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Structural Mechanisms and Therapeutic Solutions of the SARS-CoV2 Frameshifting Element

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ABSTRACT

The Severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV2) has caused the coronavirus disease 19 (COVID-19) worldwide pandemic. Despite the relative success of the various vaccines developed, emerging variants such as the delta and omicron strains present the need to find a more effective therapeutic solution. One such target is the frameshifting element, which is responsible for controlling the balance between proteins necessary both for defending against the host's immune responses and for reproduction of the retrovirus. As it is one of the most highly conserved sequences in all strains of SARS-CoV2, the purpose of this review article is to summarize what is known about the structural and functional mechanisms of the frameshifting element, and the current advancements towards developing therapeutic solutions to this attractive target.

Introduction

Since November of 2019, the severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV2) has caused the coronavirus disease 19 (COVID-19) worldwide pandemic. As of late May 2022, there have been nearly 530 million total cases that have been reported worldwide, and well over six million reported deaths (CSSEGISandData. n.d.). As the cases and death count continue to rise, the development of vaccines has had a high success when given to the populous. It is reported that out of the 3 most taken vaccines, the Pfizer vaccine is 95% effective at preventing infection of COVID-19, the Moderna vaccine has recently dropped from 95% to 90% effectiveness since December of 2020, and the Johnson & Johnson vaccine has only shown 66.3% effectiveness (Center for Disease Control and Prevention, n.d.; Katella, 2022).

However, the emergence of new variants of SARS-CoV2 such as the delta and omicron variants have caused the effectiveness of all vaccines to drop, having forced researchers to develop booster shots to nullify the new variations found within these new strains of COVID-19. As vaccines are only preventative measures, there are very limited solutions and options for treatment outside of ventilators, experimental drugs, and quarantining for those that contract or test positive for COVID-19. Furthermore, while the delta variant's effects may be reduced or nullified by becoming vaccinated, no such protective effects exist for the Omicron variant, clearly highlighting the need for better solutions against newer strains. One potential target is the highly conserved frameshifting element of the SARS-CoV2 genome.

SARS-CoV2 is a positive single-stranded RNA retrovirus with a 30-kb genome (Figure 1). Similar to other coronaviruses, two thirds of its genome consists of 2 open reading frames, ORF1a and ORF1b, which partially overlap one another (Zhang, 2021). Once the retrovirus enters the cell, the ORF1a translates and releases 11 nonstructural polyproteins including RNA-dependent RNA polymerase to disrupt the host cell's immune response, while ORF1b translates and releases 15 replicase enzymes that then stimulate transcription and translation of the virus' genome (Lan, 2022). When the translation of the newly formed ORF1a is finished, a

proportion of translating ribosomes undergo -1 programmed ribosomal frameshifting (-1 PRF) to synthesize proteins of ORF1b.

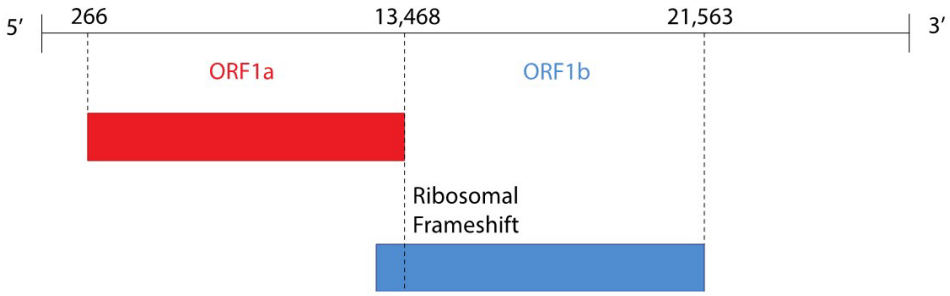

Figure 1. Genome organization of SARS-CoV2

The SARS-CoV2 genome consists of 2 open reading frames and needs to undergo frameshifting in order to produce ORF1b.

The -1 PRF mechanism is stimulated by a structured RNA motif at the 3' end of ORF1a called the frameshift stimulation element (FSE), which pauses elongating ribosomes and directs them to shift their reading frames back one base in the 5' direction to bypass a stop codon at the end of ORF1a and allow for translation of ORF1b. The FSE consists of a 5' heptanucleotide sequence (UUUAAAC) dubbed the "slippery site" where elongating ribosomes pause, followed by a RNA element which is hypothesized to form a three-stem pseudoknot, a structure that forms upon the base-pairing of a single-stranded region in an RNA loop to a stretch of complementary nucleotides elsewhere in the RNA chain. In many viruses other than SARS-CoV2, the mechanical stress that pseudoknots create on elongating ribosomes has been shown to induce frameshifting. (Zhang, 2021; Manfredonia, 2020). Regulation of the frameshifting element is very precise, and small variations in the ribosomal frameshifting rate leads to substantial differences in genomic RNA production and infection of the host cell.

As the frameshifting element is both one of the most highly conserved sequences in all strains of SARS-CoV2 and necessary for its biological functions, the purpose of this review article is to summarize what is known about the structural and functional mechanisms, and the current advancements towards developing therapeutic solutions to this attractive target.

Structural and Functional Mechanisms of the Frameshifting Element

The SARS-CoV2 frameshift stimulation element is composed of multiple elements that are seen to directly interact with one another (Figure 2). The FSE begins with the UUUAAAC slippery site at the 5' end followed by a linker sequence that engages a paused ribosome's exit channel. After this motif, two stems, a long helical stem 1 found in all coronavirus FSEs and a smaller 5 bp helix that surmises stem 2, stack upon one another while a third stem, stem 3, extends outward from this stacked configuration. Additionally, two independent linker sequences exist between stems 1 and 2 and between stems 2 and 3 to allow for structural flexibility within the pseudoknot. As an elongating ribosome begins to unwind stem 1 of the pseudoknot, supercoiling in stem 2 hinders the ribosome's progress so that a point is reached where the forward translation of the ribosome is countered by the unwinding of the pseudoknot because of stem 1 (Giedroc, 2009).

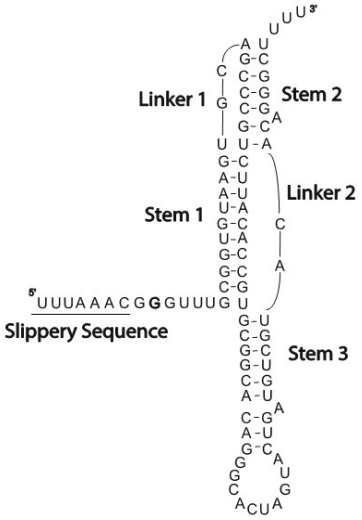

Figure 2. Predicted secondary structure of the three-stemmed pseudoknot

The secondary structure of the predicted three-stemmed pseudoknot consists of 3 stems and 2 linker sequences. The ribosome undergoes frameshifting at the slippery sequence due to the predicted pseudoknot.

Visibly, all SARS-CoV2 strains contain an extended stem 3 because of elongation from the ribosome, but with different orientations of the packing of stems 1 and 2 (Zhang, 2021; Kelly, 2021). This is because stem 3 is not directly stacked with the stem 1-stem 2 regions of the pseudoknot, providing the ability to develop distinct tertiary arrangements of the pseudoknot. One such arrangement from a recent cryo-EM structure shows the orientation of stem 3 positions stems 1 and 2 to form a circular ring with a visually apparent hole in the middle, with a linker sequence of nucleotides that essentially "threads" through the ring such tertiary bonding (Zhang, 2021). The different orientations of the packing of stems 1 and 2 as a result of stem 3's positioning, which along with 5' conformational heterogeneity, causes significant variation amongst the various strands of SARS-CoV2 (Zhang, 2021). This may explain why the SARS-CoV2 3 stem pseudoknot is still theorized, and no exact structure has yet been identified.

Despite the numerous scientists who believe in the 3 stemmed pseudoknot hypothesis, in vivo structural studies using chemical reagents that modify RNA only at single stranded RNA regions show high modifications within helical regions of the 3 stemmed pseudoknot. Therefore, it is possible that other elements within the SARS-CoV2 genome interact with the FSE, providing additional conformations that may be important to stimulating the -1 PRF strategy (Lan, 2022; Omar, 2021). For example, a recent study using dimethyl sulfide (DMS), which modifies adenosines and cytosines in single-stranded or open regions of RNA, shows that a shorter FSE containing a 92-nucleotide region only frameshifts around 17% and folds into the 3-stemmed pseudoknot found by NMR and cryo-EM studies. (Zhang, 2021; Lan, 2022; Bhatt 2021). However, when the ribosome encounters the FSE placed in the middle of an approximately 3000 nucleotides of the SARS-CoV2 native sequence context, frameshifting levels jump drastically in comparison to those of shorter FSE to about 40% (6-Lan2022).

In such longer constructs, DMS modifications occur in stem one of the predicted pseudoknot (6). These results suggest that the predicted pseudoknot structure of the FSE may not be the conformation that causes frameshifting. In addition, other areas of the genome might be involved in controlling frameshifting as the minimal FSE does not seem to recapitulate the mechanism of ribosomal frameshifting on the full-length virus during infection (Lan, 2022). Overall, all the structural studies highlight that the FSE is highly dynamic, and it seems that the conformational heterogeneity of this region is important for -1 PRF. However, the FSE may

not be the only region of the retrovirus which directly contributes to the high-level frameshifting we see in current strains of SARS-CoV2.

Therapeutic Solutions to the Frameshifting Element

It has been reported that a total of 52 drugs increased the -1 PRF frequency based on a recent screening system, with the three most effective drugs being (-)-huperzine A, erythromycin, and trimethobenzamide (Chen, 2020). In addition, fluoroquinolone merafloxacin was recently reported to inhibit -1 PRF of SARS-CoV2 and other betacoronaviruses (Wacker, 2020). However, the mRNA expression in many of these screenings was not changed by the drug treatment itself (Chen, 2020). These results strongly suggest that the -1 PRF frequency of SARS-CoV2 is sensitive to such chemical reagents.

Indeed, the signals emitted by the -1 PRF has been identified as an attractive drug target for a plethora of reasons: 1) there is no known case of -1 PRF promoted by a three-stemmed pseudoknot structure in host cellular mRNAs, 2) the -1 PRF signal is highly conserved with over 97% sequence conservation, and 3) the structure of the pseudoknot is complex enough to contain well defined binding pockets for drug binding (Wacker, 2020). As such, scientists have attempted to create new drugs and develop therapeutic solutions to inhibit the -1 PRF in SARS-CoV2. Many theorize that the use and discovery of mutations, ligands, small molecules, and locked nucleic acids all are potential options that can be used in the creating such drugs.

For scientists who use the pseudoknot as a framework for the development of small molecules and ligands, the general strategy is to attack pockets embedded within the structure of the pseudoknot to then target the -1 PRF signal. One example involves the use of locked nucleic acids (LNAs), RNA nucleotides modified within the ribose ring to prevent degradation by enzymes. These LNAs are antisense agents designed to hybridize against complementary base pair sequences within stems 2 and 3 of the 3-stemmed pseudoknot. Cell-free in vitro frameshifting assays have confirmed that such LNAs reduce frameshifting, although the inhibition was only partial. As a result, research is still being done to define the precise regions within stems 2 and 3 of the pseudoknot for LNA targeting, and to determine the exact concentrations of LNAs required for complete inhibition (Zhang, 2021).

On the other hand, other drug studies have suggested that targeting the 3-stemmed pseudoknot may not be sufficient to suppress frameshifting and other elements outside of the FSE require targeting. One such example is the small molecule 2-methylthiazol-4-ylmethyl)-[1,4]diazepane-1-carbonyl]amino}benzoic acid ethyl ester (MTDB), which is seen to inhibit viral replication. However, frameshifting levels were not affected in both in vitro and in vivo experiments in the presence of MTDB (Wacker, 2020). Additionally, MTDB appears to be resistant to natural variants of the -1 PRF stimulating pseudoknot. Even though interactions with the pseudoknot may not be sufficient to explain MTDB's inhibitory effects, it is possible that it inhibits SARS-CoV2 replication by a different mechanism. There are theories that the inhibition effect is enhanced through the presence of ribosomes, whether it be through direct contact or is ribosome induced (Lan, 2022; Wacker, 2020). Overall, the therapeutic targeting of multiple structural possibilities could be combined to attack multiple structural conformations of the FSE.

Conclusions

Overall, current studies reveal that the FSE of SARS-CoV2 can exhibit multiple conformations both in vitro and in vivo. While the 92 base-paired sequence of the predicted 3-stemmed pseudoknot represents an attractive target due to its highly conserved nature, studies that have used this as a template for drug targeting have not been able to completely ameliorate frameshifting levels. This may be due to the region's potential to form

alternative structures besides the pseudoknot and/or elements within the genome outside of the FSE that have been shown to interact with this sequence. Further studies need to be done to investigate the determinants that lead to variation in the configurations of the FSE, with the end goal being to elucidate the minimal elements required to create the conformationally permissive state(s) that control frameshifting.

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