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## Ambisense polarity of genome RNA of orthomyxoviruses and coronaviruses

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### Abstract

Influenza viruses and coronaviruses have linear single-stranded RNA genomes with negative and positive sense polarities and genes encoded in viral genomes are expressed in these viruses as positive and negative genes, respectively. Here we consider a novel gene identified in viral genomes in opposite direction, as positive in influenza and negative in coronaviruses, suggesting an ambisense genome strategy for both virus families. Noteworthy, the identified novel genes colocalized in the same RNA regions of viral genomes, where the previously known opposite genes are encoded, a so-called ambisense stacking architecture of genes in virus genome. It seems likely, that ambisense gene stacking in influenza and coronavirus families significantly increases genetic potential and virus diversity to extend virus-host adaptation pathways in nature. These data imply that ambisense viruses may have a multivirion mechanism, like "a dark side of the Moon", allowing production of the heterogeneous population of virions expressed through positive and negative sense genome strategies.

**Key Words:** Virus genome; Ambisense RNA; Influenza; Coronavirus; Virus diversity; Virus genes

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**Core Tip:** A novel genes identified in viral genomes in opposite direction, as positive in influenza and negative in coronaviruses, are considered. The identified novel genes colocalized in the same RNA regions of viral genomes, where the previously known opposite genes are encoded, a so-called ambisense stacking architecture of genes in virus genome. It seems likely, that ambisense gene stacking in influenza and coronavirus families significantly increases genetic potential and virus diversity to extend virus-host adaptation pathways in nature. These data imply that ambisense

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viruses may have a multivirion mechanism, like "a dark side of the Moon", allowing production of the heterogeneous population of virions expressed through positive and negative sense genome strategies.

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## INTRODUCTION

Orthomyxo- and coronaviruses are two families of enveloped viruses containing single stranded linear RNA genomes. Orthomyxovirus family includes seven genera: Alphainfluenzavirus, Betainfluenzavirus, Deltainfluenzavirus, Gammainfluenzavirus, Isavirus, Thogotovirus, and Quarantavirus. These viruses infect wide range of hosts including mammals, birds, rodents, fish, ticks and mosquitoes. Orthomyxoviridae viruses contain six to eight segments of negative-sense single stranded RNA with a total genome length of 10-15 Kb[1]. Coronaviridae is divided into the four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. Alpha- and betacoronaviruses infect mammals, while gamma- and deltacoronaviruses primarily infect birds. The size of genomic positive sense RNA of coronaviruses ranges from 26 to 32 kilobases, one of the largest genome among RNA viruses[2]. Here we mainly consider alphainfluenza viruses and betacoronaviruses as a typical members in both families.

## INFLUENZA A VIRUS AMBISENSE GENES

Genome of influenza A viruses is composed of 8 segments of single-stranded RNAs with mol. wt.  $0.7-2.8 \times 10^5$  kilobases/segment. Each segment encodes one or several unique polypeptides through the canonical negative sense genome strategy (Table 1). It means that genome RNA of negative sense polarity is transcribed by the virus polymerase to produce positive sense mRNAs, which recognized by ribosomes to translate individual viral proteins (Figure 1). In addition to the negative sense genes, influenza A virus genome segments were found to contain long open reading frames (ORFs, genes) in opposite positive sense orientation. These ORFs have all ribosome translation elements: canonical start codon AUG or noncanonical CUG, termination codons (UAG, UAA, or UGA), internal ribosome entry sites (IRES), and Kozak-like sequences at the initial start codon[3-9].

There are three groups of data showing *in vivo* expression potential of these negative stranded genes. (1) The template function of the full length "negative sense" genome RNA of segment 8 (NS) was demonstrated in a cell-free translation system of rabbit reticulocyte lysate. It was shown that influenza A virion RNA of segment 8 can initiate synthesis of major polypeptide negative stranded protein (NSP8) (mol.wt. 23 kD) specifically reacted with antibody to the central domain of the NSP8[10]; (2) The NSP8 encoded in the 8'th influenza A virus segment NS could be expressed *in vivo*, in insect cells (ovary cell line of *Trichoplusia ni*) infected with recombinant baculovirus (insect nuclear polyhedrosis virus) carrying influenza virus sequence NSG8 in the virus DNA genome. This gene appeared to express ~20 kD influenza-specific polypeptide NSP8, which was intracellularly stable and accumulated in the perinuclear zone of infected cells[11]. Later, it was also supported that influenza A virus NSP8 could be efficiently expressed from either a plasmid or a recombinant vaccinia virus in mammalian cells and the synthesized NSP8 was localized in the perinuclear endoplasmic reticulum (ER) and post-ER cellular compartments[12]; and (3) There are data that mice infected with influenza virus produce CTL response specific to epitopes presented in the influenza NSP8 protein[12-14]. These findings also demonstrate that translation of sequences locating on the negative RNA strand of a single-stranded RNA genome of influenza A virus can develop *in vivo* and can initiate antiviral CTL response and immunosurveillance.

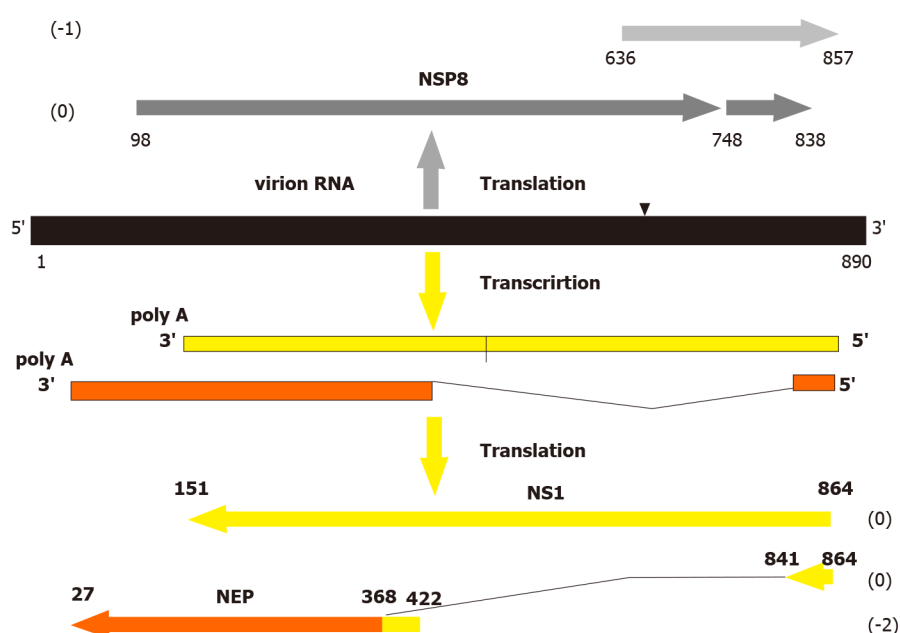
**Table 1** RNA segments of influenza A virus genome and encoded polypeptides

Viral RNA segments and their length (nt) <sup>1</sup>	Positive sense polypeptides (mol. wt., kDa) <sup>2</sup>	Negative stranded polypeptides, NSPs (mol. wt.; a.a.) <sup>3</sup>
PB1 (2341)	PB1 (86.6); PB1-N40 (89.4); PB1-F2 (10.5)	NSP1 (174, 239)
PB2 (2341)	PB2 (85.7); PB2-S1 (55)	NSP2 (116, 121, 130, 137)
PA (2223)	PA (84.2); PA-X (29); PA-N155 (62); PA-N182 (60)	NSP3 (95, 109)
HA (1778)	HA (61.5)	NSP4 (n.d.)
NP (1565)	NP (56.1); eNP (56.8)	NSP5 (117, 154)
NA (1413)	NA (50.1); NA43 (48.6)	NSP6 (91, 154)
M (1097)	M1 (27.8); M2 (11); M42 (13)	NSP7 (99, 102, 109)
NS (890)	NS1 (26.8); NEP (14.2); NS3 (21); tNS1 (17)	NSP8 (93, 167, 216)

<sup>1</sup>RNA segments and nucleotide (nt) calculations were made for the A/PR8/34 (H1N1) virus.

<sup>2</sup>Canonical influenza A virus polypeptides synthesized through the negative genome strategy (Figure 1; for review see[1]).

<sup>3</sup>Negative stranded genomic open reading frames (ORFs) and predicted negative stranded proteins (NSPs) have been calculated for A/PR8/34 (H1N1) and A/Aichi/2/68 (H3N2) viruses[3-8]. Negative stranded ORFs were identified by in silico approach using the Open Reading Frame Finder program (<https://www.ncbi.nlm.nih.gov/orffinder/>). These ORFs can be realized through the positive genome strategy. The amino acid length (a.a.) of NSPs were based on the data presented mainly in ref.[8]. A.a. values reflect variations among human, avian and other mammalian virus strains. N.d. means the absence of ORFs longer than 90 a.a. NSP: Negative stranded protein.



**Figure 1** The scheme of expression of the genome negative sense segment of influenza A virus. The negative sense (NS) segment of influenza A/Aichi/2/68 (H3N2) virus is displayed. The horizontal arrows show the open reading frames (ORFs) of the negative strand protein 8, non-structural anti-interferon protein (NS1), and nuclear export protein (NEP). Numbers in brackets indicate the ORF translation phase. Numbers under the lines indicate nucleotide positions from the 5' end of the virion genome RNA. The broken line shows the splicing segment of the NEP gene mRNA. Triangle in the virion RNA molecule shows a site position of possible translation frameshifting[10]. NS: Negative sense; NSP8: Negative strand protein 8; NS1: non-structural anti-interferon protein.

The mature product of the NSP8 gene has not been yet identified in biological systems such virus-infected cells and animals. The failure to detect NEG8 protein could be due to a number of factors other than the complete absence of translation from genomic RNA. The properties of the NSP8 as an “escaping protein” may be explained either by its low synthesis and a short period of life or/and strong tissue-specific expression in certain cell types containing factors which are necessary for the regulation of expression of these “negative sense” genes. It would not be surprising if negative polarity genes are only expressed physiologically under special circumstances *in vivo* determining host cell tropism of influenza viruses.



## NOVEL NEGATIVE SENSE GENES IN THE RNA GENOME OF CORONAVIRUSES

Recently, similar ambisense polarity has been revealed in coronaviruses genomes[15]. It is well known that these viruses possess a linear positive sense genome RNA of  $25\text{--}29 \times 10^3$  kb length[2]. The coronavirus genome RNA contains two groups of genes expressing proteins through the positive sense strategy. The first ones (nonstructural genes for nsp1-nsp19 proteins) are localized at the 5'-region of the virion genome RNA and directly translated by host ribosomes. The second ones (mostly the structural proteins genes N, S, HE, M, E and several accessory proteins, such as 3a/b, 6, 7a/b, 8a/b, 9b, *etc.*) occupy a 3'-region of the virion RNA and express proteins through the translation of subgenomic mRNAs, which was transcribed on the anti-genomic RNA template[16] (Figure 2A). In addition to the positive sense genes, we have identified numerous long open reading frames in negative sense orientation (Table 2; Figure 2B). Like in the case of the ambisense genes of flu viruses, coronavirus negative sense genes have all elements characteristic of the mRNA molecules which are recognized by host ribosomes: classical AUG or alternative CUG[17] start codons, termination codons, IRES, and Kozak-like sequences at the start area[18,19]. However, unlike to influenza A viruses, coronavirus ambisense polarity has opposite configuration: a positive sense genome strategy and a negative sense orientation of the novel negative sense genes, so called a negative sense genes or negative gene proteins (NGPs).

The identification of coronavirus negative-polarity genes implies two possible mechanisms of their expression and synthesis of the corresponding mRNAs and proteins. These mechanisms include either direct translation of a replicative (-)copy of genomic (+)RNA (replication pathway II) or the transcription of genomic (+)RNA by viral polymerase with the formation of subgenomic mRNAs of "negative polarity" for their subsequent translation to synthesize specific viral polypeptides (transcription pathway I). To realize pathway I coronavirus genome contains poly A sequence (positions 11935-1194 nt) functioning as a viral polymerase binding site and transcription initiation signal (Figure 2B).

## BIOLOGICAL SIGNIFICANCE OF THE AMBISENSE GENES

The function and role of the newly discovered ambipolar viral genes have not yet been determined. In the case of influenza viruses, there are indirect data that the identified new ambisense genes can be involved in the regulation of the host immune response against viral proteins and/or in the regulation of the stability of viral proteins in infected cells through the protein deubiquitinating system[5,12]. The possible functional significance of the novel ambisense genes is not yet generally clear. However, the stability and retaining of these type of genes in field viruses genomes for more than 100 years at the high variability of virus population suggest the functional necessity of these genes and their biological evolutionary determination[20]. Notably, the influenza NSP8 has high synonymous/nonsynonymous (dN/dS) mutations rate ( $> 1.5$ ), which was similar to that one for the most variable surface virus glycoproteins HA and NA representing major target for antiviral host adaptive immune response. The elevated variability of the NSP8 implies that it undergoes positive selection and host adaptation, which influence its evolution[5].

The discovery of new ambisense genes has raised a number of important questions regarding its origin, functions, and evolutionary variability. One of the essential questions is how the novel genes have emerged in the genomic region to encode two opposite sense genes. The appearance of the ambipolar gene suggests the existence of yet unknown correspondence principle (or reverse determination rule) for the expression of oppositely directing genes locating in the same region of RNA molecule. This principle implies that a certain pre-existing gene can predetermine the emergence mechanism and the properties of a new ambipolar gene[5]. Without this rule, chaotic accumulation of mutations will result in the appearance of a new functional gene and its further evolutionary selection, that seems to be unlikely. Moreover, the probability for such chaotic event is low, considering the ambipolar overlapping of several preexisting genes, when changes in one of them would cause changes in the coupled ambipolar genes. In this case, gene variability and selection of mutations should be interconnected in all opposite viral genes (in the case of influenza virus for NS1, NEP, and NSP8). These considerations incline to the assumption of the existence of a rule of reverse determination, when both ambipolar genes can have linked structural motives and functions. Further studies are necessary to clarify this idea.

Table 2 Negative sense genes in genomes of coronaviruses

Virus genera	Viral genomes	Number of NSGs in virus genome <sup>1,3</sup>	M.W. range of the NGPs <sup>2</sup>
Alpha-coronaviruses	HCov-229E: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_002645.1">https://www.ncbi.nlm.nih.gov/nucleotide/NC_002645.1</a>	29/1/29/5	12.4-14.4
Beta-coronaviruses	SARS-CoV-1: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_004718.3">https://www.ncbi.nlm.nih.gov/nucleotide/NC_004718.3</a>	34/0/35/2	11.5- 15.0
	SARS-CoV-2: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/MT635445.1">https://www.ncbi.nlm.nih.gov/nucleotide/MT635445.1</a>	21/1/26/4	10.9- 17.2
	MERS: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_019843.3">https://www.ncbi.nlm.nih.gov/nucleotide/NC_019843.3</a>	32/8/23/3	11.1- 18.6
	Pangolin-CoV: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/MT040335.1">https://www.ncbi.nlm.nih.gov/nucleotide/MT040335.1</a>	29/3/17/4	10.8-19.9
	HCov-HKU1: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_006577.2">https://www.ncbi.nlm.nih.gov/nucleotide/NC_006577.2</a>	15/1/13/2	11.5- 15.0
	Bat coronavirus RATG13: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/MN996532.1">https://www.ncbi.nlm.nih.gov/nucleotide/MN996532.1</a>	17/2/29/1	10.9- 19.7
	Bovine coronavirus BCoV-ENT: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_003045.1">https://www.ncbi.nlm.nih.gov/nucleotide/NC_003045.1</a>	25/1/26/0	20.8
	Murine hepatitis virus A59: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/FJ884687.1">https://www.ncbi.nlm.nih.gov/nucleotide/FJ884687.1</a>	29/5/42/7	11.2-36.8
Gamma-coronaviruses	Avian infectious bronchitis virus: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_001451.1">https://www.ncbi.nlm.nih.gov/nucleotide/NC_001451.1</a>	20/6/8/3	12.7- 26.5
Delta-coronaviruses	Porcine coronavirus HKU15: <a href="https://www.ncbi.nlm.nih.gov/nucleotide/NC_039208.1">https://www.ncbi.nlm.nih.gov/nucleotide/NC_039208.1</a>	26/5/29/3	11.2- 17.4

<sup>1</sup>Negative sense genes (NSGs) were identified by in silico approach using the Open Reading Frame Finder program (<https://www.ncbi.nlm.nih.gov/orffinder/>). First and second digits show overall and numbers of the large gene open reading frames (ORFs) starting with classical AUG, respectively. Third and fourth numbers show overall and large gene numbers ORFs having noncanonical CUG, respectively. Large genes were assumed to have more than 300 nt long. GenBank ac.n. of the viral genomes are indicated.

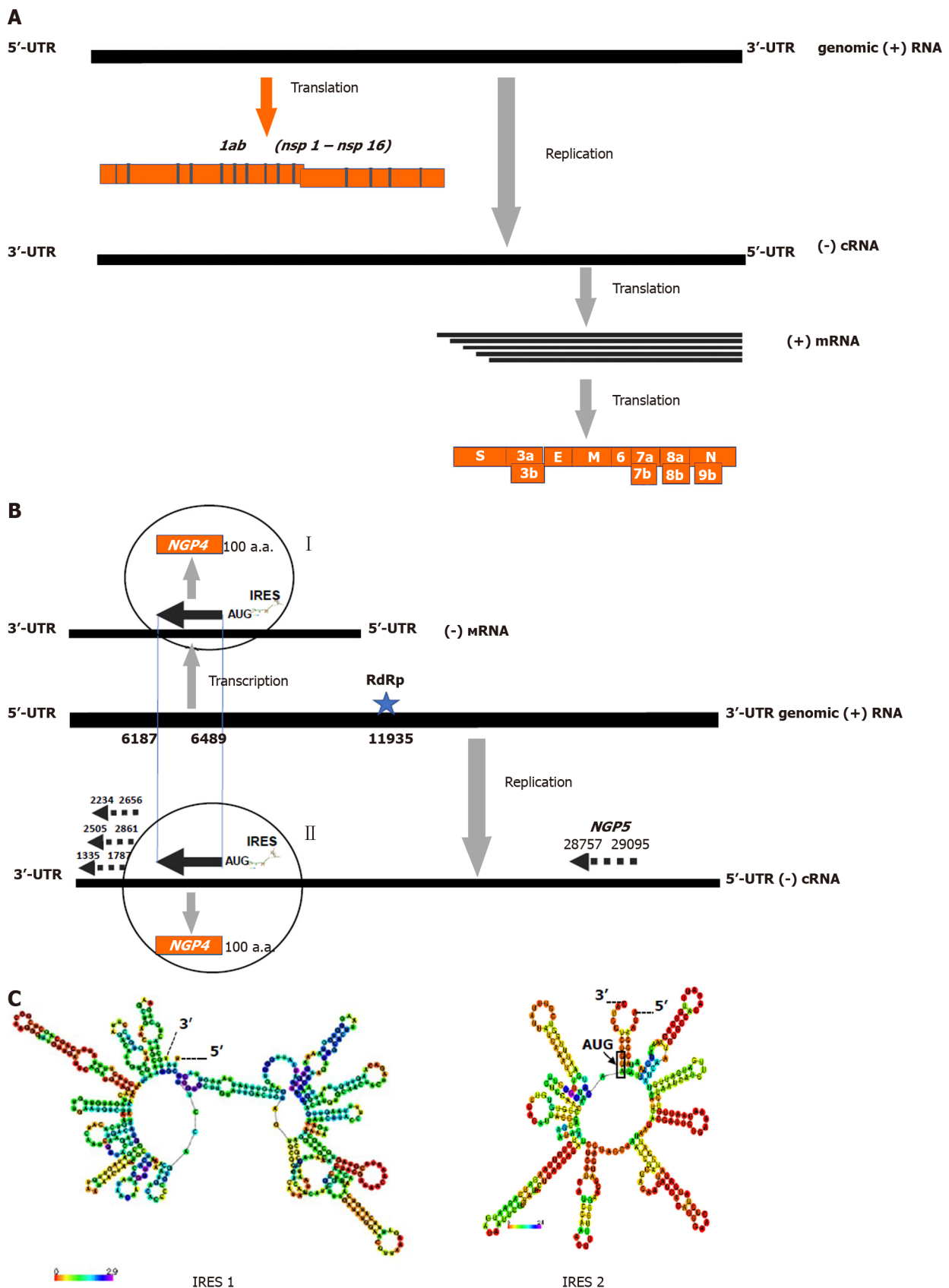
<sup>2</sup>A range of mol. wt. (kDa) of negative gene proteins encoded by the large negative sense genes ( $\geq 300$  nt) starting either with AUG or CUG codons are outlined.

<sup>3</sup>The data were partially presented in [15]. These partial elements were used here with the Publisher's permission. NSGs: Negative sense genes; SARS-CoV: Severe acute respiratory syndrome coronavirus.

Ambisense stacking of genes revealed in coronavirus and influenza virus genomes significantly increases virus diversity, genetic potential and extend virus-host adaptation pathway possibilities. Existence of numerous ambisense genes opens up a new avenue for virus reproduction where one virus genome can produce a multiple progeny population of virions possessing identical genome RNA and different protein compositions. In this case, a part of virions decorated with one of the NGPs proteins (in the case of coronaviruses) could be hidden from us, as "the dark side of the Moon". The expression of coronavirus "negative" and flu "positive" genes may have a host (tissue)-dependent regulation facilitating immune escape of overcovered virions and specific pathogenetic pathways in the host(s) where the up-expression of the virus NGP or NSP genes occurs. Further studies will shed light on this ambisense concept of human and animal orthomyxo- and coronaviruses.

For the current time, there are four ambisense virus genera (phlebo-, tospo-, arena-, and bunyaviruses), which are well known to realize both positive- and negative-sense genome RNA strategies to encode viral proteins [12,21]. Ambisense genes of these virus genera locate in separate areas of the genome RNA without their overlapping and stacking. The ambisense genes locating in the genome in the stacking manner were found in influenza viruses, in which, similarly to coronaviruses, direct expression of these genes has not yet been identified, but there are indirect signs of such expression during natural viral infection *in vivo* [12-14]. Location of genes with opposite polarity in the same region of the RNA molecule makes it possible to significantly increase the genetic capacity of the viral genome and opens new ways for virus diversity, increasing virus adaptability to the host and biological evolution in nature [15]. The presence of potential ambisense genes in genomes of influenza and coronaviruses raises the question of the classification of these families. The detection in infected cells or infected organisms of protein products expressed by the ambisense manner will give grounds for classifying the coronavirus and orthomyxovirus families as the ambisense viruses with a bipolar genome strategy.





**Figure 2 Positive sense genome strategy and translation cassette unit at the 3' end of the negative sense complimentary RNA of coronavirus severe acute respiratory syndrome coronavirus 2 genome.** A: Replication scheme of the RNA genome of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) coronavirus (ac.n. MT890462.1). UTR means untranslated RNA region; B: A 3' end area of the subgenomic (-) cRNA complimentary to the virus genome 5' end (+) vRNA of SARS-CoV-2 (ac.n. MT635445.1) is displayed. Five ORF containing cassette for NGP1-NGP5 beginning either with classical AUG (NGP4) or noncanonical CUG (NGP1-3, NGP5) codons are shown by arrows. Nucleotides counting from the 5' end of (+) vRNA are shown for each ORFs. Phases of the translation frame (fr) are estimated regarding the frame of NGP4 (fr.0) as follows: NGP1 and 2 (fr. +1), NGP3 (fr.0). Poly A tract (11935-

11940 nt) functioning as a viral RNA dependent RNA polymerase binding site is shown by star; C: IRES-like structures enriched with 16 and 10 canonical "hair-pins" RNA elements in the regions 8100-8599 nt (IRES 1) and 6488-6792 nt (IRES 2), respectively, were predicted by the IRESpred program[22]. The IRES-like structures 1 and 2 have significant free energy value as low as -99,4 and -73,8 kkal/mol, respectively. The data were partially presented in[15]. These partial elements were used here with the Publisher's permission.

## CONCLUSION

The manuscript data suggest that ambisense gene stacking in influenza and coronavirus families significantly increases genetic potential and virus diversity to extend virus-host adaptation pathways in nature. These data imply that ambisense viruses may have a multivirion mechanism, like "a dark side of the Moon", allowing production of the heterogeneous population of virions expressed through positive and negative sense genome strategies.

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