Unheeded SARS-CoV-2 protein? Look deep into negative-sense RNA

Martin Bartas[1,#,*], Adriana Volná[2,#], Ebbe Toftgaard Poulsen[3], Jiří Červeň[1], Václav Brázda[4], Vladimír Špunda[2,5] & Petr Pečinka[1,*]

[1]Department of Biology and Ecology, University of Ostrava, Ostrava 710 00, Czech Republic

[2]Department of Physics, University of Ostrava, Ostrava 710 00, Czech Republic

[3]Deparment of Molecular Biology and Genetics, Aarhus University, 8000 Aarhus, Denmark

[4]Institute of Biophysics, Czech Academy of Sciences, Brno, 612 65, Czech Republic

[5]Global Change Research Institute, Czech Academy of Sciences, Brno, 603 00, Czech Republic

*Correspondence should be addressed to Petr Pečinka or Martin Bartas

Abstract

SARS-CoV-2 is a novel ssRNA+ virus from the *Coronaviridae* family, which has caused the COVID-19 pandemic. The genome of SARS-CoV-2 is one of the largest of RNA viruses, comprising of 28 known protein-coding loci. This study aims to explore the coding potential of negative-sense RNA intermediate for its potential to contain additional protein coding-loci. We have found several putative ORFs and one very promising locus which we have called *Avo1* (Ambient viral ORF1). *Avo1* locus bona fide coding for 81 amino acid residues long protein and contains a strong Kozak sequence for translation on eukaryotic ribosomes. Altogether, our results suggest the existence of still undescribed SARS-CoV-2 protein, which may play an important role in the viral lifecycle and COVID-19 pathogenesis.

Contact: petr.pecinka@osu.cz; martin.bartas@osu.cz

1 Introduction

Coronavirus SARS-CoV-2, as a causative agent of COVID-19 disease, is intensively studied worldwide [1–3]. It is generally considered that the SARS-CoV-2 genome is coding for 16 non-structural proteins (NSP1-16), 4 structural proteins (surface glycoprotein, membrane glycoprotein, envelope protein, nucleocapsid phosphoprotein), and 6 – 9 "accessory factors" (still designated as "open reading frames", ORFs) [4–6]. The length of the SARS-CoV-2 proteins varied from 1945 aa to 13 aa [7–9]. The shortest SARS-CoV-2 protein has only 13 aa (nsp11) and its function is unknown [10], followed by ORF10 (38 aa) [11], ORF7b (43 aa) [12], ORF6 (61 aa) [13], and envelope protein (75 aa) [3]. The longest SARS-CoV-2 protein is a multi-domain protein nsp3 (1945 aa) [9]. All to date described SARS-CoV-2 proteins are coded by positive-sense viral RNA strand. Overall, positive-sense RNA viruses are generally accepted to encode proteins solely on the positive strand, but in the current work by Dinan et al., they demonstrated that also negative-sense viral RNA strand has the potential to be protein-coding [14]. We have decided to inspect the negative-sense intermediate viral RNA strand of SARS-CoV-2 and look for new potential ORFs. As an indicator for *in vivo* validity, we have chosen a combination of complementary bioinformatics approaches, e.g. the presence of the so-called Kozak sequence, which is considered strong supportive evidence for being translated on host ribosomes [15], transcription factor binding site analysis [16], sequence and domain-based homology searches [17,18], and 3D structure prediction [19]. Our analyses propose a novel SARS-CoV-2 ORF, which has a high probability to be translated in host cells.

2 Methods

The SARS-CoV-2 reference genome (NC_045512.2) was searched by NCBI ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/), to predict all potential ORFs longer than 200 nucleotides (we have chosen an arbitrary threshold of 200 nt to make this analysis feasible and to minimize false-positive results). We have focused especially on the negative strand intermediate (i.e. frames -1, -2, and -3). Using ATGpr web-based tool (https://atgpr.dbcls.jp/) [20], we have inspected, if they contain the so-called Kozak sequence, which is considered an important signal in the 5´end of mRNAs to be translated on eukaryotic ribosomes [21]. To compute Mw and isoelectric point (pI), we used the Expasy Compute pI/Mw tool (https://web.expasy.org/compute_pi/) [22]. For the transcription factor binding site analysis, the RegRNA 2.0. webserver was used [23].

To find possible domain homologs we used NCBI´s Conserved Domains Database (CDD) webserver (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [17] with an E-value cut-off set to 10. For *ab initio* structural modeling, the QUARK tool (https://zhanglab.ccmb.med.umich.edu/QUARK/) was used [19] and the resulting structures were visualized within UCSC Chimera 1.15 workflow [24]. Supplementary materials contain all nucleotide and predicted protein sequences (SM1). Predicted PDB structures are enclosed in SM2. CDD domain hits are included in SM3. Prediction of Kozak sequences is enclosed in SM4. Predicted negative-sense RNA ORFs longer than 200 aa (searched in the whole group of coronaviruses https://www.ncbi.nlm.nih.gov/genome/?term=coronavirus) are enclosed in SM5 together with estimated pI values [22] and N-glycosylation prediction http://www.cbs.dtu.dk/services/NetNGlyc/) [25].

Assessment of Avo1 expression was performed by re-searching LC-MS/MS data from two previously published SARS-CoV-2 studies looking at the infection of human alveolar macrophages [26] and green monkey Vero E6 cells [27]. Data were either search against the human or green monkey SWISS-PROT [28] reference proteomes (Human db: 09-2020, 20609 sequences; Green monkey: 08-2020, 19229 sequences) and the UNIPROT [29] SARS-CoV-2 database (12-2020, 16 sequences) to which the putative Avo1 protein sequence had been included. Data were searched using the Mascot search engine (Matrix Science, v.2.5) or by using Proteome Discoverer (Thermo Scientific, v.2.4) employing the Sequest HT and MS Amanda 2.0 search engines. Extended search criteria are included in SM6.

The whole workflow used above is accessible at https://www.ibp.cz/local/data/avo.

3 Results and discussion

We have predicted all putative SARS-CoV-2 ORFs longer than 200 nucleotides (nt), resulting in a set of 8 protein candidates (all ORFs are enclosed in SM1, the longest was 303 nt long, and the shortest 201 nt in size). Then we have inspected if they contain the so-called Kozak sequence. We have found one highly promising candidate locus and called it *Avo1* (Ambient viral ORF1). *Avo1* nucleotide sequence (Figure 1 Top) contains a very well-conserved Kozak signal in the form of RYMRMVAUGGC [21], which is a nucleic acid motif that functions as the protein translation initiation site in most mammalian mRNA transcripts [21]. The strongest Kozak signals found in the SARS-CoV-2 genome are depicted in Figure 1 Bottom (five of six Kozak signals are located in RNA positive-sense strand, one is located on the RNA negative-sense strand).

3.1 Sequence and position of *Avo1* locus

Avo1 locus is situated on nucleotide positions 422 – 667 of the reference SARS-CoV-2 genome (NC_045512.2) and has an unusually high GC content (47.97 %) - the whole SARS-CoV-2 average value is much lower (37.98 %) [30,31]. Putative protein Avo1 is translated in reading frame -2 (i.e. only from negative-sense SARS-CoV-2 RNA intermediate). The protein product would be 81 amino acid (aa) residues long and its molecular weight was computed to be 9586.06 Da with a predicted isoelectric point (pI) equal to 9.22.

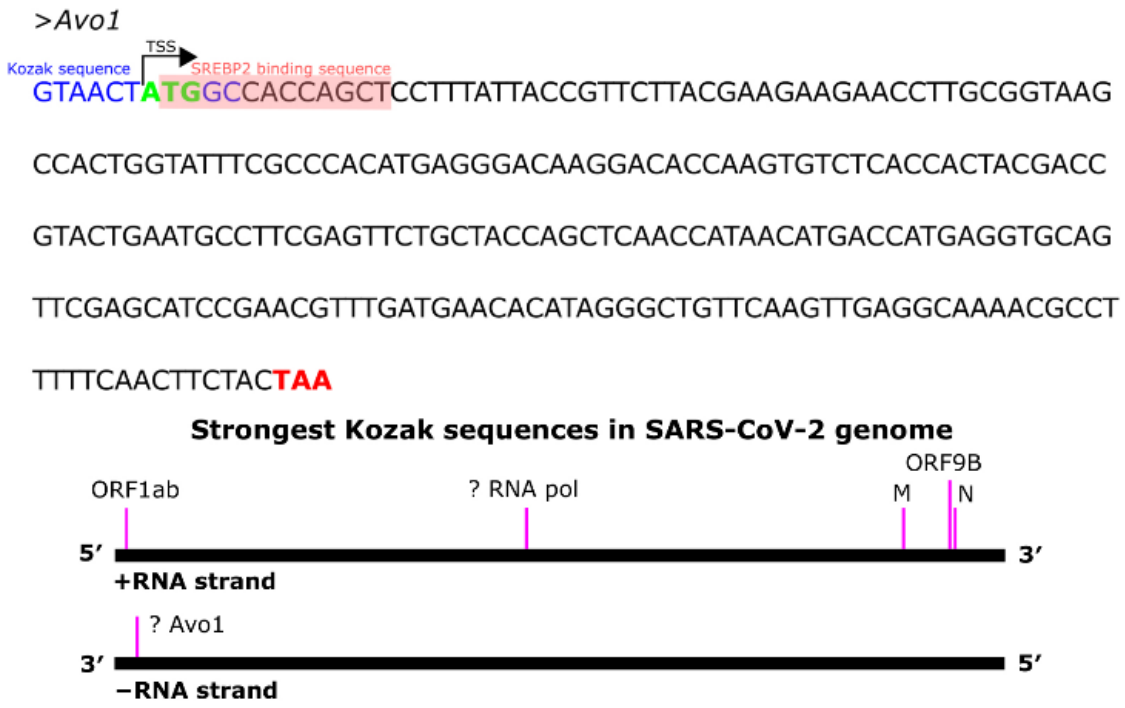

Fig. 1. (Top) **Genomic sequence of putative *Avo1* gene**. Kozak sequence is depicted in blue color, start codon ATG in green, stop codon TAA in red. SREBP2 binding sequence is highlighted by pink. TSS stands rather for "translation start site" because we are dealing already with viral RNA. (Bottom) Six highly significant Kozak sequences in the SARS-CoV-2 genome, the Avo1 signal is the second strongest one (see SM4).

Interestingly, the proposed *Avo1* gene contains a 12 nt long SREBP2 binding site (Figure 1 Top). Recently it was found that activated SREBP2 in COVID-19 patients disturbs cholesterol biosynthesis and leads to a cytokine storm [32], therefore the SREBP2 binding site in the *Avo1* locus may play an important role in the process of SREBP2 activation and regulation [33]. In addition, cytosine methylation of cytosine-rich *Avo1* locus (27.24 %) may play vital roles in SARS-CoV-2 regulation and can serve as a target for possible therapeutic intervention using

e.g. folate supplementation (which would change methylation status of viral RNA and inhibit viral replication), as proposed earlier [34,35].

Homology searches revealed that *Avo1* ORF is evolutionarily conserved in several coronaviruses, e.g. in bat coronaviruses RaTG13, RpShaanxi2011, BtRs-BetaCoV/HuB2013, Rc-o319, or in pangolin coronavirus GX/P2V (Figure 2 Top). On the other side, human SARS-CoV and MERS-CoV *Avo1* loci are truncated by stop codons - dynamic loss or gain of specific accessory proteins may be an explanation for different viral properties (virulence, pathogenesis, etc.). Three weak domain homology signals were found in the CDD database: B-12 dependent methionine synthase, Herpes envelope superfamily, and adenosine deaminase (Figure 2 Top).

3.2 Avo1 protein structure

Another evidence for possible Avo1 protein biological significance is that instead of some disordered clumps, an independent *ab initio* prediction of tertiary structures of SARS-CoV-2 Avo1 and RatG13 Avo1 has shown five alpha-helices forming conserved structures (Figure 2 Bottom).

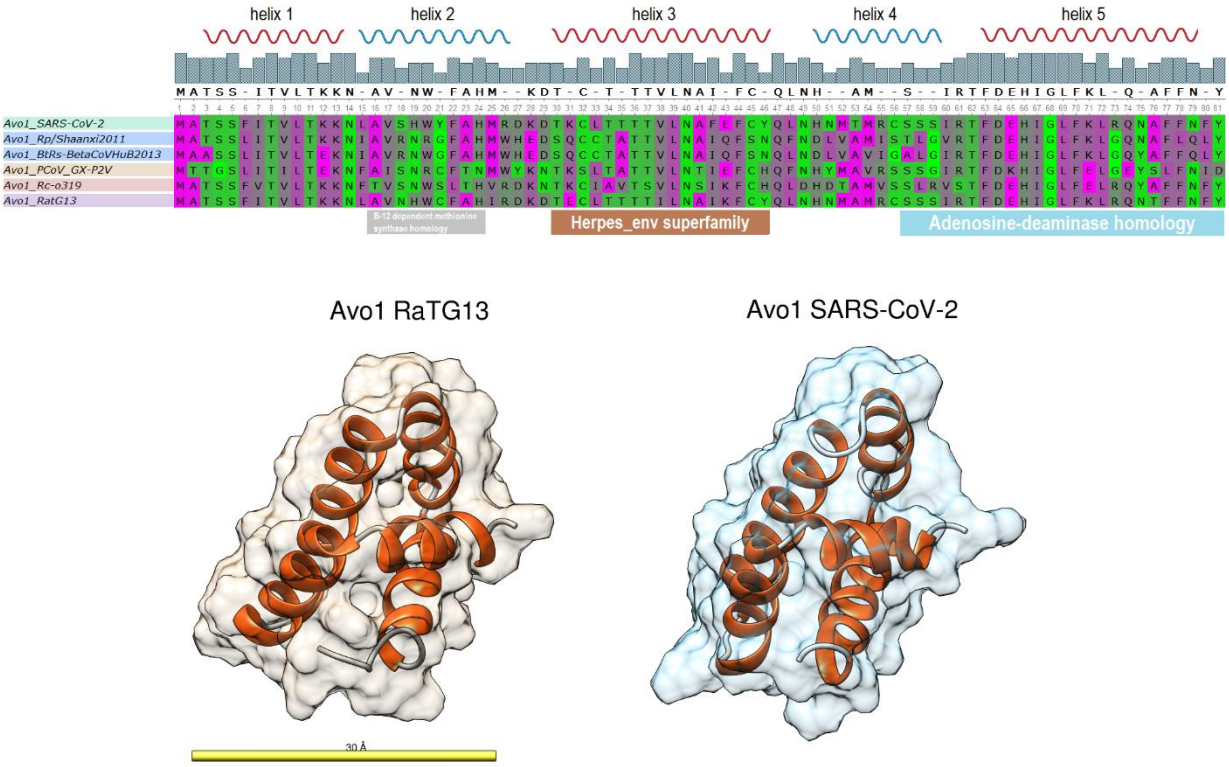

Fig. 2. (Top) **Alignment of Avo1 putative proteins from homologous coronaviruses**. Coloring expresses helix propensity, magenta – high, green – low. The consensus sequence inferred from the alignment is bar plotted. Domain hits for the SARS-CoV-2 sequence are inside the rectangles. (Bottom) *De novo* modeled Avo1 structure using QUARK workflow [19], yellow scale bar is 30 Å long.

For detection of Avo1 at the protein level, we re-searched bottom-up mass spectrometry data from two other studies involving SARS-CoV-2 infection of human primary alveolar macrophages and Vero E6 cells [26,27]. However, Avo1 remained unidentified, but on the other side, only nucleoprotein, spike glycoprotein, membrane protein, ORF3a, ORF7a, ORF9b, and replicase polyprotein 1a were identified as viral proteins. The fact that only a subset of the viral genome can be detected at the protein level point towards low expression levels or high turnover rate of the remaining viral proteins may also be valid for Avo1. A way to detect remaining SARS-CoV-2 proteins *in vivo* would be an immunoprecipitation using artificially produced antibodies [36].

3.3 Coding potential of the negative-sense RNA strand

Our subsequent searches reveal a couple of interesting ORFs located on negative-sense RNA in other Coronaviruses, so it seems that the negative-sense coronaviral RNA can generally serve as an additional pool of their coding potential. For example, Miniopterus bat coronavirus HKU8 (NC_010438.1) has a 438 aa long ORF and Alphacoronavirus Bat-CoV/P.kuhlii/Italy/3398-19/2015 (NC_009988.1) has a 313 aa long ORF on its negative-sense RNA. All the longest 13 hits (over 200 aa) are listed in SM5. Analyzes of their predicted pIs revealed, that all of them have pI higher than 8.4 (with an average value of 10.07) - it is very surprising because there was no significant sequence homology between them. In contrast, SARS-CoV-2 proteins encoded by the positive-sense RNA have an average pI value close to seven (6.84) [37] and only the SARS-CoV-2 nucleoprotein (N) has a pI greater than 10. Thus, it is possible, that proteins encoded by coronaviral negative-sense RNA later bind/interact with viral or host RNA. Furthermore, pIs of viral proteins give us an important clue about their interaction with host proteins – it was found that in human-viral interactomes, the viral basic proteins have the lowest average of interactions while the greatest one in intraviral (inside virion) [38]. Furthermore, nine of the long ORFs mentioned above also contain potential nuclear localization signal(s) (NLSs) (SM5). To date, 14 of 28 SARS-CoV-2 proteins were detected both in the nucleus and cytoplasm, suggesting a complicated interaction of SARS-CoV-2 with host cells to achieve a successful infection [5]. The following prediction of N-glycosylation suggests that the vast majority (12/13) of these ORFs are posttranslationally modified by N-glycosylation. N-glycosylation is a common phenomenon, how viral proteins can be recognized by an innate immune response, which is the first line of defense against infection [39], and could be utilized as an additional therapeutic target [40–42]. Our findings are summarized and depicted in the figure below (Figure 3).

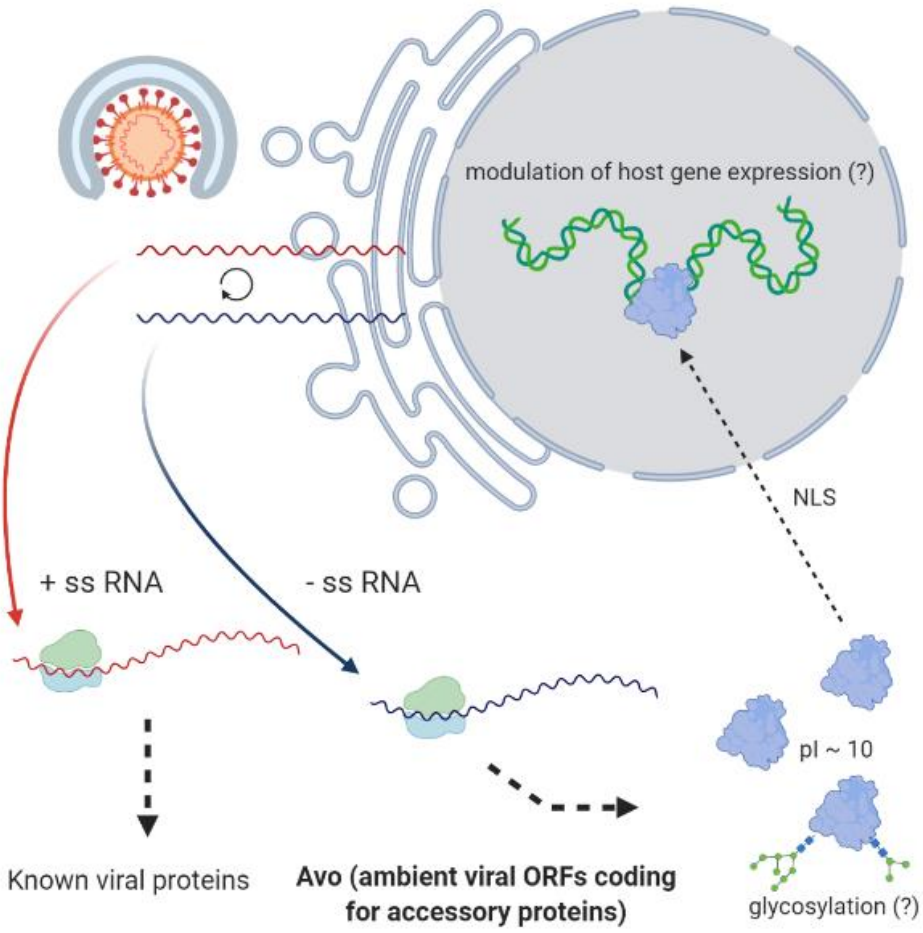

Fig. 3. Proposed hypothesis about accessory viral proteins coding on negative-sense RNA strand. The replication of coronaviral RNA takes place inside special compartments connected to the endoplasmic reticulum. We propose the hypothesis, that also negative-sense RNA has a coding potential and most of the resulting proteins are likely to be glycosylated. Besides, some of them are possibly translocated into the nucleus, where can modulate host gene expression via nucleic acid-binding. The scheme was created with BioRender.com.

It was found that the SARS-CoV-2 transcriptome is very diverse and rich [43] and therefore, it is likely that also SARS-CoV-2 proteome will follow this trend and still unheeded viral proteins or protein variants wait for its discovery. Every newly described SARS-CoV-2 protein (and its host interacting proteins) could be a potential target for a drug repurposing [4,44] or development of novel therapies [45], for instance, it was found, that SARS-CoV-2 accessory proteins ORF6 and ORF8 inhibit type I interferon signaling pathway, and thus play a critical role in innate immune suppression during viral infection [8]. Altogether, our results

suggest the existence of a still undescribed SARS-CoV-2 protein, which may play an important role in the viral lifecycle and COVID-19 pathogenesis. We hope, that this study will stimulate further research in the field of developing more specific and sensitive approaches to detect the complete SARS-CoV-2 proteome *in vivo*.

Acknowledgements

We would like to thank our institution, the University of Ostrava, for an inspiring working environment and academic freedom. M. B. was supported by the project SGS01/PřF/2020 by the University of Ostrava. E. T. P was supported by the VELUX Foundation (00014557) and the Novo Nordisk Foundation (BIO-MS).

Funding

This work has been supported by the SGS01/PřF/2020 by the University of Ostrava. E. T. P was supported by the VELUX Foundation (00014557), and the Novo Nordisk Foundation (BIO-MS).

Conflict of Interest: none declared.

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