus, key questions remain as to the function of these short yet influential untranslated segments of alphavirus RNAs.

1. Introduction

The alphaviruses (family *Togaviridae*, genus *Alphavirus*) include 30 identified viral species. Most alphaviruses are transmitted by mosquito vectors to various vertebrate hosts, especially birds, rodents, and primates. Many alphaviruses, such as chikungunya (CHIKV) and Venezuelan equine encephalitis (VEEV) viruses, cause disease in vertebrate animals with the potential to emerge into humans or domesticated animals *via* epidemic and epizootic cycles. However, a few alphaviruses have fundamentally different host range and transmission modes. The recently discovered Eilat virus infects mosquitoes but not vertebrates (Nasar et al., 2012), and salmonid alphaviruses within the species *Salmon Pancreas Disease Virus* are transmitted among fish without the known involvement of a vector (Fringuelli et al., 2008). Similarly, the transmission cycle of southern elephant seal virus (SESV) is unclear, although it has been isolated from sea lice that parasitize seals (La Linn et al., 2001).

1.1. Overview of alphavirus 5′ and 3′ UTRs

Alphaviruses contain non-segmented, single stranded RNA genomes of 11–12 kb, with a type 0 cap (N7mGppp) at the 5′ end and a poly(A) tail at the 3′ end (Fig. 1). These characteristics make alphavirus genomes appear to the host cell as messenger RNA (mRNA) for immediate translation upon entry into the cytoplasm (Strauss and Strauss, 1994). There are two open reading frames (ORFs) in alphavirus genomes, encoding nonstructural and structural polyproteins, respectively. Flanking the ORFs are UTRs located at the 5′ and 3′ ends of the genome, as well as between the ORFs

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http://dx.doi.org/10.1016/j.virusres.2015.01.016
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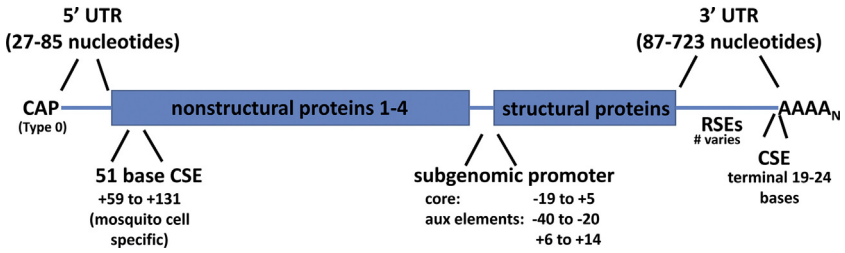


Fig. 1. Organization and regulatory landmarks of the alphavirus genome. Major RNA regulatory elements and open reading frames are indicated. The positions of the 51 base CSE and subgenomic promoter are given relative to the start site of their associated open reading frame. These latter elements are based solely on studies performed in Sindbis virus.

(the subgenomic 5′ UTR). Both the 5′ UTR and its complement in the 3′ UTR of the minus-strand RNA comprise parts of the promoters recognized by the alphavirus replication complex, including the RNA-dependent RNA polymerase (RdRp), or nonstructural protein 4.

The alphavirus 5′ and 3′ UTRs contain distinct core promoter elements for both minus- and plus-strand RNA synthesis. Shorter sequences within these UTRs form cis-acting sequence elements for viral replication. In addition to the UTRs, conserved sequence elements (CSE) can be found in two additional regions of the Sindbis virus (SINV) genome. First, a conserved 51-nucleotide element important for virus replication exists near the start of the coding portion of the non-structural protein 1 (nsP1) gene. This element is dispensable for replication in vertebrate cells, but important for replication in mosquito cells. Analysis of adaptive mutations that arise to compensate for mutations in the 51-nucleotide conserved sequence element suggests that nsP2, nsP3, and the 5′ UTR in some way functionally interact with this element (Fayzulin and Frolov, 2004). Additional analysis of these functional elements indicates that the 5′ and 3′ ends of genomic RNAs interact with each other and also interface with host factors to effectively initiate replication (Frolov et al., 2001). The second conserved sequence element lies at the junction between the nonstructural and structural polyprotein ORFs and forms the 5′ UTR of subgenomic RNA. This conserved sequence element also appears to interact with host factors (Wielgosz et al., 2001). In summary, these elements interact in host-dependent manners to regulate genome replication.

2. Alphavirus UTRs – evolution and implications for pathogenesis

Overall, key information about the functions of the alphavirus UTRs remain obscure, including: (1) their specific interactions with viral or cellular factors; (2) the molecular basis of their function; and (3) the evolutionary forces that shape the UTR sequence heterogeneity among viral species and strains. Here, we summarize related information based on recent studies.

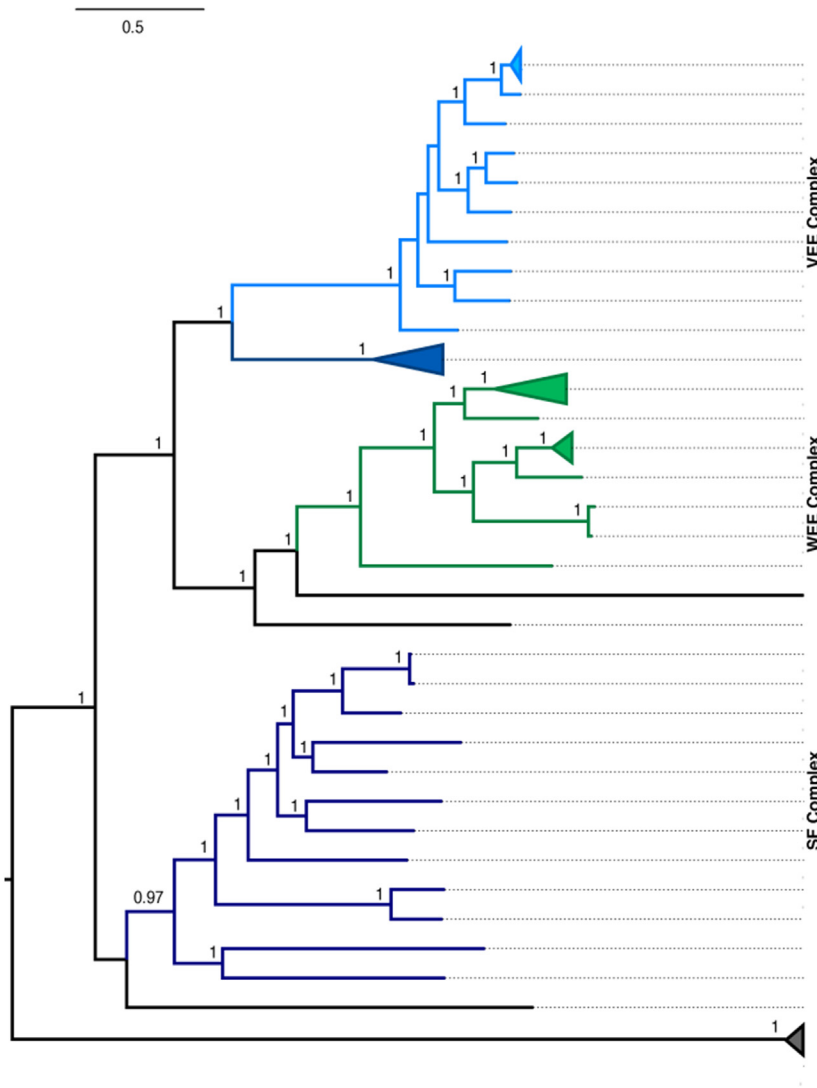
2.1. Length variation among the alphavirus 5′ and 3′ UTRs

Relieved of the constraints of protein coding, the UTRs vary greatly in length and sequence structure, both within a single alphavirus species as well as among strains of some species. Indeed, the 5′ UTRs range in length from 27 (Salmon Alphavirus (SAV)) to 85 (Semliki Forest Virus (SFV)) nucleotides, and the 3′ UTRs range from 87 (SAV) to 723 (CHIKV) nucleotides (Fig. 2). Specifically, aquatic alphaviruses without a known arthropod vector contain the shortest UTRs (5′-: 27 nucleotides; 3′-: 87–130 nucleotides), followed by VEE complex viruses (5′-: 42–48 nucleotides; 3′-: 78–184 nucleotides). Alphaviruses in the Semliki Forest (SF) complex, the most divergent complex, contains the longest UTRs with the greatest diversity in length (5′-: 76–85 nucleotides; 3′-: 227–713 nucleotides).

The difficulty in obtaining meaningful alignments among the alphavirus UTRs suggests that they evolve quickly, probably due to different evolutionary pressures. In addition to point mutations, frequent insertions, deletions, and sequence duplications are observed in alphavirus 3′ UTRs at both inter- and intra-species levels. Nonetheless, most alphavirus 3′ UTRs share a common core structure, with short repeated sequence elements (RSEs), and a 19–24 nucleotide CSE at the 3′ end of the genome immediately adjacent to the poly(A) tail (Pfeffer et al., 1998). Notably, RSEs of similar sequence are observed in closely related viruses (such as in VEE and the Eastern Equine Encephalitis (EEE) complex alphaviruses), although often with different copy numbers, indicating that these 3′ UTRs diverged from a common ancestor. Despite the significant variation among diverse SF complex members, as manifested by the different number of RSEs, each virus maintains relatively long 3′ UTRs. However, the trend toward UTR variability is not consistent as some alphaviruses, such as EEEV North American strains, maintain extremely stable UTR sequences with little difference observed between the first 1930s viral isolate and current isolates. A detailed summary of the RSEs in alphavirus 3′ UTRs has been published (Pfeffer et al., 1998).

2.2. Evolution and fitness effects of the chikungunya virus 3′ UTR

The CHIKV 3′ UTR contains a number of direct repeats (DRs 1, 2, and 3), which occur in lineage-specific patterns (Fig. 3) and probably resulted from historical duplication events (Chen et al., 2013). The Asian endemic lineage 3′ UTR, which diverged from the East/Central/South African (ECSA) enzootic lineage about 100 years ago, differs from the other two major CHIKV lineages in: (1) a duplication of DR3 and (2) point mutations, duplications and replacements of the DR1 and DR2 regions, namely DR (1 + 2). Both *in vitro* and *in vivo* studies suggest that deletion of these DRs reduces CHIKV fitness in mosquito vectors but can increase fitness in mammalian models. Presumably, the CHIKV 3′ UTR enhances viral replication in mosquitoes by interacting with mosquito cell-specific factors. However, it is not known if the deleterious effect of the longer 3′ UTR for replication in mammalian cells is caused by negative interactions with mammalian host factors or simply the replication costs of a longer genome. Overall, the evolution of the CHIKV 3′ UTR is probably shaped by the fitness trade-offs between mammalian and mosquito hosts. Reciprocal exchange of the 3′ UTR of ECSA and Asian strain and then competing each chimera with the wild-type (wt) viruses suggested that, in either genetic backbone, the fitness of the Asian lineage 3′ UTR is significantly lower than that of its ancestor, the ECSA lineage 3′ UTR, in both mammalian and mosquito hosts. As with other studies examining the Asian genotype (Tsetsarkin et al., 2011), these findings suggest that a population bottleneck resulted from a founder effect when CHIKV was



Virus	5'UTR	Subgenomic 5'UTR	3'UTR
VEE IAB, IC, ID	44-46	35	122-132
Everglades (VEE II)	43	35	118
VEEV IE	44	35	124
Tonate (VEE IIIB)	43	35	184
Mucambo (VEE IIIA)	42	35	184
VEE_71D_1252	42	35	127
Pixuna (VEE IV)	42	32	78
VEE IF	44	37	195
Rio Negro (VEE VI)	44	35	195
Cabassou (VEE V)	48	35	184
Eastern Equine Encephalitis	46	63	323-363
Sindbis	58-59	45	268-323
Whataroa	60	49	323
Western Equine Encephalitis	42	32	305-308
Highlands J	45	41	235
Buggy Creek	46	49	210
Fort Morgan	46	49	198
Aura	77	50	465
Eilat	56	82	520
Trocara	N/A	60	587
Getah	78	44	312-414
Sagiyama	78	44	410
Ross River	80	44	348-525
Bebaru	79	50	609
Semliki Forest	85	38	227-517
Mayaro	76	26	269-293
Una	N/A	37	514
Middelburg	52	46	487-566
O'nyong-nyong	79	45	413-425
Chikungunya	76-77	65-66	498-723
Barmah Forest	62	28	446
Ndumu	48	40	547
Southern Elephant Seal	N/A	0	130
Salmon Alphavirus	27	35	87-89

Fig. 2. Evolution of alphaviruses and the lengths of their untranslated regions. The phylogenetic tree is modified from the Bayesian tree based on structural protein coding regions (Nasar et al., 2012). Posterior probabilities higher than 0.9 are labeled along the branches. UTR lengths were determined from alignments of available viral sequence information (as of September 2014) in GenBank.

introduced into Asia by an infected traveler (as has been detected thousands of times since 2005 (Lanciotti et al., 2007; Panning et al., 2008)), resulting in the fixation of a lower fitness Asian lineage 3′ UTR with deletion of a fragment including a copy of DR1 and DR2. Due to the loss of structural/functional constraints, mutations and insertions accumulated in the regions surrounding the 5′ most copies of DR1 and DR2, forming the later DR (1 + 2) in the Asian lineage 3′ UTR. This founder virus presumably replicated better in humans but relatively poorly in mosquitoes compared to the ancestral ECSA wt virus; because of this, it subsequently adapted for more efficient mosquito infection and transmission by duplicating DR (1 + 2) and DR3 elements.

In summary, fitness trade-offs and population bottlenecks likely have contributed to the evolution of the CHIKV 3′ UTR. Studies identifying the host-specific factors affecting replication of CHIKV and other alphaviruses are needed to understand the functional role of the viral 3′ UTRs and the constraints for evolutionary change. This information potentially could be used to disrupt alphavirus transmission.

3. Modification and function of the 5′ UTR

3.1. Capping

The alphavirus genomic and subgenomic RNAs are modified at the 5′ termini by the addition of a 7-methylguanosine (m7G) cap structure (Cap 0), which promotes RNA stability and translation of viral transcripts. Alphavirus m7G capping is facilitated by nsP1 in a manner that is distinct from host mRNA capping (Ahola and Kaariainen, 1995; Mi et al., 1989) (Fig. 4). In eukaryotic cells, mRNA capping proceeds as follows: (1) RNA triphosphatase cleaves nascent RNA (pppNp-RNA) at the gamma phosphate position to yield diphosphate RNA (ppNp-RNA); (2) guanylyltransferase then transfers GMP from hydrolyzed GTP to the diphosphate RNA to yield GpppNp RNA; (3) this intermediate is methylated by (guanine-N7)-methyltransferase at the terminal guanosine to yield Cap 0 RNA. In contrast to N7 capping of eukaryotic and certain viral RNAs (*e.g.*, vaccinia virus and reovirus), N7-methylated GMP (and not GMP) is transferred by the viral guanylyltransferase to

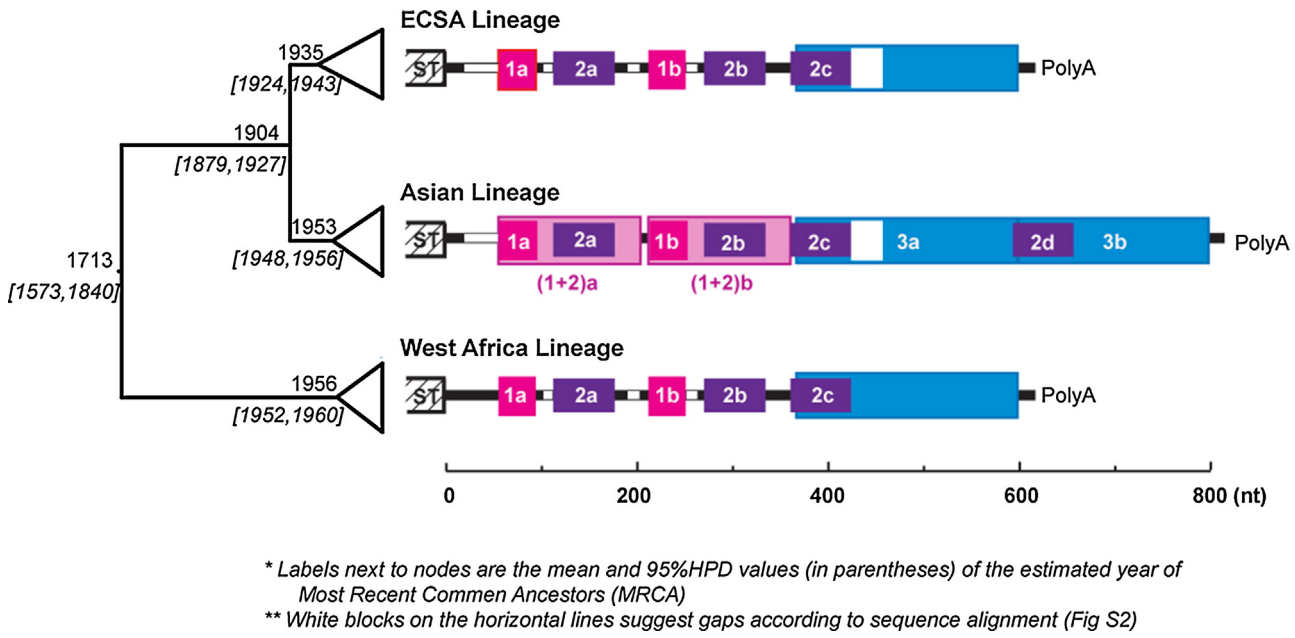


Fig. 3. Evolutionary history and lineage-specific structures of the CHIKV 3′ UTR, from (Chen et al., 2013). On the left is the Maximum Clade Credibility tree based on the complete concatenated ORF sequences, with the branches in each lineage collapsed. The estimated year of the most recent common ancestor (MRCA: mean and the 95% HPD values) of each clade is labeled left to the node. The 3′ UTR structures, based on sequence alignment, are shown next to each lineage. Direct repeats are illustrated by different colored blocks, and each of the four colors represents a different homologous sequence region. Sequence gaps in the alignment are indicated by white blocks. In the Asian lineage, two distinct derived differences are observed: (1) duplication of DR3, and; (2) duplication the of DR(1 + 2) region.

diphosphate RNA during capping of alphavirus RNAs. Messenger RNA of higher eukaryotes and many viruses that utilize cap-dependent translation also contain additional 2′-O-methylation of the first transcribed nucleotide to create a Cap 1 structure. Although alphavirus RNA is translated in a cap-dependent manner, it lacks this additional 2′-O-methylation modification. Moreover, the 5′ UTR sequence or structure is not reported to modulate the efficiency of alphavirus m7G capping, in contrast to that seen with distantly related flaviviruses (Dong et al., 2007).

3.2. Translation

While the presence of the m7G cap is required for translation of alphavirus RNAs, both the sequence and structure of the 5′ UTR can regulate this process. Indeed, RNAs containing the genomic or subgenomic 5′ UTRs of SFV and SINV are translated with different efficiencies despite containing identical 5′ terminal caps (Berben-Bloemheuvel et al., 1992; Castello et al., 2006). The key host translation initiation factors eIF4E and eIF4F differentially

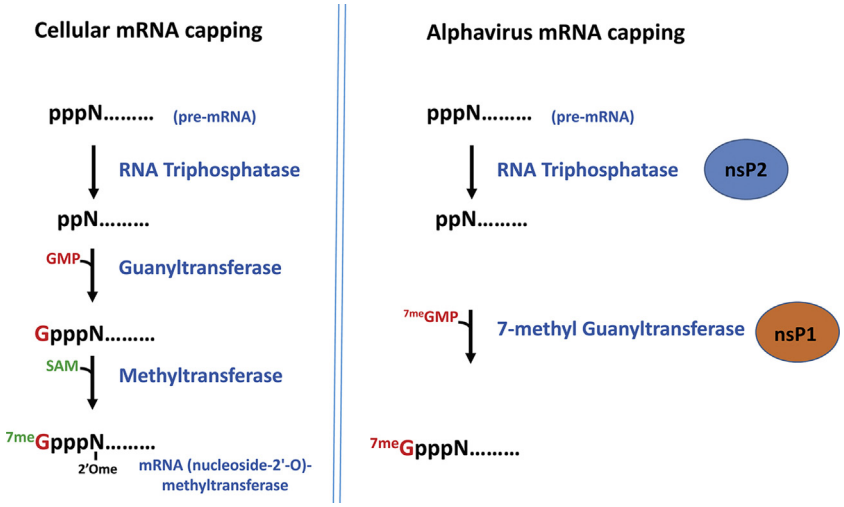


Fig. 4. A comparison between mRNA capping of cellular and alphavirus mRNAs. The methyl donor S-adenosyl methionine (SAM) is indicated in green. In alphavirus capping, the nsP1 protein methylates GMP prior to covalently attaching the modified GMP to the diphosphate at the 5′ end of the pre-mRNA generated by triphosphatase activity associated with nsP2. Cellular mRNAs generally contain a 2′-O-methyl modification (2′-O me) of the first nucleotide downstream of the N-7^{me}Gppp cap that is not seen in alphavirus transcripts.

associate with the genomic and subgenomic 5′ UTRs, which form distinct secondary structures that likely mediate this interaction. This differential pattern of binding also explains why subgenomic RNA is translated efficiently during host shut-off, whereas genomic RNA is not. During virus-induced host shut-off, eIF4G is cleaved by host (caspase-3) or viral proteins (Marissen and Lloyd, 1998), and eIF2α is phosphorylated by PKR (Gorchakov et al., 2004a; Ryman et al., 2002), leading to global inhibition of mRNA translation. However, the alphavirus subgenomic RNA retains efficient translation rates because its 5′ UTR does not require eIF4G for translation (Berben-Bloemheuvel et al., 1992; Castello et al., 2006). Thus, the alphavirus subgenomic 5′ UTR sequence and structure facilitate translation in the absence of host factors, which are inactivated or absent during host transcriptional shut-off. Recent data obtained from mutational analysis of the 5′ UTR of the subgenomic RNA also indicate that subgenomic RNA translational efficiency and quantity play a role in host translational shut off (Patel et al., 2013). The first 20 bases of the 5′ UTR of the SINV subgenomic RNA contain sequences that influence internal promoter activity. Indeed, mutation of sequence blocks within this region results in small plaque phenotypes.

3.3. Replication

The alphavirus 5′ UTR and its complementing sequence in the 3′ UTR of the negative strand are critical for RNA synthesis, and contain core promoter elements that function together with other cis-acting elements downstream and within the 3′ UTR to regulate positive- and negative-strand synthesis (Frolov et al., 2001; Gorchakov et al., 2004b; Kulasegaran-Shylini et al., 2009a). The alphavirus 5′ UTR forms stable secondary structures and mutagenesis studies have shown that both the sequence and structure of the UTR modulates replication (Kulasegaran-Shylini et al., 2009a,9; Nickens and Hardy, 2008; Niesters and Strauss, 1990). The AU dinucleotide at the very terminus of the 5′ UTR is required for replication, independent of its ability to base pair (Kulasegaran-Shylini et al., 2009a), and may be needed for binding of the viral RdRp (nsP4) to the 3′ end of the negative-sense strand for initiation of plus-strand synthesis (Shirako et al., 2003). Accordingly, this sequence is highly conserved in alphaviruses. Both the sequence and the thermodynamic stability of the 5′ stem structure regulate replication, as mutations that alter the thermodynamic stability of the stem or change the sequence but not the stability of the stem compromise replication (Kulasegaran-Shylini et al., 2009a). This suggests that host and viral factors required for replication associate with the 5′ UTR in both a sequence- and structure-dependent manner. Disruption of the native 5′ UTRs of SINV and VEEV results in emergence of pseudorevertant viruses containing AU rich sequences at the 5′ terminus, which restore replication efficiency (Gorchakov et al., 2004b; Kulasegaran-Shylini et al., 2009a). The addition of AU-rich sequences to the 5′ terminus is mediated by the RdRp, which also possesses a template-independent 3′ terminal adenylyltransferase activity (Rubach et al., 2009; Tomar et al., 2006). The revertants containing AU-rich sequences may arise from either the adenylyltransferase or error-prone polymerase activity of nsP4, or the synergistic activity of both (Kulasegaran-Shylini et al., 2009a). Although the precise role of 5′ UTR sequence and structure in RNA synthesis is not fully understood, its motifs are important for the coordinated recruitment of viral and host factors required for replication, as mutations in the 5′ UTR often give rise to compensatory mutations within the viral replicase proteins (nsPs) that alter binding of host factors to the UTR (Berben-Bloemheuvel et al., 1992; Castello et al., 2006; Pardigon et al., 1993; Pardigon and Strauss, 1992; Shirako et al., 2003).

Despite their conserved features, the 5′ UTRs among alphaviruses form distinct secondary structures, which may affect function and compatibility. As an example, studies with chimeric viruses have shown that SFV 5′ UTR sequences do not support efficient replication of SINV defective interfering genomes containing heterologous (SINV) 3′ UTR sequences (Gorchakov et al., 2004b). Incompatibility of the SFV 5′ UTR with the SINV 3′ UTR explains this observation, as interaction between the 5′ UTR and a 19-nucleotide conserved sequence element in the 3′ UTR is required for negative strand synthesis (Frolov et al., 2001). 5′ UTR and 3′ UTR incompatibility among different alphaviruses also is suggested by replication experiments with VEEV/SINV and EEEV/SINV chimeric viruses, which encode nsPs and structural proteins of heterologous alphaviruses but contain 5′ UTR and 3′ UTR sequences derived from the same virus (Garmashova et al., 2007; Petrakova et al., 2005). It remains possible that replicase proteins from different alphaviruses lack promiscuity, and may interact differentially with RNA sequences and/or structural elements found within the native 5′ UTR and its complement (Shirako et al., 2003).

3.4. Pathogenesis and innate immunity

Sequence and structural changes within the alphavirus 5′ UTR also impact immune restriction and viral pathogenesis. The role of the 5′ UTR in modulating pathogenesis was established by the observation that a single point mutation at nucleotide position 3 in attenuated strains of VEEV (TC-83 and V3043) compromised virulence in immunocompetent mice (Kinney et al., 1993, 1989; White et al., 2001). This mutation reduces infectivity of VEEV both *in vitro* and *in vivo*, but not in cells or animals deficient in the receptor for type I interferon (IFN) (Spotts et al., 1998; White et al., 2001). Sequence deletions and point mutations at nucleotides 5 and 8 within the 5′ UTR of SINV, and analogous point mutations at positions 21, 35, and 42 within the 5′ UTR of SFV also alter pathogenicity and neurovirulence in rodents (Klimstra et al., 1999; Kobiler et al., 1999; Kuhn et al., 1992; Logue et al., 2008). Recently, it was demonstrated that attenuating mutations in the 5′ UTR of VEEV and SINV increase the association of viral RNA with Ifit1, an IFN-stimulated gene that recognizes and restricts translation of non-self RNA (Hyde et al., 2014). Ifit1 is up-regulated rapidly and to high levels following type I IFN treatment or viral infection, and preferentially recognizes Cap 0 RNA transcripts which lack 2′-O-methylation of the penultimate nucleotide present in Cap 1 structures. To overcome Ifit1 restriction of non-2′-O-methylated mRNA, many cytoplasmic viruses generate Cap 1 structures on their mRNA by encoding their own viral 2′-O-methyltransferases (*e.g.*, Flaviviruses, Coronaviruses, Poxviruses), snatching caps from host mRNA (*e.g.*, Orthomyxoviruses and Bunyaviruses), or utilizing cap-independent translation (*e.g.*, Picornaviruses, Caliciviruses, and hepatitis C virus) (reviewed in Diamond, 2014). Viruses encoding a mutated non-functional 2′-O-methyltransferase are restricted *in vitro* and *in vivo*, but not in *Ifit1*$^{-/-}$ cells and animals (Daffis et al., 2010; Kimura et al., 2013; Menachery et al., 2014; Szretter et al., 2012; Zust et al., 2011). Alphaviruses are unique in that they are relatively resistant to Ifit1-mediated inhibition, despite possessing Cap 0 genomic and subgenomic mRNA and utilizing cap-dependent translation. Instead, the structure of the alphavirus 5′ UTR antagonizes the antiviral function of Ifit1 by inhibiting binding to viral RNA (Hyde et al., 2014). The thermodynamic stability of the 5′-terminal stem-loop structure contributes to Ifit1 antagonism, as VEEV and SINV mutants containing less stable structures became more susceptible to Ifit1 restriction. Ifit1 restricts viral replication, in part, by competing with host translation factors eIF4E and eIF4F for binding to viral RNA (Kumar et al., 2014).

3.5. Knowledge gaps and future directions in 5′ UTR structure/function

In summary, the 5′ UTRs of alphavirus RNAs have major roles in promoter function, translational initiation, translational shut-off and avoidance of cellular innate immune mechanisms. This is an impressive feat for a very short (85 bases or less in different alphaviruses) untranslated region. Given this complexity of function, numerous questions remain for future investigation. What is the full array of cellular factors that interact with the alphavirus genomic and subgenomic 5′ UTRs and do they change as infection progresses? As it is clear that structure as well as sequence influences 5′ UTR function, what structure(s) are formed by the alphavirus 5′ UTR in the environment of the infected cell. Approaches such as dimethyl sulfate-based mapping of RNA structure in living cells (Rouskin et al., 2014) may prove valuable for such studies. These structural findings could then be integrated with protein–RNA interaction studies and the analysis of directed mutations to provide a detailed structure–function relationship of regions of the 5′ UTR with translational initiation factors and other cellular proteins. Whether interaction with the abundant viral 5′ UTR also usurps the natural function of these cellular proteins, perhaps by 'sponging', as has been inferred for translation factors by the 5′ UTR of the subgenomic RNA (Patel et al., 2013), also should be explored. Finally, these analyses could be integrated with cellular gene expression changes, viral replication/translation, cytopathology, and pathogenesis studies to provide a comprehensive picture of the influence of this region of viral non-coding RNA in alphavirus biology.

4. Role of the 3′ UTR

4.1. Role of 3′ UTR in viral replication and polyadenylation

For positive sense RNA viruses, synthesis of a negative-strand intermediate is a required step in viral replication. A 19-nucleotide CSE at the end of the alphavirus 3′ UTR immediately prior to the poly (A) tail serves as the promoter for negative strand synthesis (Kuhn et al., 1990; Ou et al., 1981). This CSE is highly conserved in sequence and location in all alphaviruses (Pfeffer et al., 1998). Mutations in the SINV CSE reduced plaque size, and reduced viral replication or induced temperature sensitive mutations (George and Raju, 2000; Kuhn et al., 1990). Specifically, nucleotides −5 to −1 (with −1 representing the last base on the 3′ end of the genomic RNA prior to the poly(A) tail) are required for initiation of negative strand synthesis as mutations reduce the efficiency of negative strand synthesis (Hardy and Rice, 2005). The cytosine at −1 nucleotide has been identified as the site of initiation for negative strand synthesis as mutations or deletions of this nucleotide prevent RNA synthesis (Hardy, 2006; Hardy and Rice, 2005). Insertion of 3 or more uridylate residues in absence of the cytosine at −1 can restore negative strand synthesis (Hardy and Rice, 2005).

The poly(A) tail functions in conjunction with the CSE to support negative strand synthesis as well as efficient translation. A minimum of 11–12 residues in the poly(A) tail is required for efficient production of negative strand RNA (Hardy and Rice, 2005), due to a necessary interaction with the poly (A) binding protein (PABP) (Hardy and Rice, 2005). In mammalian cells, the PABP bound to the poly(A) tail of mRNAs interacts with translation initiation factors bound to the cap structure at the 5′ UTR, resulting in circularization of mRNA and protein translation (Lemay et al., 2010). A similar model of translation of the alphavirus viral genome has been proposed based on the length requirement of the poly(A) tail and requirement of the 5′ UTR in initiation of negative strand synthesis (Frolov et al., 2001; Hardy and Rice, 2005).

In each alphavirus 3′ UTR, there are RSEs that vary in number (2–5 RSEs) and length (18–102 nucleotides) (Ou et al., 1982; Pfeffer et al., 1998). The exact role of the RSEs in viral replication has not been determined for each alphavirus but evidence suggests that they contribute to virus replication in mosquito cells. Deletion of the EEEV RSEs reduces replication in mosquitoes without altering infectivity in mammalian cells (Trobaugh et al., 2014; Weaver et al., 1999). As mentioned above for CHIKV, adaptation to replication in mosquito vectors drives the retention of the RSEs in the 3′ UTR, and deletions in the RSEs result in the poor replication of the Asian lineages in mosquito vectors (Chen et al., 2013). The RSEs also may contain repeated miRNA or protein binding sites (see below) that regulate viral replication in mosquito cells (Trobaugh et al., 2014).

Polyadenylation of newly synthesized cellular mRNA occurs in the nucleus immediately after transcription. For alphaviruses, viral replication occurs in the cytoplasm and separate mechanism exists to polyadenylate the viral RNA (Griffin, 2007). One line of evidence suggests that poly(U) sequences in the 5′ end of the negative strand of SFV result in the addition of a poly(A) tail during synthesis of the subsequent positive strand (Sawicki and Gomatos, 1976). However, subsequent experiments with SINV did not confirm the presence of a sufficient poly(U) sequence in the UTR that would promote addition of a poly(A) tail (Hardy, 2006). Instead, the nsP4 protein, in addition to its RdRp function, contains a terminal adenylyltransferase activity that adds poly(A) residues to the end of the viral RNA (Tomar et al., 2006).

The number of nucleotides between the 19-nucleotide CSE and the last nucleotide at the 3′ end of the viral structural protein genes may not be important for efficient negative strand synthesis. Outside the 19-nucleotide CSE, little conservation is seen between alphaviruses in both nucleotide sequence and length of the UTR (Pfeffer et al., 1998). Deletion of 31–293 nucleotides in the 3′ UTR of SINV has no effect on viral replication in chicken embryonic fibroblasts (Kuhn et al., 1990). Similarly, deletion of 260 nucleotides from the 3′ UTR of EEEV has no effect on viral replication in baby hamster kidney (BHK) or Vero cells (Trobaugh et al., 2014; Weaver et al., 1999). Serial passage of EEEV in BHK cells results in a similar deletion in the 3′ UTR (Weaver et al., 1999) demonstrating that maintenance of this EEEV genome region is due to other selection factors (described below).

4.2. Interaction of the 3′ UTR with cellular miRNAs

MicroRNAs (miRNAs) are small endogenous RNAs that regulate protein translation through inhibition and/or degradation of mRNA. A 7–8 nucleotide seed sequence in the 5′ end of the miRNAs binds to complementary sequences in the 3′ UTR of cellular mRNAs as part of the RNA-induced silencing complex (Bartel, 2009). MicroRNA expression is regulated transcriptionally in a cell-specific manner, resulting in cell-type specific potential interactions with viral RNAs (Landgraf et al., 2007). Since alphaviruses have a positive sense RNA genome that mimics host mRNA, the incoming viral genome has potential to interact with cellular miRNAs, which may provide an important regulatory step in determining cellular tropism and disease pathogenesis.

One example of how a host miRNA can interact with a viral genomic RNA is that of EEEV and the hematopoietic specific miRNA, miR-142-3p. In hematopoietic/myeloid cells, miR-142-3p binds to miR-142-3p binding sites in the 3′ UTR of EEEV, blocking translation of the viral genome (Trobaugh et al., 2014). By blocking viral translation and replication in myeloid cells *in vivo*, miR-142-3p repression minimizes induction of type I IFN and other innate immune effectors and limits prodromal disease. This allows EEEV to replicate essentially undetected by host defense responses, which exacerbates disease in animal models. Limited type I IFN production and prodrome during the early stages of infection is a hallmark

of human EEEV disease and correlates with a poor clinical outcome in children (Silverman et al., 2013). Removal of the miR-142-3p binding sites rescues viral translation and replication in myeloid cells *in vitro* and *in vivo* resulting in enhanced systemic type I IFN production, prodromal signs of disease, and attenuation of the virus *in vivo* (Trobaugh et al., 2014). The interaction of miR-142-3p and EEEV demonstrates how a host miRNA can influence viral replication through cellular tropism, greatly affecting the pathogenesis of RNA viruses. Three of the four miR-142-3p binding sites in the 3′ UTR have perfect seed sequence matches to miR-142-3p, whereas the fourth is considered non-canonical due to one nucleotide mismatch in the seed sequence (Trobaugh et al., 2014). The number of miRNA binding sites, the location of miRNA binding sites in the secondary structure, and seed sequence complementarity all regulate miRNA:mRNA interactions (Peterson et al., 2014). Both canonical and non-canonical seed sequence miRNA binding sites have the potential to restrict translation of viral RNA in a cell-specific manner (Helwak et al., 2013). Including non-canonical binding sites in the prediction algorithms increases the number of potential miRNA binding sites in viral 3′ UTRs. However, the role of non-canonical miRNA binding sites in miRNA-mediated repression of viral RNA has yet to be determined.

Alphavirus 3′ UTRs range from 77 to 609 nucleotides yet only a small fraction of nucleotides including the CSE and HuR binding sites (described in detail in Section 4.4) are essential for replication of some alphaviruses in mammalian cells (George and Raju, 2000; Kuhn et al., 1990; Ou et al., 1981; Pfeffer et al., 1998; Sokoloski et al., 2010; Trobaugh et al., 2014). Clearly, those with longer UTRs have the potential for a greater number of miRNA binding sites. Predictive algorithm analysis of the comparatively long 3′ UTRs of CHIKV or SINV, for example, yields numerous canonical and non-canonical miRNA binding sites. However, the presence of highly specific sequences that result in restriction of RNA viruses presents a conundrum, as viruses should readily mutate to eliminate these sites and enhance replication in some cells. As was the case with EEEV, identification of functional cellular miRNA:viral RNA interactions requires understanding of virus and host biology in addition to identification of binding seed sequences (Gardner et al., 2008; Trobaugh et al., 2014). Most likely, miRNAs, while restricting virus replication in certain cells, will provide a growth advantage for the virus either at the vertebrate organism level or, in the case of arboviruses, in the mosquito vector or reservoir host. In addition, miRNA-virus genome interactions could promote virus replication directly as seen with the hepatitis C virus 5′ UTR (Jopling et al., 2005; Shimakami et al., 2012). Indeed, absent knowing that the lack of a prodrome during EEEV infection is due to restriction of virus genome translation in myeloid cells, the interaction of miR-142-3p and EEEV 3′ UTR may not have been identified (Gardner et al., 2008; Trobaugh et al., 2014). Thus down-regulating the activity of the miRNA systems in single cell types is unlikely to reveal the complex effects of miRNAs on virus tropism and disease (Bogerd et al., 2014).

4.3. Role of 3′ UTR in determining host range of alphaviruses

Increasing evidence suggests the alphavirus 3′ UTR also has an important role in viral replication and adaptation to new replication environments in mosquito and mammalian cells. As discussed above, deletion of 31–293 nucleotides in SINV reduces viral replication in mosquito cells but not in chicken cells (Kuhn et al., 1990). For EEEV, deletion of the 260 nucleotides in the 3′ UTR reduces replication in C6/36 mosquito cells (Trobaugh et al., 2014; Weaver et al., 1999). Also, this region of the 3′ UTR is important for vector infection as infection rates of *Aedes taeniorhynchus* mosquitoes are lower after deletion of the 3′ UTR compared to WT EEEV viruses (Trobaugh et al., 2014). The requirement of the 3′ UTR for efficient mosquito infection suggests a potential mechanism for maintaining sequences in the viral 3′ UTRs and, possibly, specific miRNA binding sites. As yet, there is no evidence that the RNA interference system of mosquitoes facilitates alphavirus replication. As discussed in depth in Section 2.2 above, similar conclusions have been made by large scale sequencing and mutagenesis analysis of the 3′ UTRs of different CHIKV lineages associated with geographical and temporal changes in virus occurrence (Chen et al., 2013). From these data, Weaver and coworkers have proposed that elements in the 3′ UTR, such as repeated sequences, can be associated with virus adaptation to new reservoir hosts but are primarily dictated by constraints in replication in particular mosquito vectors. Even though miR-142-3p is not expressed in mosquitoes, the fact that a small number of substitution mutations in the miR-142-3p binding sites in EEEV markedly affected mosquito replication competence supports the concept of the importance of 3′ UTR elements in viral replication in mosquitoes.

4.4. Functional implications of 3′ UTR interactions with cellular proteins

As mentioned above, the 3′ UTR of alphaviruses can interact with the cellular HuR and La proteins (Sokoloski et al., 2010; Pardigon and Strauss, 1996). Although the functional implications of the interactions with the cellular La protein are unknown, binding of the cellular HuR protein to high affinity sites in the alphavirus 3′ UTR region impacts both cellular and viral gene expression during infection. Most alphaviruses, except CHIKV, o'nyong-nyong virus (ONNV), and Ross River virus (RRV), contain a U-rich sequence (~40 nucleotides in length) preceding the 19-nucleotide CSE that is the site of interaction with the cellular protein, HuR. This interaction stabilizes the viral RNA in the cytoplasm in mammalian and mosquito cells preventing deadenylation and viral RNA decay (Dickson et al., 2012; Garneau et al., 2008; Sokoloski et al., 2010). In ONNV and RRV that lack a U-rich sequence in their 3′ UTRs, HuR protein interacts with the RSEs with high affinity through alternative sequence elements (Dickson et al., 2012). In addition, HuR becomes sequestered in the cytoplasm by interactions with the 3′ UTRs of these highly expressed viral transcripts (Dickson et al., 2012; Barnhart et al., 2013). This 'sponging' of cellular HuR protein impacts post-transcriptional regulation of gene expression in infected cells. Cellular mRNAs that normally rely on HuR protein for stability are destabilized and fail to accumulate. In addition, nuclear processes such as splicing and polyadenylation that are influenced by HuR binding are dysregulated in infected cells. These effects are due primarily to the viral 3′ UTR and can be reproduced by transfection of RNAs containing the SINV 3′ UTR with no additional viral factors (Barnhart et al., 2013). Thus, the presence of large amounts of an alphavirus 3′ UTR in infected cells can cause substantial changes in cellular gene expression that likely impact cellular homeostasis. Whether additional cellular factors are sponged/sequestered by the alphavirus 3′ UTR remains to be determined.

5. Conclusions

The 5′ and 3′ UTRs in alphavirus RNA transcripts have important and diverse roles in the virus life cycle and influence key aspects of viral pathogenesis. Recent work has expanded our understanding of these non-coding regions beyond the traditional areas of viral promoter function and translational regulation into aspects of immune evasion, host cell tropism, and viral pathogenesis. As our knowledge of the role of RNA-based regulatory mechanisms in cells increases, it is likely that our appreciation of how alphavirus UTRs interface with, usurp, or disenable these mechanisms will increase as well.

Acknowledgements

S.C.W. is supported by NIH grants AI071192 and AI069145, and grant DEAC5207NA27344-DTRA from the Defense Threat Reduction Agency. W.B.K. is supported by NIH grants R01 AI095436 and R21 AI111115. M.S.D. is supported by NIH grants U19 AI083019, R01 AI104972, and R01 AI104002. J.W. is supported by U54 AI065357 and NSF MCB-1301983.

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