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In silico structure analysis of alphaviral RNA genomes shows diversity in the evasion of IFIT1-mediated innate immunity

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The IFIT (interferon-induced proteins with tetratricopeptide repeats) family constitutes a major arm of the antiviral function of type I interferon (IFN). Human IFIT1, the earliest discovered member of this family, inhibits several viruses of positive-strand RNA genome. IFIT1 specifically recognizes single-stranded RNA with canonical 7-methylguanylate cap at the 5′ end (Cap0), and inhibits their translation by competing with eIF4E (eukaryotic initiation factor 4E), an essential factor for 5′Cap recognition. Recently, a novel viral mechanism of IFIT1 suppression was reported, in which an RNA hairpin in the 5′ untranslated region (5′UTR) of the viral genome prevented recognition by IFIT1 and enhanced virus growth. Here, I have analyzed the *in silico* predicted structures in the 5′UTR of the genomes of the *Alphaviruses*, a large group of enveloped RNA virus with positive-sense single-stranded genome. The results uncovered a large ensemble of RNA secondary structures of diverse size and shape in the different viruses, which showed little correspondence to the phylogeny of the viruses. Unexpectedly, the 5′UTR of several viral genomes in this family did not fold into any structure, suggesting either their extreme sensitivity to IFIT1 or the existence of alternative viral mechanisms of subverting IFIT1 function.

Keywords. 5′Cap; 5′UTR; IFIT1; methyltransferase; positive-sense RNA; RNA fold; RNA virus

1. Introduction

The antiviral activity of interferon (IFN) results from its ability to induce the expression of a large number of cellular genes, collectively termed ‘interferon-inducible genes’ (ISGs) (Schneider *et al.* 2014). The IFIT family genes constitute one of the most highly induced ISGs, of which IFIT1 is the first discovered and best studied member with strong inhibitory effect on diverse viruses (Wathelet *et al.* 1986; Guo *et al.* 2000; Fensterl and Sen 2011; Vladimer *et al.* 2014; Fensterl and Sen 2015). Understandably, the antiviral mechanism of IFIT1 and its potential suppression by RNA viruses have been subjects of intense research. Collective evidence suggests that IFIT1 inhibits translation primarily by binding to 5′-capped RNA and competing out eIF4E, the rate-limiting subunit of the eukaryotic translation initiation complex (Kumar *et al.* 2014). Recent studies have shown that IFIT1 specifically recognizes the 7-methylguanylate cap (Cap0) structure, required for translation initiation on essentially all eukaryotic mRNA (Daffis *et al.* 2010; Kimura *et al.* 2013; Kumar *et al.* 2014; Abbas *et al.* 2017). Additional RNA-binding studies and recent crystal structures revealed that optimal IFIT1 binding also requires four nucleotides of single-stranded RNA following the 5′Cap0, and that methylation at the 2′ positions of ribose in the adjoining nucleotides (often referred to as N1 and N2), generating Cap1 or Cap2 structures, prevents IFIT1 binding (Kumar *et al.* 2014; Abbas *et al.* 2017). Nuclear methyltransferases (MTases) of the eukaryotic cell catalyzes 2′-O-methylation of the cellular mRNA, which also shields the mRNA from self-recognition by IFIT1 in the cytoplasm (Daffis *et al.* 2010; Kumar *et al.* 2014). Several cytoplasmic RNA viruses, recently exemplified by West Nile virus (WNV) have imitated this strategy to protect their own RNA (Daffis *et al.* 2010); however, as their RNA resides exclusively in the cytoplasm, they cannot use the host nuclear MTase. These viruses, therefore, encode a viral MTase that 2′-O-methylates the viral RNA in the cytoplasm and protects viral translation from inhibition by IFIT1 (Daffis *et al.* 2010). Interestingly, the alphaviruses lack such a MTase activity, and therefore, contain unmethylated Cap0 RNA genome (Griffin 2007). Nonetheless, these viruses are still relatively resistant to IFIT1 and the antiviral effects of IFN.

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Pioneering studies with pathogenic strains of Venezuelan equine encephalitis virus (VEEV), an emergent alphavirus, indicated that its IFIT1-resistance is due to the formation of a cap-proximal RNA hairpin in the 5′UTR of the viral genome, which prevented IFIT1 binding (Hyde *et al.* 2014).

In view of the importance of alphaviruses in public and animal health (Griffin 2007; Suhrbier *et al.* 2012; Weaver and Forrester 2015), I have interrogated the generality of this viral evasion mechanism by analyzing the *in silico* folding potential of all known alphaviral RNA 5′UTR sequences, using a universally accepted RNA structure analysis tool, which was also used in the VEEV study mentioned above (Hyde *et al.* 2014). These studies revealed that the majority of these sequences could indeed generate thermodynamically stable RNA folds; however, notable exceptions were found, which called for alternative mechanisms of IFIT1 evasion.

2. Materials and methods

2.1 *Virus sequence retrieval*

A comprehensive list of all alphaviruses and their taxonomic branches (table 1) was compiled from multiple sources, including published papers alphavirus phylogenomics (Powers *et al.* 2001; Luers *et al.* 2005; Forrester *et al.* 2012), GenBank (using related search terms such as Togaviridae, alphavirus and individual virus names followed by search for similar sequences), Wikipedia, Microbewiki, ICTV and UniProt. All sequences were obtained from GenBank (NCBI), and the 5′UTRs were retrieved as the full RNA sequence upstream of the AUG start codon of the first coding sequence, that of the polyprotein, nsp1234.

2.2 *RNA structure prediction*

The RNA structural folds were determined essentially as described recently (Barik 2017). In brief, computations based on minimum free energy (MFE) (Mathews *et al.* 2004) was used, since this was the exact suite that was used in the previous study that found the RNA hairpin in question (Hyde *et al.* 2014). Folding was performed in the RNAstructure suite, version 5.8.1 (University of Rochester) (Reuter and Mathews 2010), using the maximum % energy difference of 10 and window size 0. The resultant CT files were drawn into secondary structures using the Java applet VARNA (Visualization Applet for RNA) (Darty *et al.* 2009). Additionally, independent confirmation was obtained by using the RNA Mfold web server (Zuker 2003), although all RNA folding suites currently share the modified Turner parameters or some variations thereof (Mathews

Table 1. Major members of the *Alphavirus* genus of positive-strand RNA viruses

Name of complex	Abbreviation	GenBank Acc#
Classified alphaviruses		
Barmah forest virus complex		
Barmah forest virus	BFV	NC_001786.1
Eastern equine encephalitis complex		
Eastern equine encephalitis virus	EEEV	NC_003899.1
Madariaga virus	MRV	NC_023812.1
Middelburg virus complex		
Middelburg virus	MIDV	NC_024887.1
Ndumu virus complex		
Ndumu virus	NDUV	NC_016959.1
Semliki forest virus complex		
Chikungunya virus	CHIKV	NC_004162.2
Mayaro virus (Una)	MAYV	NC_003417.1
Una virus	UNAV	Sequence incomplete
O'Nyong Nyong virus (Igbo-Ora)	ONNV	NC_001512.1
Ross river virus (Bebaru, Getah, Sagiama)	RRV	NC_001544.1
Bebaru virus	BEBV	NC_016962.1
Geta virus	GETV	NC_006558.1
Sagiyama virus	SAGV	AB032553.1
Semliki Forest virus (Me Tri)	SFV	NC_003215.1
VEEV complex		
Cabassou virus	CABV	Sequence incomplete
Everglades virus	EVGV	AF075251
Mucambo virus	MUCV	Sequence incomplete
Pixuna virus	PIXV	AF075256
Trocara virus (Bijou Bridge)	TRCV	Sequence incomplete
VEEV	VEEV	NC_001449.1
Western equine encephalitis complex		
Aura virus	AURV	NC_003900.1
Sindbis virus	SINV	NC_001547.1
Babanki virus (Sindbis-like)	BBNV	HM147984
Kyzylagach virus (Sindbis-like)	KZV	KF981618.1
Ockelbo virus (Sindbis-like)	OKV	M69205.1
Whataroa virus	WHAV	NC_016961.1
Fort Morgan virus	FMV	NC_013528.1
Buggy Creek virus (FMV-like)	BCV	HM147986.1
Highlands J virus	HJV	NC_012561.1
Western equine encephalitis virus	WEEV	NC_003908.1
Unclassified alphaviruses		
Eilat virus	ELV	NC_018615.1
Salmon pancreatic disease virus	SPDV	NC_003930.1
Sleeping disease virus	SDV	NC_003433.1
Southern elephant seal virus	SESV	NC_016960.1
Taï forest virus	TFV	NC_032681.1
Tonate virus	TNV	Sequence incomplete

The *Alphavirus* genus is classified into seven antigenic 'complexes' (Powers *et al.* 2001; Luers *et al.* 2005; Forrester *et al.* 2012), and a few unclassified viruses, that has not yet been assigned into any 'complex'. 'Sequence incomplete' means that the genome sequence of the virus is described as 'partial' in the GenBank, which is often reflected by the lack of the polyprotein start codon (AUG) at the 5′ end of the genome. The 5′UTR of the three underlined viruses did not fold into any secondary structure (supplementary material 2).

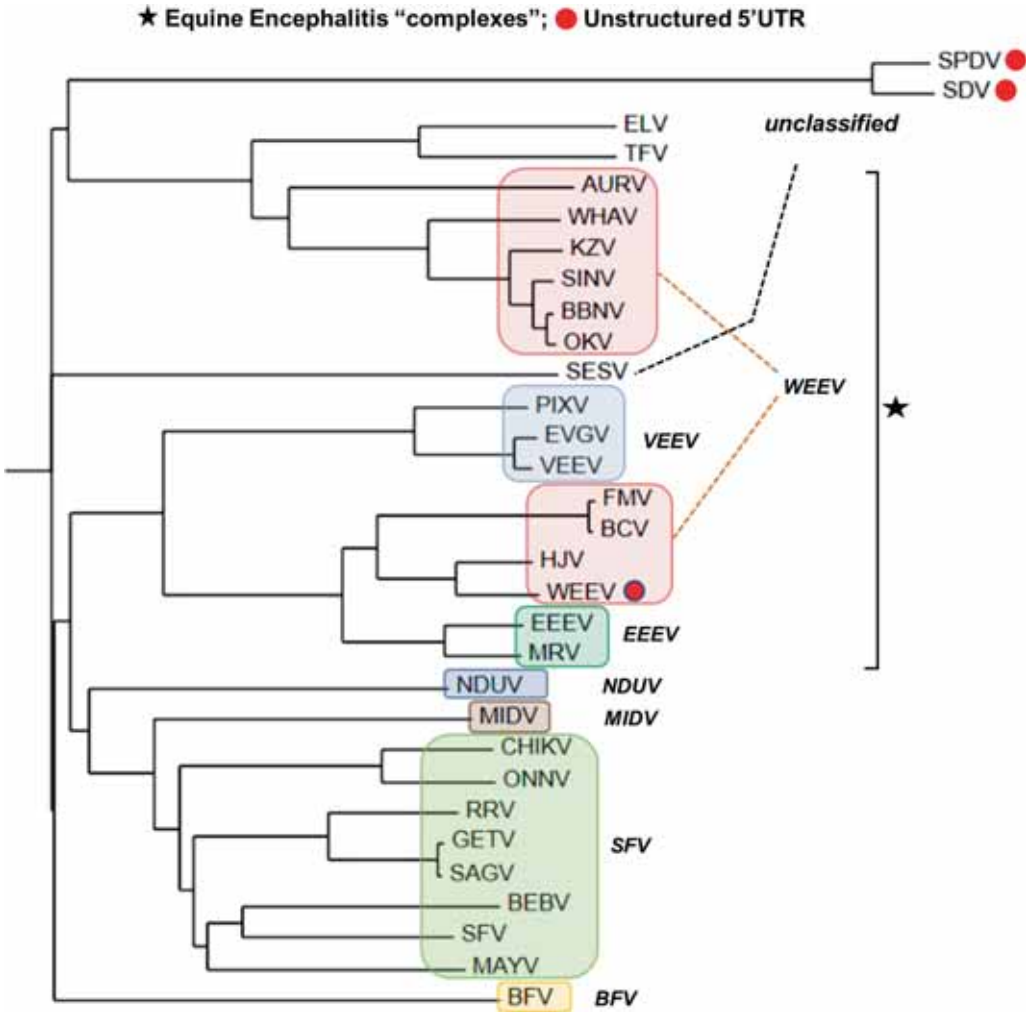

Figure 1. Close similarity among alphavirus nsp1 sequences. The close relationships are displayed as a rooted phenogram generated from multiple alignment of nsp1 amino acid sequences (supplementary material 1), as described in Materials and methods. Individual amino acid sequences, showing the conservation of amino acids, essential for the capping function, are detailed in supplementary material 1. The abbreviations of the viral names are listed in table 1, which also lists the various antigenic 'complexes' that are color-coded here. The three alphaviruses with unstructured 5′UTR (see figure 3) are marked by red dots.

et al. 2004; Seetin and Mathews 2012). In all cases, the two programs generated the same structure.

2.3 *Prediction of ubiquitination sites*

The putative ubiquitination sites in IFIT1 sequences were identified using three different web-based programs, available at the following sites: *http://bdmpub.biocuckoo.org/prediction.php*, *http://csb.cse.yzu.edu.tw/UbiSite/predict.php*, *http://iclab.life.nctu.edu.tw/iclab_webtools/ESAUbiSite/*.

While the first two use the Bayesian discriminant method (Xue *et al.* 2006), the third uses an evolutionary screening algorithm (ESA) to select effective negatives among non-validated ubiquitination sites and an ESA-based prediction method (Wang *et al.* 2017).

3. Results

3.1 *Members of the Alphavirus genus and their conserved capping enzyme sequence*

Alphavirus is the largest genus in the *Togaviridae* family that belongs to group IV of the Baltimore scheme of virus classification (Powers *et al.* 2001; Maclachlan *et al.* 2010). Their genome is linear, single-stranded, non-segmented, positive sense RNA, of ∼12 kb in length, with a 5′Cap0 structure and 3′poly(A) tail. The genome is translated by the cap-dependent translational machinery of the host, which includes eIF4F mentioned earlier. As listed in table 1, the *Alphavirus* genus is comprised of 30 known viruses, but six additional viruses, listed here as 'unclassified', are also currently considered alphaviruses although their exact

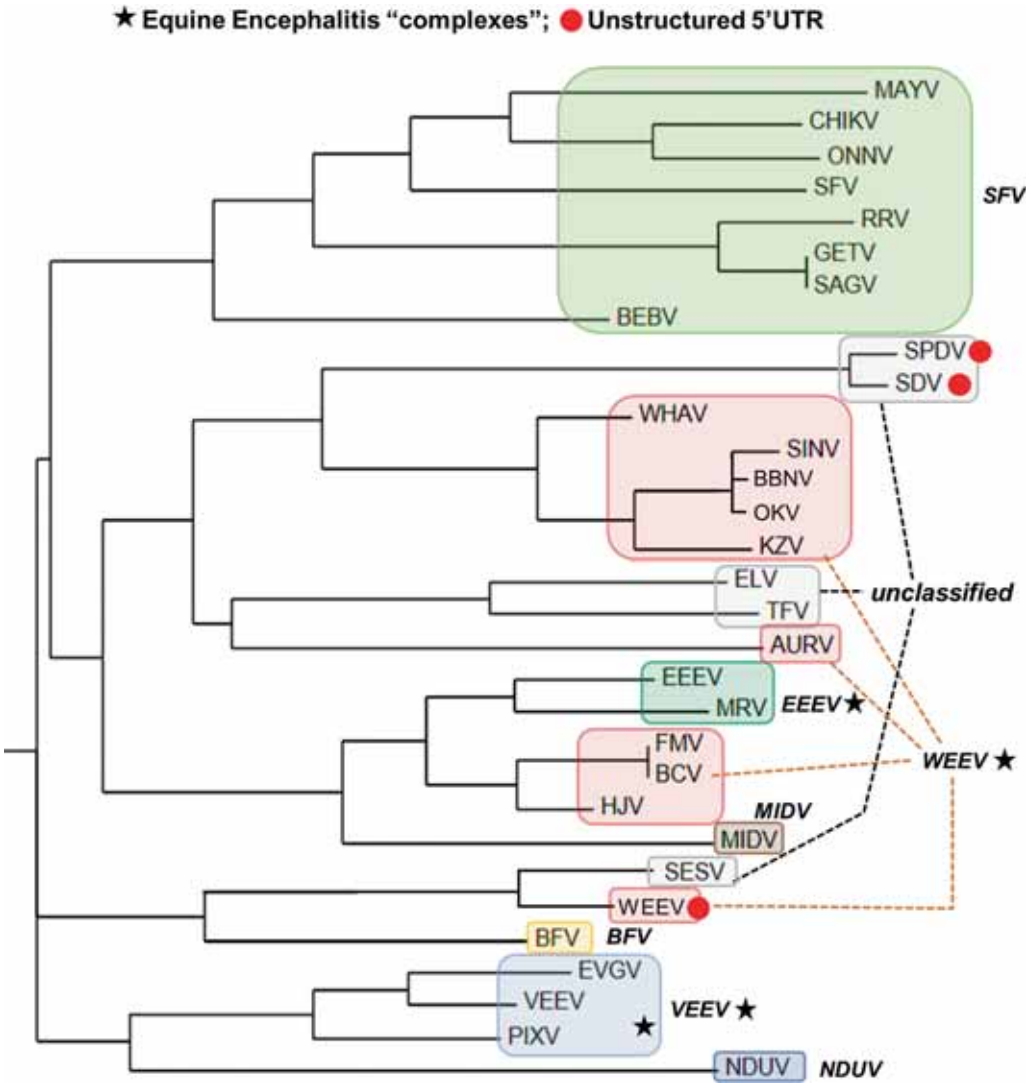

Figure 2. Relationship among the 5′UTR sequences of alphaviral genomes. Multiple alignment of the 5′UTR nucleotide sequences (supplementary material 2) and the resultant plot were done as described in Materials and methods. As in figure 1, the 'complexes' are color coded and the red dots indicate unstructured 5′UTR (see figure 3).

phylogenetic placement awaits further characterization (Powers *et al.* 2001; Luers *et al.* 2005; King *et al.* 2012).

The initial assignment of the alphaviruses into 'complexes' was based on antigenic reactivity (table 1) (Powers *et al.* 2001; Luers *et al.* 2005; Forrester *et al.* 2012), determined primarily by virion surface glycoproteins rather than genome sequence homology. Moreover, the 5′ cap structure or its methylation status has remained determined for most alphaviruses. Since the postulated role of the alphaviral 5′UTR hairpin is to inhibit viral Cap0-dependent translation, it was important to at least determine if all alphaviruses in fact encode methyltransferase sequences, necessary to generate Cap0. Without the Cap0 structure, IFIT1 will not be recruited in the first place, and therefore, there would be no need for the 5′UTR hairpin anyway. In the alphaviral genome, the first coding sequence at the 5′ end codes for a polyprotein (nsp1234), which undergoes self-cleavage, forming the individual polypeptides (Griffin 2007). Of these, the most N-terminal polypeptide (nsp1) encodes both guanylyltransferase and methyltransferase activities, responsible for 5′-capping of the newly transcribed RNA, thus generating the Cap0 structure mentioned earlier.

The conceptually translated nsp1 sequences of all alphaviruses were subjected to multiple sequence alignment by ClustalW. The results (supplementary material 1) reveal that all 15 catalytically important residues that were experimentally demonstrated to be essential for capping activity (Li *et al.* 2015), and found in VEEV nsp1, are also conserved in all other alphaviruses, even in those that have remained antigenically 'unclassified' (table 1), concurring with an essential role of this capping enzyme in alphaviral translation. The phylogenetic tree (figure 1), generated from the multiple alignment, also revealed a high degree of overall sequence similarity among all nsp1 proteins, in spite of their diverse antigenic nature (table 1). The phenogram also revealed that

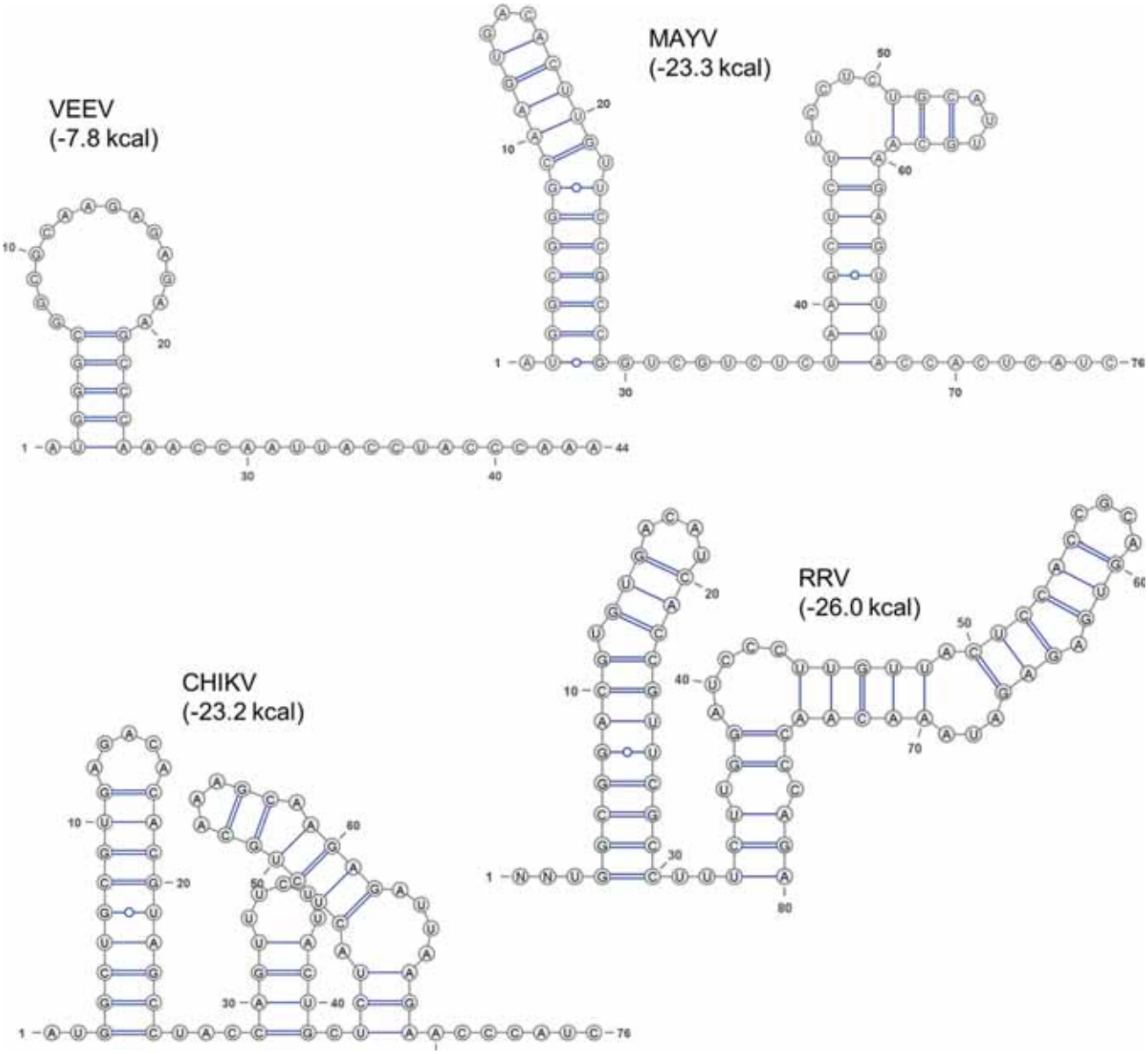

Figure 3. Representative alphavirus 5′-UTR structures. The 5′UTR sequence was folded *in silico* and displayed as described in Materials and methods. The thermodynamic ΔG values for each structure are shown within parentheses. Only four structures are shown here to illustrate the variety of folds; all structures are presented elsewhere (supplementary material 3).

all equine encephalitis viruses – Eastern, Venezuela and Western – were in a single large cluster (figure 1; marked with asterisk), even though they were antigenically in separate branches (table 1). Overall, these results strongly suggested the presence of a functional capping enzyme in all alphaviruses and their close phylogenetic relationship, against which the 5′UTR sequences were then compared.

3.2 *In silico predicted RNA secondary structures of alphavirus 5′UTR*

Having shown that all alphavirus genomes encode an authentic and conserved capping enzyme sequence, I then tested if they also contain 5′UTR sequences similar to the one shown for VEEV (Hyde *et al.* 2014). I first compared the primary structures of the alphavirus 5′UTRs in multiple alignment (supplementary material 2), which clearly revealed that the sequences are in fact highly diverse. The phenogram plot (figure 2) also indicated substantial diversity, although a few clusters could be recognized, notably the WEEV, SFV complexes. Thus, the primary structures of the 5′UTR provided little clue to the relatedness of the viruses for innate immune evasion, which led to examination of their secondary structures.

To this end, the 5′UTR sequence of all alphaviruses were subjected to minimum free energy-based folding *in silico*, using the same algorithm previously used for VEEV

Table 2. Predicted secondary structures in *Alphavirus* 5′UTRs: distance from the genome 5′-terminus and thermodynamic stability

Virus name (abbrv.)	Distance from 5′-terminus (nt)	ΔG (kcal/mol)
SPDV, SDV	No structure	NA
ELV	2	− 10.6
TFV	2	− 8.3
AURV	1	− 15.3
WHAV	0	− 11.5
KZV	0	− 4.3
SINV	0	− 6.7
BBNV, OKV	0	− 11.9
SESV	1	− 9.9
PIXV	0	− 8.2
EVGV	1	− 8.6
VEEV	1	− 7.8
FMV, BCV	1	− 12.3
HJV	1	− 8.1
WEEV	No structure	NA
EEEV	3	− 12.9
MRV	0	− 9.0
NDUV	1	− 12.2
MIDV	2	− 13.4
CHIKV	2	− 23.2
ONNV	2	− 25.1
RRV	3	− 26.0
GETV, SAGV	2	− 22.1
BEBV	1	− 22.2
SFV	1	− 19.7
MAYV	2	− 23.3
BFV	1	− 11.8

Virus names are in order of their location in the phylogenetic tree, based on nsp1 amino acid sequence homology (figure 2). NA = not applicable, since there is no structure.

procedures, as described in Materials and methods. In addition, it was tempting to test the Rubella virus 5′UTR, as it is the lone member of the sister Rubivirus genus in the *Togaviridae* family, also with a positive-sense genome, likely capped by 5′Cap0 structure. To conserve space, only a few representative structures are displayed (figure 3), but all structures are presented elsewhere (supplementary material 3) and their major features tabulated (table 2). As shown, the majority of the alphaviruses (and the rubivirus) generated thermodynamically stable secondary structures in their 5′UTR (table 2); however, there was little or no structural similarity among them, and the Δ*G* values also differed widely. It is notable that all structures were very close to the 5′ end, ranging only 0–3 nucleotides away from the 5′ end. This must play a critical role in IFIT1 evasion since crystal studies have clearly shown that IFIT1 requires four nucleotides of cap-proximal RNA for efficient binding and hence to compete out eIF4E (Abbas *et al.* 2017). However, the RNA-binding pocket of IFIT1 exhibits some adaptability (Abbas *et al.* 2017), and therefore, it is a curious question whether RNA structures that are three nucleotides away, namely in EEEV and RRV, are only partially effective in inhibiting IFIT1, i.e. whether these two alphaviruses exhibit an intermediate level of IFN sensitivity. Lastly, and most importantly for this paper, three 5′UTRs, namely those of SDV, SPDV and WEEV, did not produce any secondary structure from the retrieved sequences, as listed (supplementary material 2); in fact, none could be generated for these viruses using up to 200 nt of RNA sequence from the 5′ end, which included substantial lengths of the nsp1 coding sequence. In WEEV, only when ≥300 nt of 5′ RNA sequence was used, nt 2–4 hydrogen bonded with nt 294–296 to form a complex structure of questionable significance (data not shown). In conclusion, these three alphaviral 5′UTRs did not generate any RNA structure using the exact algorithm that generated RNA folds for other alphaviral 5′UTRs, including VEEV, in which the predicted RNA fold and its function could be confirmed and functionally validated (Hyde *et al.* 2014).

4. Discussion

The main conclusion in this short bioinformatic paper is that the RNA secondary structural fold, originally found in the 5′UTR of VEEV and shown to prevent IFIT1 binding (Hyde *et al.* 2014), is not exactly conserved in all alphaviruses. In fact, in three alphaviruses, the analysis did not generate any RNA fold. Although the results presented here were derived from *in silico* studies, they were based on established procedures and with VEEV as the 'control' virus. It is hoped that these studies will generate interest in comprehensive experimental analysis of 5′UTR RNA structure of multiple alphaviruses, using approaches similar to those used for VEEV (Hyde *et al.* 2014), such as determination of RNA structure by chemical probing (Smola and Weeks 2018), combined with IFIT1-binding and translational reporter assays. One should also bear in mind that variants of these sequences may occur in nature, especially due to the high mutational rate of RNA viral genomes, and such variants may have diverse RNA folds that may or may not bind IFIT1. Nevertheless, the simplest corollary of the current analysis is that alphaviral evasion of translational inhibition of IFIT1 by viral RNA secondary structures may not apply equally to all members of this genus. To put the implications of this finding in proper perspective, I present a schematic model highlighting the mutually exclusive interaction of the alphaviral genome 5′UTR with either IFIT1 or eIF4E, while focusing on the kinetics of the choice between the two proteins (figure 4). To start with, the majority of the input (incoming) alphavirus genomes, because of their 5′UTR secondary structure, will not be hindered by IFIT1; however, since eIF4E binding does not require an interaction with the cap-downstream RNA sequence (Marcotrigiano *et al.* 1997), the genome will be readily engaged by eIF4E and hence translated (figure 4A). The minority of viral genomes with unstructured 5′UTR (figure 1) can be engaged by either protein, the proportion being dependent on the relative binding efficiency and kinetics, which is largely unknown at this point. Lastly, the *de novo* synthesized nascent genome

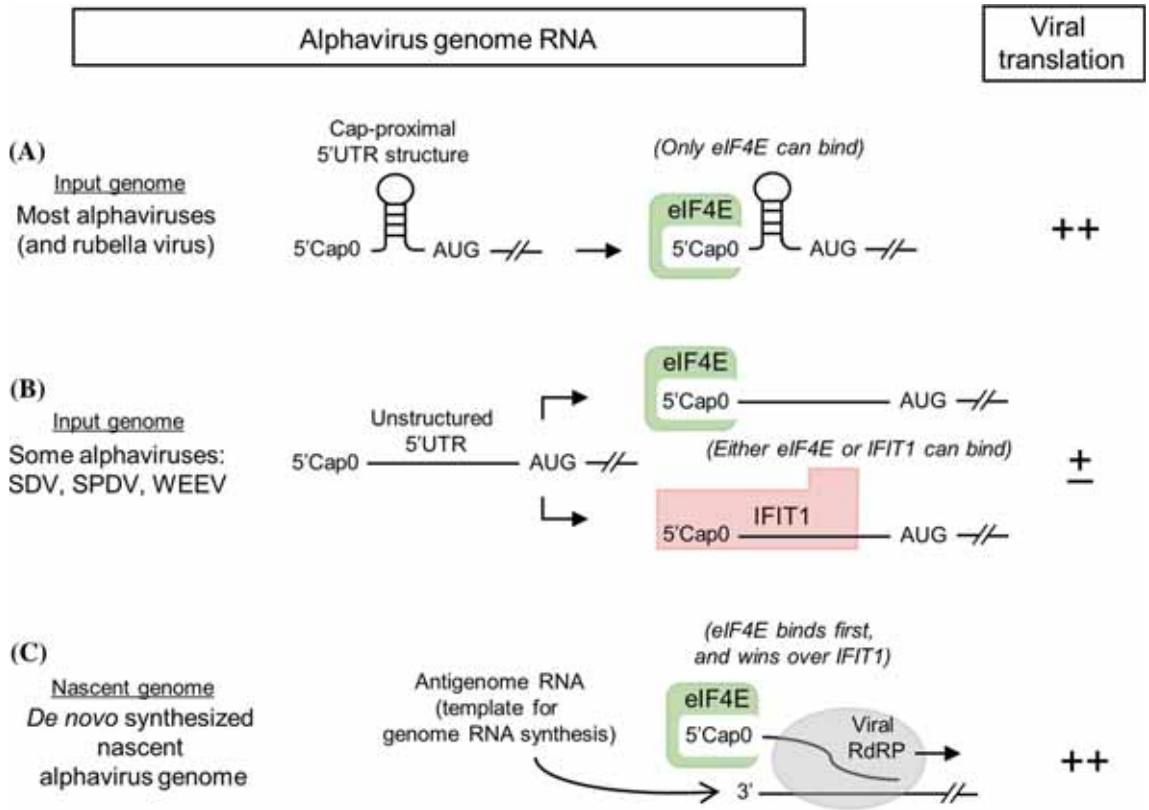

Figure 4. Schematic model for mutually exclusive binding of IFIT1 or eIF4E to alphaviral genome RNA 5′ end. Preference for eIF4E and IFIT1 binding are shown for alphaviral genomes with (**A**) structured or (**B**) unstructured 5′UTR. The nascent 5′ end of the viral genome (**C**) may recruit eIF4E only; in this panel, the viral RdRP is shown along with its direction of movement on the antigenome template. The plus or minus signs are qualitative indicators of the extent of viral translation. The rationale and outcome of these interactions are detailed in Results.

presents an interesting scenario for kinetic competition between eIF4E and IFIT1 (figure 4C). Since eIF4E only requires the 5′cap structure to engage (Marcotrigiano *et al.* 1997), whereas IFIT1 requires four additional nucleotides of the downstream RNA (Abbas *et al.* 2017), eIF4E will engage the nascent genome first, when the cap has just emerged out of the leading RNA-dependent RNA polymerase (RdRP). Note that the engagement of eIF4E and IFIT1 is mutually exclusive, as both proteins cannot bind simultaneously to the same RNA end. Thus, regardless of the ability of 5′UTR to form a secondary structure, all *de novo* synthesized alphaviral genome will be translated (figure 4C). Once eIF4E engages, downstream RNA structures will not present a translational block because of the RNA helicase activity of the initiation complex, particularly that of the eIF4A subunit (Andreou and Klostermeier 2013). Subsequent rounds of initiation will physically cover the RNA with translating ribosomes, generating the polysome, further consolidating the translational take over and exclusion of IFIT1.

It is thus conceivable that the relevance of the 5′UTR structure in IFIT1 interaction is only an issue for the input alphaviral genome, but not for the newly made genome. However, *de novo* synthesis of viral proteins, as a rule, is needed for large-scale viral genome replication, and thus, the initial success of IFIT1 over eIF4E will essentially shut off the entire viral life cycle, explaining the strong antiviral effect of IFIT1 and underscoring the importance of its evasion by the 5′UTR RNA structure (Hyde *et al.* 2014).

The alphaviruses with unstructured 5′UTR may in fact fail to evade IFIT1 and hence exhibit extraordinary sensitivity to IFN, which will be known only when their sensitivity is quantified and directly compared to a virus with well-characterized IFIT1-evasive role of 5′UTR, such as VEEV. Alternatively, these viruses may have evolved novel mechanisms to subvert IFIT1, such as ribose 2′-O-methylation of the Cap0 to generate Cap1 or Cap2 structures that are known to be IFIT1-resistant (Daffis *et al.* 2010; Abbas *et al.* 2017), in which case these viruses may need to be reclassified. It is also unlikely that these alphaviruses use the currently known cap-independent translation initiation mechanisms, such as a covalently attached viral protein or specific 5′ RNA structures known as IRES (internal ribosome entry site), found in picornaviruses (Martínez-Salas *et al.* 2008).

Finally, several RNA viruses of the *Paramyxoviridae* family are known to directly target the cognate inhibitory innate immune factor. For example, pneumoviruses promote

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hIFIT1  (211) RLNPDNGYIKVLLALKLQDEGQEAEGE
mIFIT1b (202) RLNPEDPYLKVLLALKLQDLGEHVEAE
              ****:: *:********** *...*.*
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Figure 5. Predicted conserved ubiquitination site of mouse and human IFIT1. The conceptually translated amino acid sequences of human (hIFIT1) and mouse IFIT1 (mIFIT1b) (Daugherty *et al.* 2016) were obtained from GenBank (accession numbers NP_001539.3 and NP_032357.2, respectively). The ubiquitination sites were predicted as described in Materials and methods, and the single highest-scoring common site – Lys226 in human and the equivalent Lys218 in mouse – is indicated in bold. The conserved residues were marked using Clustal Omega alignment.

sequestration and/or ubiquitin-mediated proteasomal degradation of several IFN-pathway proteins (Ling *et al.* 2009; Swedan *et al.* 2009; Dhar and Barik 2016), and many rubulaviruses and morbilliviruses promote degradation of STAT proteins (Horvath 2004). In contrast, it is currently unknown whether any member of the IFIT family is regulated by ubiquitination or degradation. Covalent linkage with ISG15, a ubiquitin-like protein, has been demonstrated for all four human IFIT isoforms, including IFIT1 (Zhao *et al.* 2005), but the physiological function of this modification has remained unknown. In an *in silico* attempt to explore ubiquitination of IFIT1, I used three major web-based prediction algorithms for ubiquitination sites, and one particular residue, namely Lys226, scored highly in all three predictions (figure 5). As shown, this residue was also highly conserved in a number of species, including mouse and human. Clearly, further research is needed to establish if IFIT1 is a substrate of ubiquitin-based proteasomal system and whether this is co-opted by viruses to evade the host's innate immune response.

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