**Intragenomic rearrangements in SARS-CoV-2, other betacoronaviruses, and alphacoronaviruses**

Roberto Patarca, MD, PhD, and William A. Haseltine, PhD

ACCESS Health International, 384 West Lane, Ridgefield, Connecticut 06877, USA

**Corresponding author:** William A. Haseltine, PhD; e-mail: william.haseltine@accessh.org

**Abstract**

Variation of the betacoronavirus SARS-CoV-2 has been the bane of COVID-19 control. Documented variation includes point mutations, deletions, insertions, and recombination among closely or distantly related coronaviruses. Here, we describe yet another aspect of genome variation by beta- and alphacoronaviruses. Specifically, we report numerous genomic insertions of 5'-untranslated region sequences into coding regions of SARS-CoV-2, other betacoronaviruses, and alphacoronaviruses. To our knowledge this is the first systematic description of such insertions. In many cases, these insertions change viral protein sequences and further foster genomic flexibility and viral adaptability through insertion of transcription regulatory sequences in novel positions within the genome. Among human Embecorivus betacoronaviruses, for instance, from 65% to all of the surveyed sequences in publicly available databases contain 5'-UTR-derived inserted sequences. In limited instances, there is mounting evidence that these insertions alter the fundamental biological properties of mutant viruses. Intragenomic rearrangements add to our appreciation of how variants of SARS-CoV-2 and other beta- and alphacoronaviruses may arise.

**Keywords:** Alphacoronaviruses, betacoronaviruses, variants, intragenomic rearrangements

**Significance:** Understanding mechanisms of variation in coronaviruses is vital to control of their associated diseases. Beyond point mutations, insertions, deletions and recombination, we here describe for the first time intragenomic rearrangements and their relevance to changes in transmissibility, immune escape and/or virulence documented during the SARS-CoV-2 pandemic.

## Introduction

Coronaviruses (CoVs) are positive, singe stranded RNA viruses of the order Nidovirales, family Coronaviridae, subfamily Orthocoronavirinae, with four genera, namely alpha [α], beta [β], gamma [γ] and delta [δ], which have been further subdivided into 25 subgenera, including five for β-CoVs: Sarbeco-, Merbeco-, Embeco-, Nobeco- and Hibecovirus (Weiss and Navas-Martin 2005), and fifteen for α-CoVs: Luchaco-, Decaco-, Nyctacovirus, -Minunaco-, Pedaco-, Colaco-, Myotaco-, Duvinaco- Setraco-, Rhinaco-, Tegaco-, Minaco-, Sunaco-, Soracoviurs, and Amalacovirus (ICTV Coronaviridae Study Group. 2021). Seven CoVs infect humans; two of the α-genus (the Duvinacovirus hCoVs 229E & the Setracovirus NL63) and five of the β-genus: the Sarbecoviruses severe acute respiratory syndrome (SARS)-CoVs 1 and 2, the latter responsible for a pandemic since 2019 (Pollett et al. 2021; Jackson et al. 2021; VanInsberghe et al. 2021; Turkahia et al. 2021); the Merbecovirus Middle East respiratory syndrome (MERS) CoV; and the Embecoviruses hCoV-OC43 and -HKU1. Human CoVs have a zoonotic origin, with bats as key reservoir (Menachery et al. 2015) and possibly intermediate hosts (Fan et al. 2019; Pickering et al. 2021; Reusken et al. 2013; Song et al. 2005). Bat β-CoVs related to human CoVs belong to the Sarbeco-, Nobeco-, and Hibecovirus subgenera (Latinne et al. 2020; Wong et al. 2019; Woo et al. 2007).

Coronaviruses display substantial genomic plasticity and resilience (Amoutzias et al. 2022; Andersen et al. 2020) via recombination, point mutations, deletions, and insertions, which are reported to drive variant emergence, host range, gene expression, transmissibility, immune escape, and virulence (Decaro et al. 2009; Goldstein et al. 2021; Gussow et al. 2020; Simon-Loriere et al. 2011; Throne et al. 2022). The use of an RNA-dependent-RNA polymerase (RdRp)-driven template switching mechanism for transcription and control of structural and accessory gene expression in CoVs (Sawicki et al. 2007) has been reported to account for the high frequency of recombination (Amoutzias et al. 2022; Bobay et al. 2020; Boni et al. 2020; Forni et al. 2017, 2020; Lau et al. 2018; Makino et al. 1986; Simon-Loriere et al. 2011; Su et al. 2016; Yang et al. 2021).

In template switching, a leader transcription regulatory sequence (TRS-L; ACGAAC core in β-CoVs) (Wang et al. 2021) in the 5'-untranslated region (UTR) interacts with homologous TRS-body (B) elements upstream of viral genes in the last third of the genome (illustrated for SARS-CoV-2 in Figure 1A) (Bentley et al. 2013; Sawicki et al. 2007; Sola et al. 2015; Van Marle et al. 1995). Template switching renders the neighborhood of TRS-Bs, especially that for the spike gene, a recombination hotspot during viral transcription (Bobay et al. 2020; Boni et al. 2020; Forni et al. 2020; Graham et al. 2010, 2018; Goldstein et al. 2021; Lytras et al. 2022; Nikolaidis et al. 2021; Pollett et al. 2021; Yang et al. 2021).

Viral subgenomic messenger RNAs contain a 5'-leader sequence that spans from the terminal 5'-cap ($m^7G$) structure to the TRS-L and harbors three conserved stem-loop (SL1-3) regulatory elements of gene expression and replication (Figure 1B) (Madhugiri et al. 2018; Miao et al. 2021; Zhang et al. 1994). The TRS-L core sequence and the secondary structure of the leader sequence are conserved within but not among coronavirus genera (Rfam database: http://rfam.xfam.org/covid-19).

The entire 5'-leader nucleotide sequence of SARS-CoV-2, and beyond up to almost SL5 can be translated into a peptide sequence (Figure 1B), and although there is no evidence for the functionality of any open reading frame within the UTRs (Chen et al., 2010; Miao et al., 2021), the 5'-leader sequence is translated after most of it (nucleotides 8-80, including SL1-3 and TRS-L) is duplicated and translocated to the distal end of the accessory ORF6 gene of a SARS-CoV-2 variant with deleted ORFs 7a, 7b and 8 isolated from 3 patients in Hong Kong (Tse et al. 2021). We (Patarca and Haseltine 2021) also reported that a shorter portion of the 5'-leader sequence (nucleotides 50-75) is duplicated and translocated to the end of the accessory ORF8 gene of a USA variant generating a modified ORF-8 protein.

In the present study, using 5'-leader nucleotide sequences and amino acid sequences translated in the three reading frames as queries to search public databases, we document the presence of intragenomic rearrangements involving segments of the 5'-leader sequence in geographically and temporally diverse isolates of SARS-CoV-2. The intragenomic rearrangements modify the carboxyl-termini of ORF-8 (also

in *Rhinolophus* bat Sarbecovirus β-CoVs) and ORF7b; the serine-arginine-rich region of the nucleocapsid protein, generating the well characterized R203K/G204R paired mutation; and two sites of the NiRAN domain of the RdRp (nsp12).

Beyond SARS-CoV-2, we found similar rearrangements of 5'-UTR leader sequence segments including the TRS-L in all subgenera of β-CoVs except for Hibecovirus (possibly secondary to the availability of only 3 sequences in GenBank). These rearrangements are in the intergenic region between ORFs 3 and 4a, and at the carboxyl-terminus of ORF4b of the Merbecovirus MERS-CoV; intergenic regions in the Embecoviruses hCoV-OC43 (between S and Ns5) and hCoV-HKU-1 (between S and NS4); and in the Y1 cytoplasmic tail domain of nsp3 of Nobecoviruses of African *Rousettus* and *Eidolon* bats. We also found intragenomic rearrangements in α-CoVs in nsp2 (Luchacovirus subgenus), nucleocapsid (Nyctacovirus subgenus), and ORF5b or ORF4b (Decacovirus subgenus). No rearrangements involving 5'-UTR sequences were detected for the β-CoV SARS-CoV-1; the other 12 subgenera of α-CoVs including hCoV-229E and hCoV-NL63 infecting humans; or δ (Andeco-, Buldeco-, and Herdecovirus subgenera) and γ CoVs (Brangaco-, Cegaco-, and Igacovirus subgenera) for which wild birds are the main reservoir (Wille and Holmes 2020; Woo et al. 2009).

The present study highlights an intragenomic source of variation involving duplication, inversion (in two α-CoVs subgenera) and translocation of 5'-UTR sequences to the body of the genome with implications on gene expression and immune escape of α- and β-CoVs in humans and bats causing mild-to moderate or severe disease in endemic, epidemic, and pandemic settings. Genome-wide annotations had revealed 1,516 nucleotide-level variations at different positions throughout the entire SARS-CoV-2 genome (Islam et al. 2020) and a recent study documented outspread variations of each of the six accessory proteins across six continents of all complete SARS-CoV-2 proteomes which was suggested to reflect effects on SARS-CoV-2 pathogenicity (Hassan et al. 2022). The intragenomic rearrangements involving 5'-UTR sequences described here, which in several cases affect highly conserved genes with a low propensity for

recombination, may underlie the generation of variants homotypic with those of concern or interest and with differing pathogenic profiles.

## Results

Using the approaches described in the Materials and Methods section, we conducted a systematic analysis of SARS-CoV-2 and other coronaviruses and detected insertions involving 5'-UTR sequences at various locations in β- and α-CoVs, as described below by subgenus.

### Intragenomic rearrangements alter the carboxyl termini of ORF8 and ORF7b (Sarbecoviruses)

We had reported on a U.S. isolate of SARS-CoV-2 in which a segment encompassing nucleotides 50-75 of the 5'-UTR was duplicated and translocated to the end of the accessory ORF8 gene giving rise to an ORF8 protein with modified carboxyl-terminus encoded by the translocated 5'-UTR sequences (Patarca and Haseltine 2021). Figure 2 summarizes the results of our systematic search which revealed 240 similar insertions of various lengths of the same 5'-UTR sequence at various points in a stretch of 7 amino acids ($_{115}$RVVLDFI$_{121}$) of the ORF8 carboxyl-terminal sequence. As depicted in Supplemental legend to Figure 2, these internal rearrangements were detected in temporally and geographically diverse isolates, collected from March 2020 to December 2021 in 38 USA states, Bahrain, China, Kenya, and Pakistan, which is not exhaustive of what exists. All translocated 5'-UTR nucleotide sequence segments include TRS-L with variable extents of SL3 and SL2, that could affect expression of the nucleocapsid gene located immediately after the ORF8 gene (Thorne et al. 2022), and all insertions alter the carboxyl-terminus of ORF8. The analysis also revealed that the insertions in some isolates had further changes involving point mutations, deletions, and insertions. Moreover, as shown in Figure 3A, a similar 5'-UTR-derived insertion at the carboxyl-terminus of ORF8 is seen in five Sarbecovirus β-CoVs from what is considered the animal reservoir for SARS-CoV-2, the *Rhinolophus* (horseshoe) bats residing in Indochina and Southwest China (Temmam et al. 2021) all the way to England (Crook et al. 2021).

Figure 2C depicts the predicted secondary structure of ORF8 without and with (ORF8x) the 5'-UTR derived insertion. Both structures have similar predicted free minimum energy. The insertion involves the TRS-B sequence located in the intergenic region between ORF8 and N and is preceded by a uridine-adenosine (U/A)-rich region including a sequence similar to that of the torovirus attenuation sequence (Ujike and Taguchi 2021), which like TRS-B, could cause the RNA-dependent RNA polymerase to pause, thereby facilitating the intragenomic rearrangement as it is theorized to do during subgenomic RNA synthesis. Supplemental Figure 1 shows the predicted RNA structures of the most common ORF8x variants with similar predicted minimum free energy, and Supplemental Figure 2 shows an alternative RNA structure involving the interaction between the TRS-B in the intergenic region with a second and closer complementary TRS-B, yielding a similar predicted minimum free energy.

Crystal structure of ORF8 of SARS-CoV-2 had revealed a ~60-residue core like that of SARS-CoV-2 ORF7a (from which ORF8 has been postulated to originate by non-homologous recombination) (Neches et al. 2021) with the addition of two dimerization interfaces, one covalent and the other noncovalent, unique to SARS-CoV-2 ORF8 (Flores et al. 2021). In the C-terminus of ORF8 that is altered by 5'-UTR-derived insertions (i.e., $_{115}$RVVLDFI$_{121}$), R115, D119, F120, and I121 contribute to the covalent dimer interface (marked with asterisks in Figure 2) with R115 and D119 forming salt bridges that flank a central hydrophobic core in which V117 interacts with its symmetry-related counterpart (Flores et al. 2021).

How the C-terminal insertions and changes therein affect the dimerization of ORF8 remains to be determined and described functions for ORF8 remain a matter of debate (Redondo et al. 2021). However, the changes caused by insertions may contribute to immune evasion by SARS-CoV-2 by affecting the interactions of ORF8 as a glycoprotein homodimer with intracellular transport signaling, leading to down-regulation of MHC-I by selective targeting for lysosomal degradation via autophagy (Zhang et al. 2021), and/or extracellular signaling (Matsuoka et al. 2022) involving interferon-I signaling (Li et al. 2020), mitogen-activated protein kinases growth pathways (Valcarcel et al. 2021), the tumor growth factor-β1

signaling cascade (Stukalov et al. 2021) and interleukin-17 signaling promoting inflammation and contributing to the COVID-19-associated cytokine storm (Lin et al. 2021).

The carboxyl-terminal region may include T- and/or B-cell epitopes that may be affected by the variations described. To this end, approximately 5% of CD4+ T cells in most COVID-19 cases are specific for ORF8, and ORF8 accounts for 10% of CD8+ T cell reactivity in COVID-19 recovered subjects (Gordon et al. 2020; Griffoni et al. 2020). Another possible effect of the insertions stems from the fact that anti-ORF8 antibodies are detected in both symptomatic and asymptomatic patients early during infection by SARS-CoV-2 (Hachim et al. 2020; Wang et al. 2020) and diagnostic assays for SARS-CoV-2 infection that target only accessory genes or proteins such as ORF8 may be affected (Tse et al. 2021).

A shorter segment of the SARS-CoV-2 5'-UTR leader sequence (nts. 57 to 95, including TRS-L and SL3) than that described for ORF8 insertions was also duplicated and translocated to the end of ORF7b in two SARS-CoV-2 isolates (Figure 3B), one with a truncated ORF7b and the other with a truncated ORF8, which may have favored the internal rearrangements. Figure 3C shows the predicted secondary structures of the region with the intragenomic rearrangement, and as that of ORF8, involves the intergenic TRS-B sequence which is preceded and followed by a U/A-rich region, in this case also incorporating an HIV-like attenuation sequence (AAAUUU; Harrison et al. 1998). Figure 3C also shows a region of similarity between ORF8 and ORF7b which precedes the intragenomic region. The function of the SARS-CoV-2 ORF7b remains to be determined and has been suggested to mediate tumor necrosis factor-α-induced apoptosis based on cell culture data (Yang R et al. 2021) and theoretically the dysfunction of olfactory receptors by triggering autoimmunity (Khavison et al. 2020).

**Intragenomic rearrangements alter the serine-arginine-rich region of the N protein (SARS-CoV-2)**

In terms of structural proteins of SARS-CoV-2, we found a similar segment of the 5'-UTR corresponding to the leader sequence (nucleotides 56 to 76 of the Wuhan reference strain [NC_045512], including TRS-L, SL3 and part of SL2, and encoding the 7-amino acid sequence DLFSKRT) within the N gene at the

end of its SR region, as exemplified by isolate QTO33828 (USA/Texas, Figure 4A). The 5'-UTR-derived segment changes 5 of 7 positions, including R203K/G204R, which are known to be frequent co-occurring mutations in the N protein; however, the rest of the N protein sequences are well conserved with only 1 or 2 amino acid differences in the isolates identified. In another set of SARS-CoV-2 isolates, as exemplified by isolate EPI-ISL_3434731 (Brazil/Espirito Santo) in Figure 4A, the same 5'-UTR-derived sequence is present in N but without the leucine (L) residue and the phenylalanine (F) changed to serine (S), more closely approaching the Wuhan reference strain sequence.

The predicted RNA structures of the N protein without and with (Nx) the 5'-UTR-derived insertion are shown in Figure 4C, with that for Nx being more stable with almost half the minimum free energy. Although there is no TRS-B in the N region where the intragenomic rearrangement was found, there is an inverted TRS-B that can pair with a complementary inverted TRS-B, both surrounded by U/A-rich regions which could facilitate the intragenomic rearrangement.

In total, 37 SARS-CoV-2 isolates had 5'-UTR-derived sequences in their N gene, in contrast to ~336,000 isolates with either R203K or G204K as per NCBI Virus (mutations in SARS-CoV-2 SRA data); most were isolates of the variant of concern gamma GR/501Yv3 (P1) lineage (first detected in Brazil and Japan) from Brazil, Chile, and Peru, but also alpha (B.1.1.7; first detected in Great Britain) from USA and Canada (Supplemental legend to Figure 4). The R203K/G204R co-mutation has been associated with B.1.1.7 (alpha) lineage emergence, which along with variants with the co-mutation including the P1 (gamma) lineage (Franco-Muñoz et al. 2020), possess a replication advantage over the preceding lineages and show increased nucleocapsid phosphorylation, infectivity, replication, virulence, fitness, and pathogenesis as documented in a hamster model, human cells, and COVID-19 patients including an analysis of association between COVID-19 severity and sample frequency of R203K/G204R co-mutations (Johnson et al. 2021; Mourier et al. 2022; Wu et al. 2021).

The nucleocapsid is the most abundant protein in CoVs, interacts with membrane protein (He et al 2004; Lu et al. 2021), self-associates to provide for efficient viral assembly (Yao et al. 2020), binds viral RNA

(McBride et al. 2014) and has been involved in circularization of the murine hepatitis virus genome via interaction with 3'- and 5'-UTR sequences which may facilitate template switching during subgenomic RNA synthesis (Lo et al. 2019). Phosphorylation transforms N-viral RNA condensates into liquid-like droplets, which may provide a cytoplasmic-like compartment to support the protein's function in viral genome replication (Carlson et al. 2020; Lu et al. 2021).

The phosphorylation-rich stretch encompassing amino acid residues 180 to 210 (SR region) in which the 5'-UTR-derived sequences were found, serves as a key regulatory hub in N protein function within a central disordered linker for dimerization and oligomerization of the N protein, which is phosphorylated early in infection at multiple sites by cytoplasmic kinases (reviewed in Carlson et al. 2020). Serine 202 (numbering of reference Wuhan strain), which is phosphorylated by GSK-3, is conserved in the 5'-UTR-derived sequence next to the R203K/G204R co-mutation, as is threonine 205, which is phosphorylated by PKA (Kemp et al. 1977; Kennelly et al. 1991). R203 and G204 mutations affect the phosphorylation of serines 202 and 206 in turn affecting binding to protein 14-3-3 and replication, transcription, and packaging of the SARS-CoV-2 genome (Surjit et al. 2005; Tugaeva et al. 2021; Tung and Limtung 2021).

The N gene displays rapid and high expression, high sequence conservation, and a low propensity for recombination (Dutta et al. 2020; Jaroszewski et al. 2021; Nikolaidis et al. 2021). However, it can show variation driven by internal rearrangement which does not affect the length of the protein. The N protein is highly immunogenic, and its amino acid sequence is largely conserved, with the SR region being a strong immunodominant B-cell epitope (Oliveira et al. 2020) as highlighted in Figure 4A.

**Intragenomic rearrangements alter the Nidovirus RNA-dependent RNA polymerase associated nucleotidyl transferase (NiRAN) domain (SARS-CoV-2)**

Another example of intragenomic rearrangement is the presence of the translated sequence (DLFSK) of a shorter segment of 5' UTR (nts. 56-70 in Wuhan reference strain, including parts of SL2 and SL3 but not TRS-L) at amino acids 36-40 of the NiRAN domain of the viral RdRp (nsp12) in isolates QVL75820

(EPI_ISL_1209225, USA/Seattle, 2021-03-28; lineage: B.1.2 [Pango v.3.1.20 2022-02-02]) and EPI_ISL_1524008 (USA/Washington, 2021-03-28; VOC Alpha GRY (B.1.1.7+Q.*) first detected in the UK) and at amino acids 146-150 in isolates UFT72204 (EPI_ISL_6912949, USA/Colorado, 2021-10-27; VOC Delta GK [B.1.617.2+AY.*] first detected in India), EPI_ISL_1384819 (India/Maharashtra, 2021-02-12; lineage: B.1.540 [Pango v.3.1.20 2022-02-02]) and EPI_ISL_1703925 (India/Maharashtra, 2021-02-07; B.1.540 lineage), respectively (Figure 5A). The latter strains have only one amino acid change outside of the insertions relative to the Wuhan reference strain. A subsegment of 5'-UTR (nts. 62-70) translated as FSK is present at the more proximal site (amino acids 38-40) in 230 isolates isolated from diverse populations at various times (listed in Supplemental legend to Figure 5) and exemplified by isolate UHP90975 [USA/Wisconsin, 2021-12-13] in Figure 5A. Isolate QZM71485 (USA/New York, 2021-08-05) exemplifies isolates with the FSK sequence at the more distal site (amino acids 148-150). Examples of the most common single amino acid changes in overlapping segments of other isolates are listed as comparators, and they have similar or lower frequency than those of the 5'-UTR-derived segments. However, the Wuhan reference strain sequence corresponding to the areas with 5'-UTR sequences is the most abundant among SARS-CoV-2 isolates.

Genes encoding components of the replication-transcription complex, such as the RdRp (nsp12) (Hartenian et al. 2020; Lauber et al. 2013), are highly conserved and have a low propensity for recombination among CoVs (Nikolaidis et al. 2021). The nsp12 NiRAN domain is one of the five replicative peptides that are common to all Nidovirales and used for species demarcation because it is not involved in cross-species homologous recombination (Gorbalenya et al. 2020). However, as in other examples here of conserved genes, it is involved in intragenomic rearrangements of 5'-UTR-derived sequences. Figure 5B shows the predicted structure RNA structures for the proximal site of intragenomic rearrangement in nsp12 and nsp12x (with 5'-UTR-derived sequence). As in the case of the example in the intragenomic rearrangement in N there is no TRS-B at the site of intragenomic rearrangement, which is

however preceded by a torovirus-like attenuation sequence within a U/A-rich region which may facilitate pausing of the RdRp.

The NiRAN domain of nsp12 is involved in the NMPylation of nsp9 (Slanina et al. 2021) during the formation of the replication-transcription complex (interface regions [Yan et al. 2021] are shown with yellow bars and key residues therein with ochre letters in Figure 6). The 5'-UTR-derived sequence at the proximal site in the nsp12 NiRAN domain overlaps with one of the interface regions with nsp9 but does not affect key interface residues or alter the charge distribution of amino acid side chains in the overlap region. The nsp12 NiRAN domain also exhibits a kinase/phosphotransferase like activity (Dwivedy et al. 2021), is involved in protein-primed initiation of RNA synthesis (Lehmann et al. 2015) and catalyzes the formation of the cap core structure (GpppA; contact regions with GDP [Yan et al. 2021] indicated with blue boxes and key residues therein in ochre in Figure 5A) (Park et al. 2022). The 5'-UTR-derived sequence at the proximal site in nsp12 NiRAn domain is close to the first contact region with GDP.

**Intragenomic rearrangements in β-CoVs of Merbecovirus, Embecovirus, and Nobecovirus subgenera**

Merbecovirus

As shown in Figure 6A, a segment of the 5'-UTR of the β-CoV Merbecovirus MERS-CoV including TRS-L and part of the second of the two stem-loops is present in the intergenic region between p3 and p4a in isolate MG923473 (Burkina Faso, 2015) and at the carboxyl-terminal end of p4b in isolate MK564475 (Ethiopia, 2017). In the latter case, the last 4 amino acids (HPGF) of p4b in the reference MERS-CoV sequence (NC_019843) are replaced by two amino acids (QL). The Q residue is encoded by a cytosine present in the reference sequence (indicated in orange in Fig. 6A) and two adenosines incorporated by the 5'-UTR-derived sequence. Figure 6B depicts the predicted RNA secondary structures without and with the insertion corresponding to the intragenomic rearrangement between p3 and p4b. The

structures have similar predicted free minimum energy, and the rearrangement involves the intergenic TRS-B sequence which is preceded and succeeded by torovirus-like attenuation sequences.

p4a, a double stranded RNA-binding protein, as well as p4b and p5 of MERS-CoV are type-I IFN antagonists (Liu et al. 2014; Matthews et al. 2014; Niemeyer et al. 2913; Siu et al. 2014). p4a prevents dsRNA formed during viral replication from binding to the cellular dsRNA-binding protein PACT and activating the cellular dsRNA sensors RIG-I and MDA5 (Niemeyer et al. 2013; Siu et al. 2014). p4a is the strongest in counteracting the antiviral effects of IFN via inhibition of both its production and Interferon-Stimulated Response Element (ISRE) promoter element signaling pathways (Yang et al. 2013). Therefore, the intragenomic rearrangements found in MERS-CoV may facilitate immune evasion by bringing regulatory sequences to the intergenomic regions preceding the 4a and 5 genes and facilitating their expression.

Embecoviruses

The β-CoV Embecovirus hCoV-HKU1 is a sister taxon to murine hepatitis virus and rat sialodacyoadenitis virus (Corman et al. 2018). Out of 48 HKU-1 isolates in GenBank, a 5'-UTR sequence including TRS-L, SL3 and most of SL2 (nucleotides 42-74 in hCoV-HKU-1 references NC_006577 and AY597011) is present in 31 isolates (65%) between the S and Ns4 genes (Figure 7A). The hCoV-HKU-1 NS4 protein is structurally similar to the hCoV-OC43 ns5a protein whose function is detailed below.

All 245 full genome isolates of the β-CoV Embevovirus hCoV-OC43 in GenBank had 5'-UTR-leader derived sequences (largest spanning nucleotides 35-67 of the hCoV-OC43 reference strain KJ958218) between the spike (S) and Nsp5a genes (Figure 7B). The insertions did not affect the protein sequences of either S or Nsp5a. The hCoV-OC43 5'-UTR sequence inserted is identical to that of bovine coronavirus (BCoV) 5'-UTR except for one nucleotide (an adenosine substituted by a guanosine in BCoV) up to the TRS-B, and sequences of varying length after the TRS-B show similarities to BCoV 5'-UTR ,which is consistent with a most probable bovine or swine coronavirus origin for hCoV-OC43 (Vijgen et al. 2005).

The 5'-UTR-derived insertion sequence is also present in a molecularly characterized cloned hCoV-OC43 S protein gene (Mounir et al. 1993).

hCoV-OC43 ns5a, as well as ns2a, M, or N protein significantly reduced the transcriptional activity of ISRE, IFN-β promoter, and NF-κB-RE following challenge of human embryonic kidney 293 (HEK-293) cells with Sendai virus, IFN-α or tumor necrosis factor-α (Beidas et al. 2018a). Like SARS-CoVs and MERS-CoV, hCoV-OC43 can downregulate the transcription of genes critical for the activation of different antiviral signaling pathways (Beidas et al., 2018b), and the intragenomic rearrangements described in the intergenic region preceding hCov-OC43 ns5a may facilitate immune evasion as was mentioned above for other immunomodulatory accessory proteins.

The Spike (S) gene encodes a structural protein that binds to the host receptors and determines cell tropism as well as the host range. As mentioned in the Introduction, the neighborhood of the spike gene, particularly the region before the S gene, is a hotspot for modular intertypic homologous and non-homologous recombination in coronavirus genomes (Nikolaidis 2021). In the cases described above for hCoV-HKU-1 and hCoV-OC43, intragenomic rearrangements involved the intergenic region at the end of the S gene highlighting a potential source of regulatory sequences that may affect expression of adjoining genes.

Nobecoviruses

An intragenomic rearrangement involving a 5'-UTR sequence (nucleotides 1-55) to the C-terminal cytoplasmic Y1 domain of nsp3 (nucleotides 6837-6891; amino acids 2188-2205), is seen in the β-CoV subgenus Nobecovirus of African bats, namely isolates MIZ240 (OK067321) and MIZ178 (OK067320) from *Rousettus madagascariensis* bats and isolates CMR900 (MG693169; protein: AWV67046), CMR705-P13 (MG693172, protein: AWV67070), and unclassified (NC_048212) from *Eidolon helvum* bats (Cameroon). Using the translated nucleotide sequence as query, the following additional isolates were detected: *Eidolon helvum* (Cameroon) isolates CMR704-P12 (YP_009824989 and YP_009824988),

and CMR891-892 (AWV67062). The 5'-UTR sequence involved in this intragenomic rearrangement does not include the TRS-L and includes a stem-loop structure highlighted in grey in Figure 8A. The position of the translated sequence of the 5'-UTR identical sequence is amino acids 2188-2205, which corresponds to amino acids 1567-1584 in SARS-CoV-2 nsp3. Figure 8B depicts the predicted secondary structures of nsp3 and nsp3x with the intragenomic rearrangement. Both structures have similar predicted minimum free energy. Although there are no TRS-B sequences present in this region the rearrangement takes place adjacent to an inverted complementary TRS-B within a U/A-rich region.

Nsp3 protein, the largest protein encoded by CoVs encompasses up to 16 modular domains. The N-terminal cytosolic domains include a mono-ADP-ribosylhydrolase (Alhammad et al. 2021), a papain-like protease (Lei et al. 2018), and a scaffold region that participates in replication-transcription complex assembly (Imbert et al. 2008). After the latter domains, there are two transmembrane domains (TM1 and TM2) with an endoplasmic reticulum luminal loop (Ecto3) between them, and two cytosolic domains (Y1 and CoV-Y) following TM2. The nsp3 segment encoded by the 5'-UTR-derived sequence falls in the cytosolic domain Y1. Nsp3C anchors nsp3 to the endoplasmic reticulum membrane and induces membrane rearrangement leading to double membrane vesicle formation via a yet unknown molecular mechanism (Angelini et al. 2013; Hagemeijer et al. 2014). Although there are structural data on the CoV-Y domain (Pustovalova et al. 2021), its function is unknown as is that of the Y1 domain. Therefore, although the nsp3 sequence is well conserved among bat Nobecoviruses, the significance of the nsp3 segment encoded by the 5'-UTR-derived sequence, which might affect double vesicle membrane formation, remains to be determined.

**Intragenomic rearrangement in nsp2 of rodent α-CoVs subgenus Luchacovirus**

As shown in Figure 9A, a segment of the 5'-UTR (nts. 1-119) of the Luchacovirus AcCoV-JC34 (KX964649; isolated in China, 2011-10 from the rodent *Apodemus chevieri*) was duplicated, inverted, and translocated to the genomic region encoding the nonstructural protein nsp2 (nts. 1679-1760). The latter sequence in nsp2 differs by only one nucleotide from that in the 5'-UTR (99% similarity), and it is also

present with varying degrees of similarity in other rodents. Two examples are shown in Figure 9A for isolates from rat (Lucheng Rn rat CoV isolate Ruian 83; MT820626; isolated from *Rattus norvegicus* in China, 2014, 76% similar), and mouse (Fievel mouse CoV strain FiCoV/UMN2020 (OK655840; isolated from *Mus musculus* in USA, 2018, 59% similar). Other isolates (listed by rodent of origin) with intragenomic rearrangements in nsp2 with nucleotide sequences up to 75% similar to the 5'-UTR-derived sequences include: *Apodemus chevrieri* (MT820625, China, 2015, 93% similar); *Apodemus agrarium* (MZ328302, China, 2016, 93% similar); *Eothenomys miletus* (MT820627, China, 2014, 81% similar); *Eothynomys melanogaster* (KY370054, China, 2015-12, 79% similar); *Myodes rufocanus* (KY370045, China, 2014-08, 79% similar); *Rattus losea* (KY370050, China, 2015-05, 78% similar); *Rattus norvegicus* (MK163627, United Kingdom, 2014-06-23, 78% similar; NC_032730, China, 2013; MT549854, China, 2016-12, 76% similar; MW802582, China, 2017-03-07, 76% similar); and *Brylmys bowersi* (MZ328301, China, 2016, 77% similar).

There appears to be a temporal gradient with the most similar sequence (99%) in isolate KX964649 (China, 2011-10) to the least similar (59%) in isolate OK655840 (USA, 2018). The temporal gradient of decreasing similarity holds within rodents from the same genus, which would suggest that the translocated sequence is the oldest and the rest reflect more recent mutations. This is consistent with a possible common ancestor for all rodent α-CoVs sampled so far, with phylogenetic analyses suggesting relatively frequent host-jumping among the different rodent species (Tsoleridis et al. 2019). The minimum free energy of the predicted RNA secondary structures of the intragenomic rearrangement and adjacent sequences increases from the most similar to the least similar to the 5'-UTR insertion (Figure 9B). The function of the region of intragenomic rearrangement in nsp2 remains to be determined and it does not overlap with that contributing to inflammation via NF-kB activation in the α-CoV porcine transmissible gastroenteritis virus (Wang et al. 2018).

**Intragenomic rearrangements in N of bat α-CoVs subgenus Nyctacovirus.**

As shown in Figure 10A, in this intragenomic rearrangement in bat α-CoVs subgenus Nyctacovirus, a 115-nucleotide-long segment of the 5'-UTR is duplicated, inverted (negative-sense strand) and translocated to the proximal end of the nucleocapsid gene thereby encoding the first 38 amino acids of the amino-terminus of N. Other variants share the sequence with lesser similarity to the 5'-UTR sequence. There is a TRS-B sequence (AACUAA) at the beginning of the insertion, and the negative strand 5'-UTR -derived sequence also has a AACUAA sequence, which may have mediated the intragenomic rearrangement. The functional significance of this intragenomic rearrangement remains to be determined. Although in infectious bronchitis virus, the amino terminal domain of N has been shown to interact with nucleotide sequences in the 3'-UTR which is relevant to viral RNA packaging, the amino acids that are critical for such interaction are more distally located in the amino terminus (amino acids 76 or 94) (Tan et al. 2006; Zhou and Collisson, 2000) than those encoded by the intragenomic rearrangement in this case.

**Intragenomic rearrangements in ORF5b/4b of bat α-CoVs subgenus Decacovirus.**

Orb5b and ORF4b proteins are 53% similar (including conservative substitutions) between bat alphacoronaviruses subgenus Decacovirus shown in Figure 10B. In both sets of viruses, ORF5b or ORF4b overlap the beginning of the membrane (M) gene, i.e., there is no intergenic region between them and M. However, there is a TRS-B sequence (AACUAA) within the 3' end of ORF5b and ORf4b where the intragenomic rearrangement occurs. Viruses with similar intragenomic rearrangements are for ORF5b: *Rhinolophus* bat coronavirus HKU32 strain TLC28A (MK720946), *Rhinolophus* bat coronavirus HKU32 strain TLC26A (MK720945; Hong Kong, 08-06-2005), Alphacoronavirus sp. strain bat/Yunnan/HcYN26/2020 (MZ081384; *Hipposideros cineraceus*; China, 07-29-2020), Alphacoronavirus sp. strain bat/Yunnan/RsYN12/2019 (MZ081386; *Rhinolophus sinicus*; China, 10-22-2019), Alphacoronavirus sp. strain bat/Yunnan/MmYN16/2020 (MZ081385; *Myotis muricola*; China, 04-18-2020), Alphacoronavirus sp. strain bat/Yunnan/RmYN21/2020 (MZ081387; *Rinolophus malayanus*; China, 06-03-2020); and for ORF4b: Bat coronavirus isolate BtCoV/Rh/YN2012_Rs4259 (MG916903;

China, 04-17-2013), Bat coronavirus isolate BtCoV/Rh/YN2012_Rs4125 (MG916902; China, 09-16-2012). The functional significance of this intragenomic rearrangement remains to be determined.

**Intragenomic rearrangements derived from 5'-UTR sequences were not detected in some β-or α-, or in any γ- and δ-CoVs, and no 3'-UTR-derived intragenomic rearrangements were detected in any coronavirus**

A listing of the other coronaviruses analyzed beyond the ones found to have intragenomic rearrangements is provided at the end of the Supplemental section. The directionality of translocation appears to be in the 5' to 3' direction as further underscored by the absence of 3'-UTR-derived insertions in any of the viruses analyzed.

## Discussion

We here describe intragenomic rearrangements involving 5'-UTR-derived sequences and the coding section of the genome of β- and α-CoVs. Figure 11A summarizes the locations of insertions (yellow arrows) in accessory, structural, and nonstructural genes of SARS-CoV-2, which for at least the accessory and structural genes appear to involve and/or affect the template switching mechanism by creating new regions of homology for interaction with TRS-L. We had previously reported (Patarca and Haseltine 2022) on the presence of conserved complementary sequences (CCSs) in the 5'- and 3'-UTRs potentially involved in circularization of the genome during subgenomic RNA synthesis. As shown in Figure 11B, the 5'-UTR-derived sequences involved in intragenomic rearrangements in SARS-CoV-2 shown in the present work usually include the TRS-L and span approximately half of the 5' CCS, thus potentially facilitating circularization of the genome from locations closer to the 3'-UTR.

Most of the 5'-UTR sequences duplicated and translocated include TRS-L. Extending the homology region of interaction between the TRS-L in the 5'-leader and the TRS-L introduced in a particular area of the body of the genome optimizes free minimum energy of the interaction. Such facilitation may favor

expression of certain genes over that of others, thereby altering the hierarchy in gene expression. Because insertions are in various locations of viral genes, including some encoding nonstructural proteins, they may propitiate formation of new subgenomic RNAs thereby expanding the repertoire of proteins and even transforming noncanonical subgenomic messenger RNAs, i.e., not associated with TRS homology, to canonical ones. SARS-CoV-2 and other CoVs (Bentley et al. 2013) have been reported to generate noncanonical subgenomic RNAs in abundance, accounting for up to a third of subgenomic messenger RNAs in cell culture models of infection and increasing in proportion over time (Nomburg et al. 2020).

The structural genes control genome dissemination (Lauber et al. 2013) while the accessory genes in the same region of the genome may be involved in adaptation to specific hosts, modulation of the interferon signaling pathways, the production of pro-inflammatory cytokines, or the induction of apoptosis (Cui et al. 2021; Fang et al. 2021). Gaining insight into the effect of the amino acid changes introduced by the 5'-UTR-derived sequences is likely to shed light into pathogenesis and immune evasion mechanisms. For instance, a few point mutations can have a profound effect as exemplified by the few mutations in the C-terminus of the spike protein that transform the feline CoV associated with mild disease to one, the feline infectious peritonitis virus, that is generally lethal (Rottier et al. 2005).

Intragenomic rearrangements are yet another example of the tremendous genomic flexibility of coronaviruses which underlies changes in transmissibility, immune escape and/or virulence documented during the SARS-CoV-2 pandemic.

**Limitations**

The intragenomic rearrangements involving 5'-UTR sequences were detected in all subgenera of β-coronaviruses infecting humans (i.e., Sarbecovirus, Embecovirus, and Merbecovirus) and in the Nobecovirus but not the Hibecovirus subgenera of CoVs infecting bats. There were only 3 Hibecovirus genomes in the database, which may account for the lack of detection of internal rearrangements in this subgenus most closely related to Sarbecoviruses. In this respect, the most diverse detection of

rearrangements in SARS-CoV-2 may reflect the bias generated by the presence in GenBank of SARS-CoV-2 isolates in up to 5 orders of magnitude greater number than any other CoV. However, the relative paucity of α-, γ-, or δ-CoV sequences available also applies to those of β-CoVs other than SARS-CoV-2 for which 5'-UTR rearrangements were found in notable proportions. Moreover, the present analysis included CoVs involved in large outbreaks such as the swine enteric CoVs of the α and δ genera and avian infectious bronchitis virus of the γ genus that have been studied over decades with hundreds of isolates characterized without apparent evidence for intragenomic rearrangements. The apparent absence of internal rearrangements in the latter viruses bodes well for the specificity of the findings described here for 4 of 5 subgenera of β-CoVs and 3 of 12 subgenera of α-CoVs.

Many sequences in the databases have incomplete 5'-UTRs rendering it difficult to comprehensively analyze them and to calculate more reliable proportions of variations. There are also partial genome and protein sequences, and we excluded sequences with undetermined amino acids. Nonetheless, for SARS-CoV-2, the frequency of variants with full-length insertions appears low relative to those with subsegments or other mutations relative to the reference strain in the same insertion area. One could posit that for hCoV-OC43 and hCoV-HKU-1, the apparently much higher frequency of intragenomic rearrangements involving 5'-UTR sequences might be driven by characterization of a greater number of isolates during epidemics with rearrangements possibly providing transmissibility, immune evasion and/or virulence advantages.

A limitation of the methods used for detecting these isolates is that they may not be viable, i.e., they may be associated with molecular diagnostic detection of virus but not necessarily culture conversion, or may represent artifacts of sequencing; however, their prevalence with redundancy in various locations and processing laboratories would be consistent with human-to-human transmission. Moreover, Turakhia et al. (2020), among others, have pointed out that systematic errors associated with lab-or protocol-specific practices affect some sequences in the repositories, which are predominantly or exclusively from single labs, co-localize with commonly used primer binding sites and are more likely to affect the protein-coding

sequences than other similarly recurrent mutations. Although we cannot rule out that such systematic errors may underlie some if not all the rearrangements detected, the possibility is rendered less likely by the geographic and temporal diversity of the isolates with each intragenomic rearrangement (as underscored by the data in the Supplemental section), their presence in diverse variants of concern, as well as the occurrence of rearrangements in sequences from before the pandemic era and among diverse viruses of 2 genera and various subgenera in at least three hosts (humans, bats, and rodents). Moreover, it is unlikely that the insertion in the nucleocapsid gene of SARS-CoV-2 which encodes for a common co-mutation of adjacent sites that has been shown experimentally to have functional significance reflects an artifactual event.

Intragenomic rearrangements might be more common in isolates with large deletions, as exemplified by those involving the ORF6 (Tse et al. 2021), ORF7b and ORF8 of SARS-CoV-2, which in all cases affect the C-termini of the encoded proteins. The length of the insertion does not notably affect that of the protein in isolates without major genomic deletions. For 5'-UTR segments within viral genes, such as the examples shown in N, nsp12 and nsp3, or intergenic regions, the length of the protein or intergenic region appears not to be affected.

Variation driven by internal rearrangements is distinct from the non-homologous recombination events proposed as origins of Sarbecovirus/Hibecovirus/Nobecovirus β-CoV ORF3a by gene duplication followed by rapid divergence from M (Nikolaidis et al. 2021; Ouzounis et al. 2020) or of SARS-CoV-2 ORF8 from ORF7a (Neches et al. 2021). The mechanisms underlying intragenomic rearrangements warrant further study. Understanding the variation that they introduce also is of relevance in the design of prophylactic and therapeutic interventions for all coronaviruses, including a pan-β-coronavirus vaccine.

**Materials and methods**

To assess the presence of 5'-UTR-derived insertions in the body of the genome, we used 5- to 10-amino acid stretches from the 3 reading frames of the translated 5'-UTR nucleotide sequence of SARS-CoV-2

(Wuhan reference, NC_045512) as query sequences to search the GenBank® database using the Basic Alignment Search Tool (BLAST)P® (Protein BLAST: search protein databases using a protein query (nih.gov); Altschul et al. 1997) for SARS-CoV-2 and SARS-CoV-related viral proteins encoding similar stretches. All nonredundant translated CDS + PDB + SwissProt + PRF excluding environmental samples from WGS projects were searched specifying severe acute respiratory syndrome coronavirus 2 as organism.

Using the accession number listed in PubMed (SARS-CoV-2 Resources - NCBI (nih.gov)) for the viral protein sequence, we obtained the respective nucleotide sequence and translated it using the insilico (DNA to protein translation (ehu.es) [Bikandy et al. 2004] and Expasy (ExPASy - Translate tool [Duvaud et al. 2021]) tools to determine by manual inspection and the BLASTN program (Johnson et al. 2008) if the nucleotide sequences encoding said stretches were identical to those in the 5'-UTR nucleotide sequence of SARS-CoV-2 or SARS-CoV-related viruses.

To detect isolates with similar insertions whose sequences had not been included in GenBank, we then searched the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ database of SARS-CoV-2 sequences (GISAID - Initiative; Elbe et al. 2017; Khare et al. 2021; Shu et al. 2017) using as queries the nucleotide sequences of the insertions plus adjoining 20 nucleotides on either side from the viral genomes. This approach is limited by the fact that maximum number of search results in GISAID is 30. Information on location and timing of isolate collection was obtained from the GenBank and GISAID databases.

We used the Rfam database (http://rfam.xfam.org/covid-19) with the curated Stockholm files containing UTR sequences, alignments and consensus RNA secondary structures of major genera of Coronoviridae; the representative RefSeq sequences for each genus obtained from the International Committee on Taxonomy of Viruses (ICTV) taxonomy Coronaviridae Study Group (2020 release; https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/223/coronaviridae-figures); the reference sequences in the GenBank database; and

listings in publications involving phylogenetic analyses of alpha-, delta-, and gamma-coronaviruses from NCBI Taxonomy [Nikolaidis M. et al. 2021; Tsoleridis T. et al. 2019] to derive the 5'-UTRs of various CoVs.

We then utilized the 5'-UTR segments as query sequences to search for insertions in their respective genomes (nucleotide collection [nr/nt]; expect threshold: 0.05; mismatch scores: 2, -3; gap costs: linear). The GSAID database does not include sequences of CoVs other than SARS-CoV-2 and therefore could not be used for this analysis. Using nucleotide sequences instead of translated amino acid sequences from the 5'-UTR in the 3 reading frames as query sequences was unproductive to detect insertions in SARS-CoV-2 because of the large number of SARS-CoV-2 sequences in the GenBank database and the limit of 5000 results in the BLAST algorithm settings which yielded solely 5'-UTR sequences.

If the intragenomic rearrangement detected using the 5'-UTR sequences involved a coding region, we translated the 5'-UTR insertion and adjacent segments using the insilico (DNA to protein translation (ehu.es) [Bikandy et al. 2004] and Expasy (ExPASy - Translate tool [Duvaud et al. 2021]) tools.

RNA secondary structures of the 5'-UTR-derived insertion and adjacent sequences of the intragenomic rearrangement were visualized using forna, a force directed graph layout (ViennaRNA Web services; [Kerpedjiev et al. 2015]), and the optimal secondary structures and their minimal free energies were determined using the RNAfold webserver [Gruber et al. 2008; Lorenz et al. 2011].

In terms of the locations of the insertions in the body of the genomes, the boundaries of nonstructural, structural, and accessory open reading frames were determined based on GenBank annotation and from manual inspection of multiple alignments and sequence similarities. In the results presented, we excluded matches to entries corresponding to the 5'-leader sequences in mRNAs from full viruses or defective interfering RNA particles, as well as protein sequences with >80% unknown amino acids (represented by the letter X) in GenBank. We also tested the 3'-UTR sequences using the same approaches described for the 5'-UTR ones. The Supplementary section includes the accession numbers and collection site and date,

and in some cases the SARS-CoV-2 lineages, for the isolates with intragenomic rearrangements involving 5'-UTR sequences.

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**Figure legends**

**Figure 1. A. Discontinuous synthesis of SARS-CoV-2 negative strand subgenomic RNA**. For the synthesis of subgenomic RNA, the leader transcription regulatory sequence (TRS-L, blue box) within the 5’-leader sequence interacts with homologous TRSs in the body (TRS-B) of the genome that precede

structural (red boxes) and accessory (green boxes) genes. Overlapping genes for ORF3a (namely, ORF3b-d) and N (namely, ORF9b, c) that would be translated from the sgRNAs shown for ORF3a and N, respectively, are shown at the bottom. **B. Secondary structure of the 5'-UTR including the 5'-leader sequence and translated sequence of the 5'-leader sequence and beyond until the stop codon before stem-loop (SL)5.** The secondary structure of the 5'-UTR is shown as presented in Miao et al. (2021). The 5' leader sequence extends from the cap structure ($m^7G$) to the TRS-L and encompasses SL1-3, which have been associated with viral replication and gene expression. An open reading frame spans most of the 5'-UTR (Wuhan reference strain, NC_045512 shown) except for SL5, where ORF1ab starts. The segment of the open reading frame that is translocated wholly or partially in SARS-CoV-2 variants is underlined. At the nucleotide level, the segment includes the TRS-L, and at the amino acid level, the translated 5'-leader sequence and beyond includes a predicted upstream open reading frame (uORF, grey box) which has not been shown to be functional and whose initiation methionine [M] is shown in gray.

**Figure 2. Modified carboxyl-termini of ORF8 encoded by a 5'-UTR-derived insertion in SARS-CoV-2. A.** The largest 5'-UTR segment that was duplicated and translocated as an insertion to the carboxyl terminus of ORF8 is shown at the nucleotide and amino acid levels (latter underlined). All translocated 5'-UTR nucleotide sequence segments include TRS-L (dark blue box) with variable extents of SL3 (blue) and SL2 (red). Examples are shown, and corresponding similar sequences in GenBank as of February 20, 2022, are listed in the Supplemental legend to Figure 2. The C-terminus of ORF8 in the Wuhan reference strain is depicted using orange letters with mutations in ochre; the asterisks over the C-terminus sequence designate residues contributing to the covalent dimer interface (Arg115, Asp119, Phe120, Iso121; Flower et al. 2021). The 5'-UTR insertions are shown as underlined letters in black with mutations, deletions, and insertions within them highlighted in green. **B. Secondary structures of ORF8 RNA in reference strain and in that with longest 5'-UTR-derived intragenomic rearrangement.** Nucleotide and amino acid sequences of the carboxyl termini of ORF8 from Wuhan reference (NC_0445512) and from isolate QUP34336 (USA/Minnesota, 2021-04-05) with the longest 5'-UTR-

derived sequence. ORF8 protein from the latter has modified carboxy terminus and is therefore designated ORF8x. Amino acid sequence from the reference strain is highlighted in yellow while that encoded by the duplicated and translocated 5'-UTR segment is highlighted in green. Stop codon of ORF8 is depicted with a red asterisk, and initiation codon of N is in green letters. TRS-B core sequence and complementary TRS-Bs in ORF8 and in ORF8x are highlighted in blue; and the uridine/adenosine tracks, including the torovirus-like attenuation sequence (Ujike and Taguchi. 2021) are highlighted in fuchsia. 5'-UTR nucleotide and amino acid sequences in ORF8X are in bold letters and highlighted in green.

**Figure 3. A. Modified carboxyl-termini of ORF8 encoded by an insertion of a 5'-UTR segment in SARS-related β-coronaviruses of *Rhinolophus* bats from China.** For SARS-related bat β-CoVs (BatSARSCoV Rf1/2004 and Bat CoV 273/2005 are subgroup 2b; Menachery et al. 2015), all inserted terminal sequences were the same. The nucleotide sequence of the inserted 5'-UTR segment differed from that of SARS-CoV-2 by two nucleotides: a C to U change (underlined) which translates into an amino acid change (serine [S] to phenylalanine [F]), and a U to A (underlined) which introduces a stop codon. **B. Modified carboxyl termini of ORF7b encoded by an insertion of a 5'-UTR segment in SARS-CoV-2.** The two isolates with modified ORF-7 proteins are QXH28554 (USA/Alabama, 2021/04/14), and QSV08409 (USA/California; 2021/02/26); the latter has a truncated ORF7b and the former a truncated ORF8. Color codes and abbreviations are as in Figures 1 and 2. **C. Secondary structures of RNAs for ORF7b and ORF7bx.** Color scheme is as in Figure 2B. An HIV-like attenuation sequence (Harrison et al. 1998) is also highlighted.

**Figure 4. A. Insertion of 5'-UTR segment into the serine-rich region of the nucleocapsid (N) in SARS-CoV-2.** The R203K and G204R amino acid substitutions (blue arrows) which are commonly present concomitantly are encoded in this case by the insertion of a 5'-TR segment into the SR-rich region of the N protein at the end of a strong immunodominant B-cell epitope (purple box; Oliveira et al. 2020). B. **Secondary structures of RNAs for N and Nx.** Color scheme is as in preceding Figures.

**Figure 5. A. Insertions of a 5'-UTR sequence into two sites within the Nidovirus RdRp associated nucleotidyl transferase (NiRAN) domain of the RNA-dependent RNA polymerase (nsp12) of SARS-CoV-2.** Examples of isolates with 5'-UTR-derived sequences at the proximal and distal sites are provided in the figure and a full listing is provided in the Supplemental legend to Figure 6, as is a listing of variants with single amino acid changes relative to the Wuhan reference strain in the segment corresponding to the insertion. The Wuhan reference strain sequence corresponding to the insertion areas is the most abundant among SARS-CoV-2 isolates. The nsp12-nsp9 interface regions are shown with yellow bars and key residues therein with ochre letters, while the contact regions with GDP are indicated with blue boxes and key residues therein in ochre. **B. Secondary structures for RNAs in the proximal sites in nsp12 and nsp12x.** Color scheme is as in previous figures. The site for -1 frameshifting is also highlighted.

**Figure 6. A. Intragenomic rearrangement with 5'-UTR sequences present in the intergenic regions between p3 and 4a as well as between p4b and p5 of the Merbecovirus Middle East respiratory syndrome (MERS)-CoV.** A segment of the 5'-UTR of the MERS-CoV including TRS-L and part of the second of the two stem-loops is present in the intergenic region between p3 and p4a in isolate MG923473 (Burkina Faso, 2015) and between p4b and p5 affecting the carboxyl-terminal end of ORF4b in isolate MK564475 (Ethiopia, 2017). In the latter case, the last 4 amino acids (HPGF) of ORF4b in the reference MERS-CoV sequence (NC_019843) are replaced by two amino acids (QL). The Q residue is encoded by a cytosine present in the reference sequence (indicated in orange color) and two adenosines incorporated by the 5'-UTR-drived sequence. **B. RNA secondary structures of the intergenic region between p3 and p4a in MERS-CoV without and with intragenomic rearrangement.** Color scheme is as in previous figures.

**Figure 7. A. Presence of hCoV-HKU-1 (β-CoV Embecovirus) of 5'-UTR-derived sequence in the intergenic region between the spike (S) and the Ns4 genes.** The hCoV-OC43 5'-UTR sequence inserted is identical to that of bovine coronavirus (BCoV) 5'-UTR (shown at the bottom of the figure) except for one nucleotide (an adenosine [A] instead of a guanosine [G] in BCoV). 31 out of 48 variants

(65%) in GenBank have this intragenomic rearrangement. **B. Presence in in the intergenic region between the S and Ns5a genes hCoV-OC43 (β-CoV Embevovirus) of sequences of various lengths of the same 5'-UTR region.** As in the case of hCoV-HKU1, the 5'-UTR segment translocated to the intergenic region between S and Ns5a of hCoV-OC43 variants is similar to that of BCoV. Differences among them(all 245 isolates in GenBank) are distally to the TRS-B and involve various extents of sequences similar to the 5'-UTR of BCoV.

**Figure 8. A. Presence of 5'-UTR sequence in the Bat β-CoV Nobecovirus nsp3 gene.** All variants in GenBank with this intragenomic rearrangement are listed in the Supplemental legend to Figure 8. **B. Secondary structures of the RNAs of nsp3 and nsp3x.** Color scheme is as in previous figures.

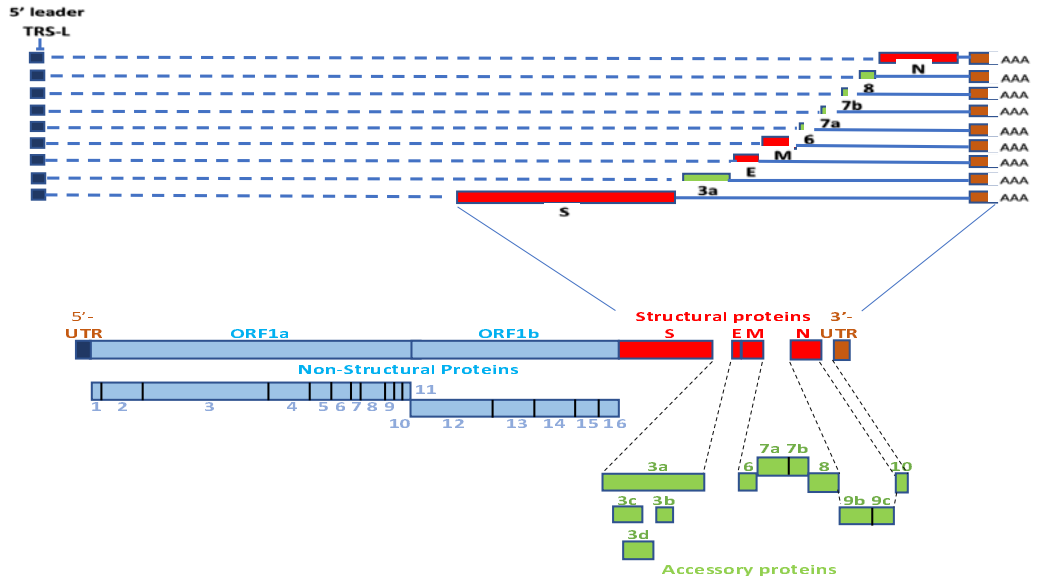
**Figure 9. Intragenomic rearrangement in nsp2 of rodent alphacoronaviruses subgenus Luchacovirus.** Nucleotide and amino acid sequences of intragenomic rearrangements are shown. Region derived from 5'-UTR (negative strand) is highlighted in green. Conservative amino acid substitutions are highlighted in blue while non-conservative ones are highlighted in red. Two examples of isolates (listed by rodent of origin) with nsp2 nucleotide sequences up to 75% similar to the 5'-UTR-derived sequences are shown. There appears to be a temporal gradient with the most similar sequence (99%) in isolate KX964649 (China, 2011-10) to the least similar (59%) in isolate OK655840 (USA, 2018). The temporal gradient holds within animals from the same genus, which would suggest that the translocated sequence is the oldest and the rest reflect more recent mutations. For the predicted secondary structures of the RNAs corresponding to the intragenomic rearrangement and adjacent sequences, the minimum free energy increases among variants from those with the most to those with the least similar sequence to the 5'-UTR insertion. Color scheme is as in previous figures.

**Figure 10. Intragenomic rearrangements in N of bat alphacoronaviruses subgenus Nyctacovirus (A) and in ORF5b or ORF4b of bat alphacoronaviruses subgenus Decacovirus.** Color scheme is as in Figure 9.

**Figure 11. A. Summary of locations of intragenomic rearrangements identified in SARS-CoV-2.** Nonstructural structural and accessory genes are represented in yellow, red, and green, respectively. **B. Partial overlap between the 5'-UTR nucleotide segment that is translocated to viral genes and a predicted sequence potentially involved in circularization of genome during viral replication.** The previously reported conserved complementary sequences (CCSs) in the 5'- and 3' UTRs potentially involved in circularization of the genome during subgenomic RNA synthesis (Patarca and Haseltine 2022) are shown. The insertion sequences usually include the TRS-L and span approximately half of the 5' CCS, thus potentially facilitating circularization of the genome from locations closer to the 3'-UTR.

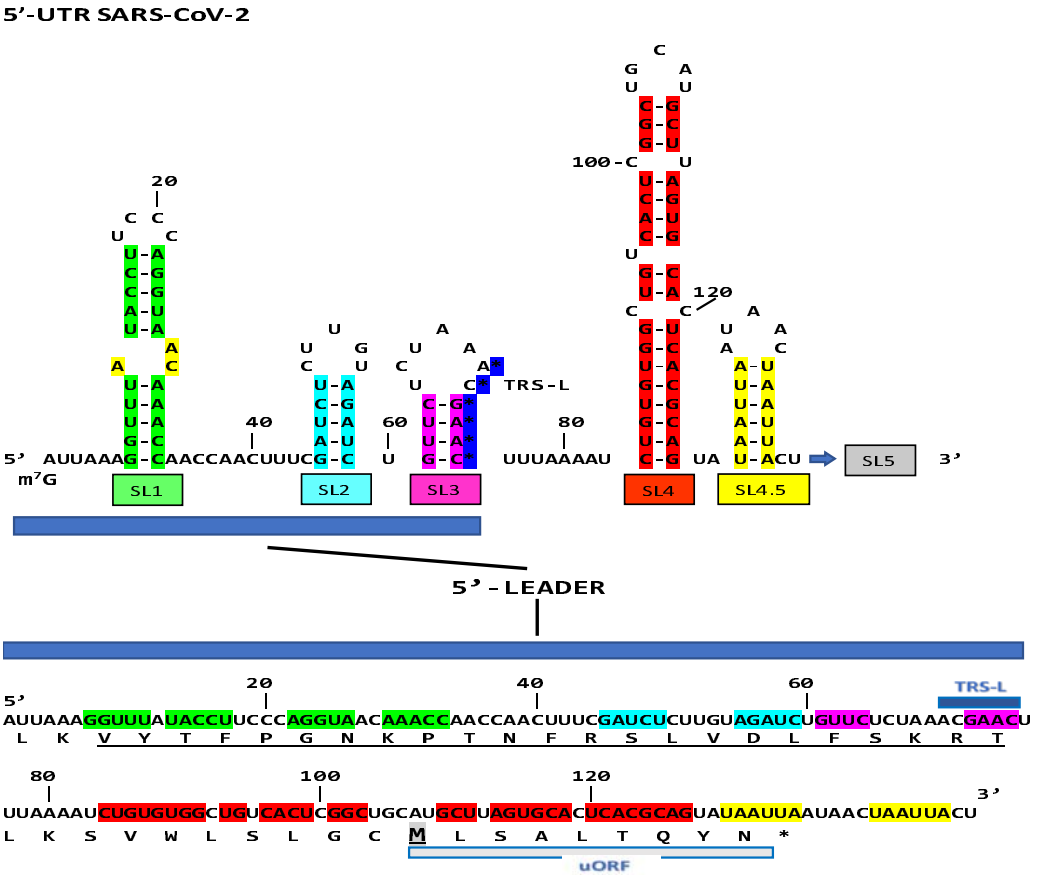
**Figure 1.**

**A.**



5' leader
TRS-L
N
8
7b
7a
6
M
E
3a
S
AAA
5'-UTR
ORF1a
ORF1b
Structural proteins
S
E M
N
3'-UTR
Non-Structural Proteins
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
3a
3c 3b
3d
7a 7b
6
8
10
9b 9c
Accessory proteins

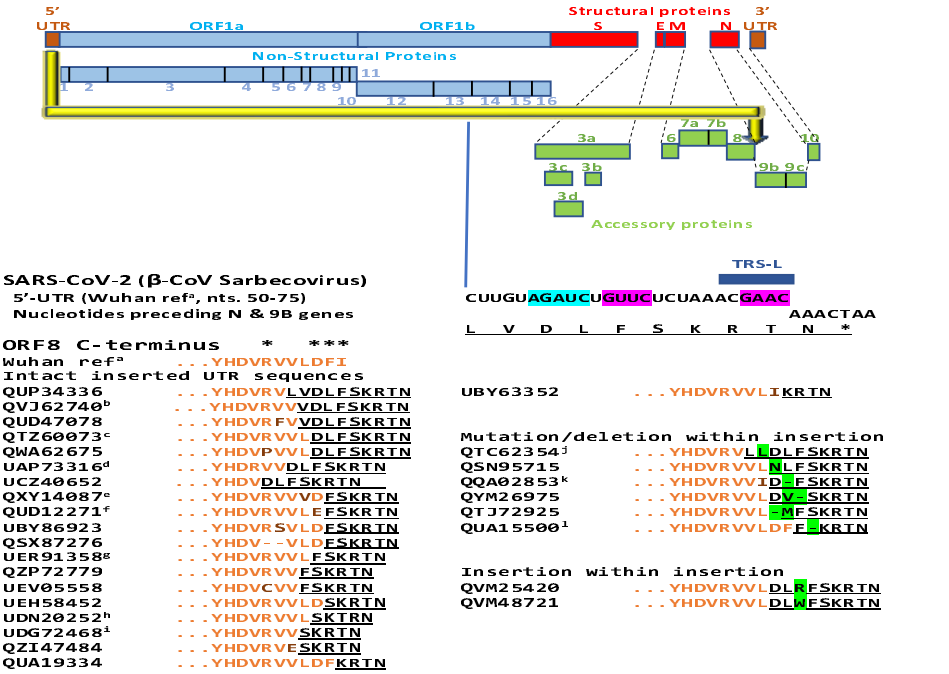

**B.**



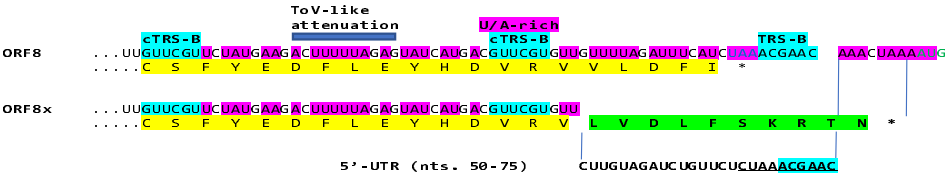
5'-UTR SARS-CoV-2
5' AUUAAAG-CAACCAACUUUCG-C U UUUAAAAU UA U-ACU
m7G
SL1
SL2
SL3
SL4
SL4.5
SL5
3'
TRS-L
5'-LEADER
5' AUUAAAGGUUUAUACCUUCCCAGGUAACAAACCAACCAACUUUCGAUCUCUUGUAGAUCUGUUCUCUAAACGAACU
L K V Y T F P G N K P T N F R S L V D L F S K R T
UUAAAAUCUGUGUGGCUGUCACUCGGCUGCAUGCUUAGUGCACUCACGCAGUAUAAUUAAUAACUAAUUACU
L K S V W L S L G C M L S A L T Q Y N *
uORF

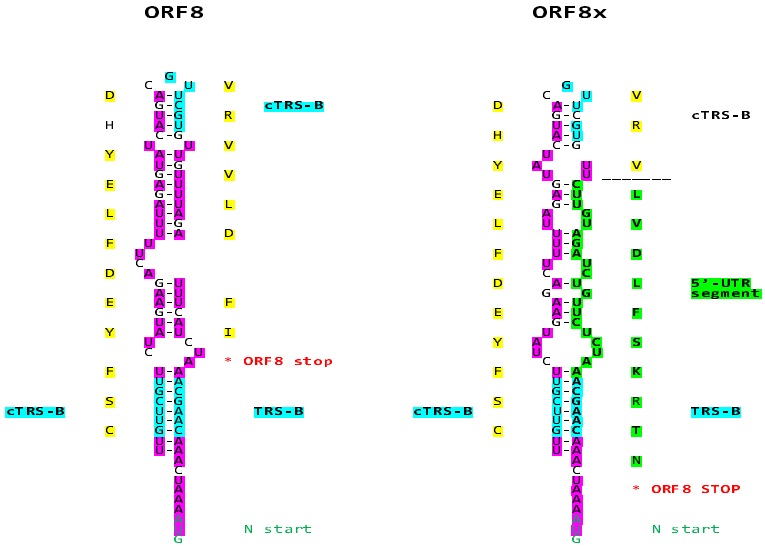
**Figure 2.**

**A.**


5' UTR
ORF1a
ORF1b
Structural proteins
S
E M
N
3' UTR
Non-Structural Proteins
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
3a
3c 3b
3d
6
7a 7b
8
9b 9c
10
Accessory proteins
TRS-L
SARS-CoV-2 (β-CoV Sarbecovirus)
5'-UTR (Wuhan ref[a], nts. 50-75)
Nucleotides preceding N & 9B genes
CUUGUAGAUCUGUUCUCUAAACGAAC
AAACTAA
L V D L F S K R T N *
ORF8 C-terminus * ***
Wuhan ref[a] ...YHDVRVVLDFI
Intact inserted UTR sequences
QUP34336 ...YHDVRVLVDLFSKRTN
QVJ62740[b] ...YHDVRVVVDLFSKRTN
QUD47078 ...YHDVRFVVDLFSKRTN
QTZ60073[c] ...YHDVRVVLDLFSKRTN
QWA62675 ...YHDVPVVLDLFSKRTN
UAP73316[d] ...YHDRVVDLFSKRTN
UCZ40652 ...YHDVDLFSKRTN
QXY14087[e] ...YHDVRVVVDFSKRTN
QUD12271[f] ...YHDVRVVLEFSKRTN
UBY86923 ...YHDVRSVLDFSKRTN
QSX87276 ...YHDV--VLDFSKRTN
UER91358[g] ...YHDVRVVLFSKRTN
QZP72779 ...YHDVRVVFSKRTN
UEV05558 ...YHDVCVVFSKRTN
UEH58452 ...YHDVRVVLDSKRTN
UDN20252[h] ...YHDVRVVLSKTRN
UDG72468[i] ...YHDVRVVSKRTN
QZI47484 ...YHDVRVESKRTN
QUA19334 ...YHDVRVVLDFKRTN
UBY63352 ...YHDVRVVLIKRTN
Mutation/deletion within insertion
QTC62354[j] ...YHDVRVLLDLFSKRTN
QSN95715 ...YHDVRVVLNLFSKRTN
QQA02853[k] ...YHDVRVVID-FSKRTN
QYM26975 ...YHDVRVVLDV-SKRTN
QTJ72925 ...YHDVRVVL--MFSKRTN
QUA15500[l] ...YHDVRVVLDFE-KRTN
Insertion within insertion
QVM25420 ...YHDVRVVLDLRFSKRTN
QVM48721 ...YHDVRVVLDLWFSKRTN

**B.**


ToV-like attenuation
U/A-rich
cTRS-B
cTRS-B
TRS-B
ORF8
...UUGUUCGUUCUAUGAAGACUUUUUAGAGUAUCAUGACGUUCGUGUUGUUUUAGAUUUCAUCUAAACGAAC AAACUAAAAAG
.....C S F Y E D F L E Y H D V R V V L D F I *
ORF8x
...UUGUUCGUUCUAUGAAGACUUUUUAGAGUAUCAUGACGUUCGUGUU
.....C S F Y E D F L E Y H D V R V L V D L F S K R T N *
5'-UTR (nts. 50-75)
CUUGUAGAUCUGUUCUCUAAACGAAC


ORF8
ORF8x
cTRS-B
cTRS-B
TRS-B
* ORF8 stop
N start
5'-UTR segment
* ORF8 STOP
ΔG=-18.1 Kcal/mol
ΔG=-11.4 Kcal/mol
1 isolate

# Figure 3.

**A.**

SARS-related β-CoVs of *Rhinolophus* bats (China)

5'-UTR (2 nts. changes vs. SARS-CoV-2)

TRS-L

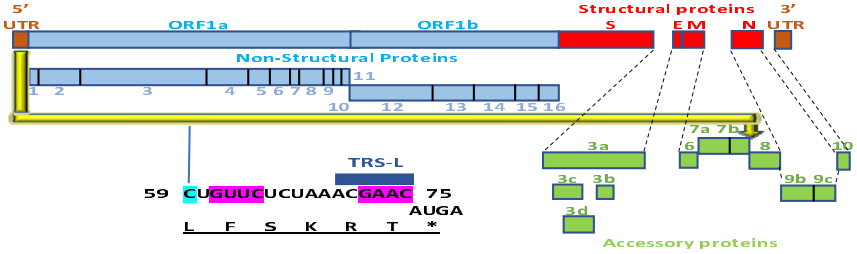
GUAGAUCUGUUCUUUAAACGAACUUAA

V D L F F K R T *

ORF8 C-terminus FRDIHVDLFFKRT

AAZ67036 Bat SARSCoV Rf1/2004
AIA62307 BtRf_BetaCoV/SX2013
AKZ19083 Bat SARS-like CoV YNLF_31C
AIA62297 BtRF-BetaCoV/HeB2013
ABG47066 BatCoV 273/2005

**B.**




SARS-Cov-2 ORF7b

Wuhan ref MIELSLIDFYLCFLAFLLFLVLIMLIIFWFSLELQDHNETCHA
QXH28554 MIELSLIDFYLCFLAFLLFLVLIMLIIFWFSLELQDHNETCLFSKRT
QSV08409 LAFLLFLVLIMLIIFWFSLELQDHNETCLFSKRT

**C.**

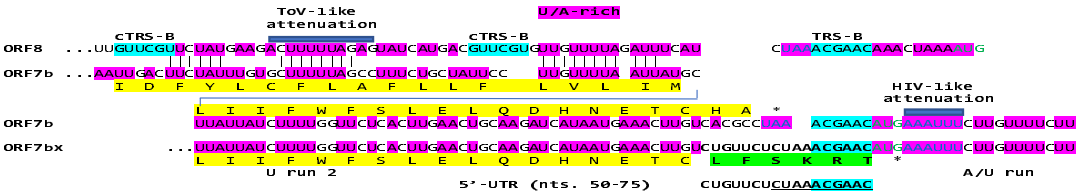
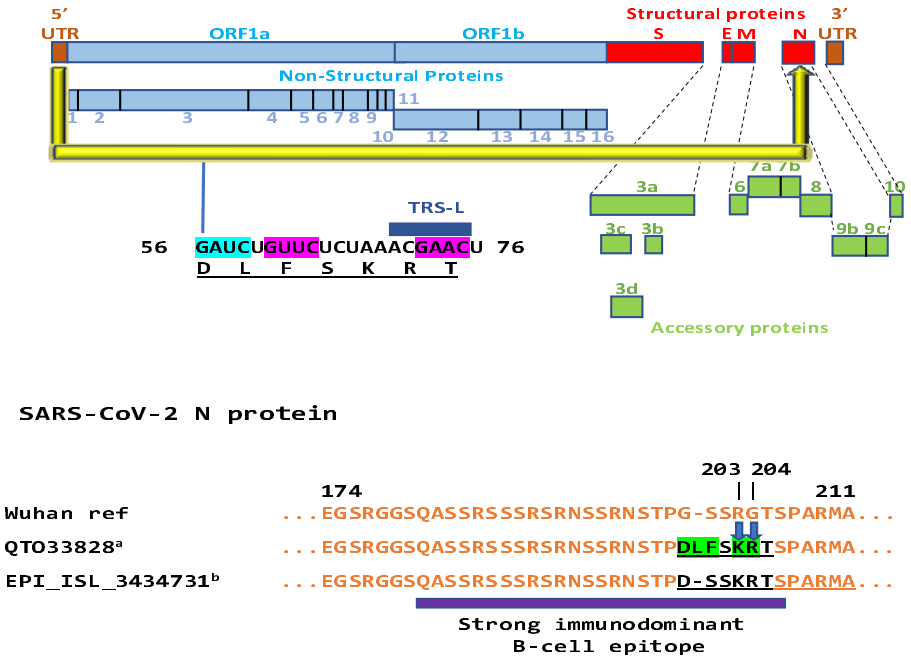


**Figure 4.**

**A.**


5′ UTR
ORF1a
ORF1b
Structural proteins
S
E M
N
3′ UTR
Non-Structural Proteins
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
TRS-L
56 GAUCUGUUCUCUAAACGAACU 76
D L F S K R T
3a
3c 3b
3d
6 7a 7b 8 9b 9c 10
Accessory proteins
SARS-CoV-2 N protein
174
203 204
211
Wuhan ref ...EGSRGGSQASSRSSSRSRNSSRNSTPG-SSRGTSPARMA...
QTO33828[a] ...EGSRGGSQASSRSSSRSRNSSRNSTPDLFSKRTSPARMA...
EPI_ISL_3434731[b] ...EGSRGGSQASSRSSSRSRNSSRNSTPD-SSKRTSPARMA...
Strong immunodominant
B-cell epitope

**B.**

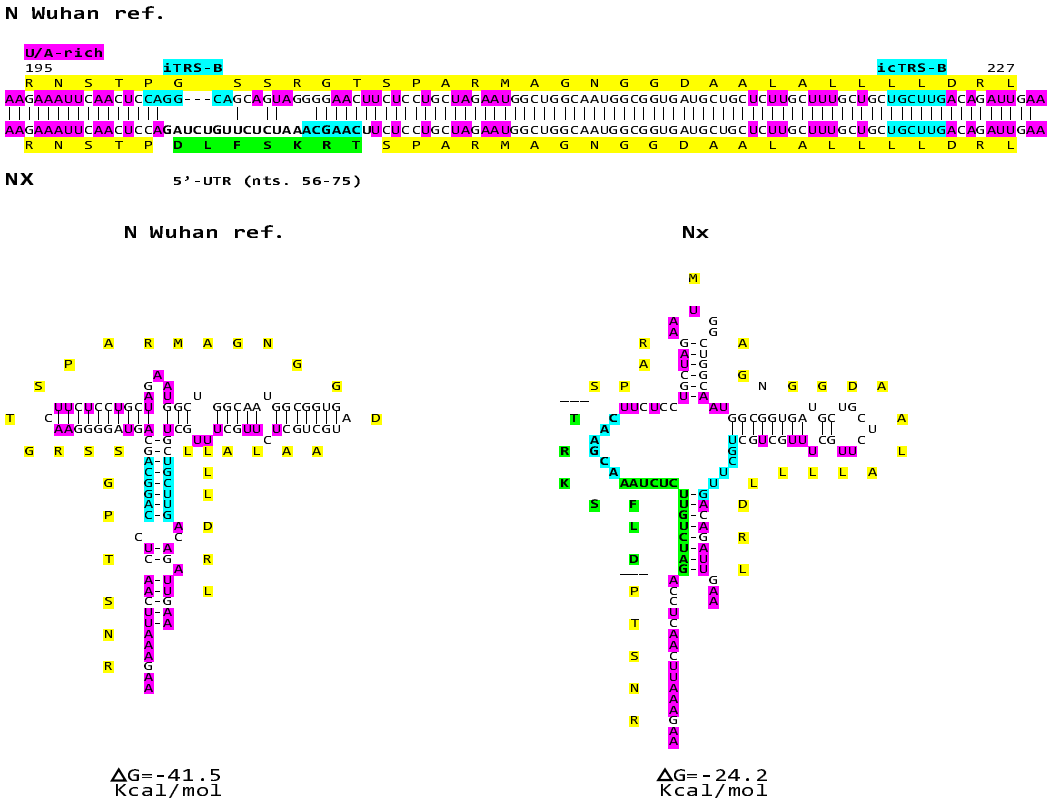
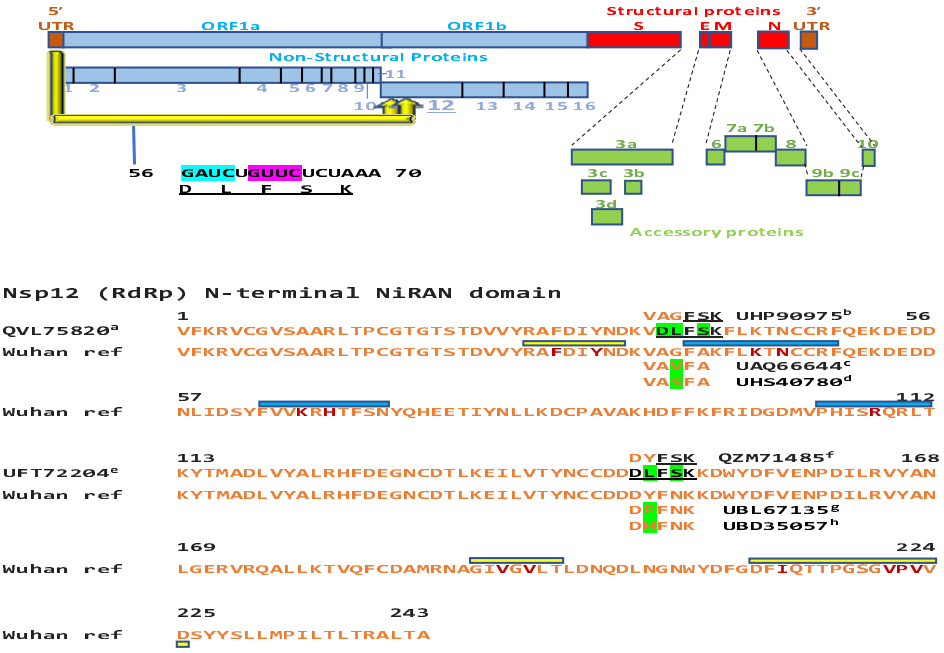

N Wuhan ref.
U/A-rich
195
iTRS-B
icTRS-B
227
NX
5′-UTR (nts. 56-75)
N Wuhan ref.
Nx
ΔG=-41.5
Kcal/mol
ΔG=-24.2
Kcal/mol

**Figure 5.**

**A.**


5' UTR
ORF1a
ORF1b
Structural proteins
S
E M
N
3' UTR
Non-Structural Proteins
56 GAUCUGUUCUCUAAA 70
D L F S K
3a
3c 3b
3d
6
7a 7b
8
10
9b 9c
Accessory proteins
Nsp12 (RdRp) N-terminal NiRAN domain
QVL75820a
Wuhan ref
UHP90975b
UAQ66644c
UHS40780d
UFT72204e
QZM71485f
UBL67135g
UBD35057h

**B.**

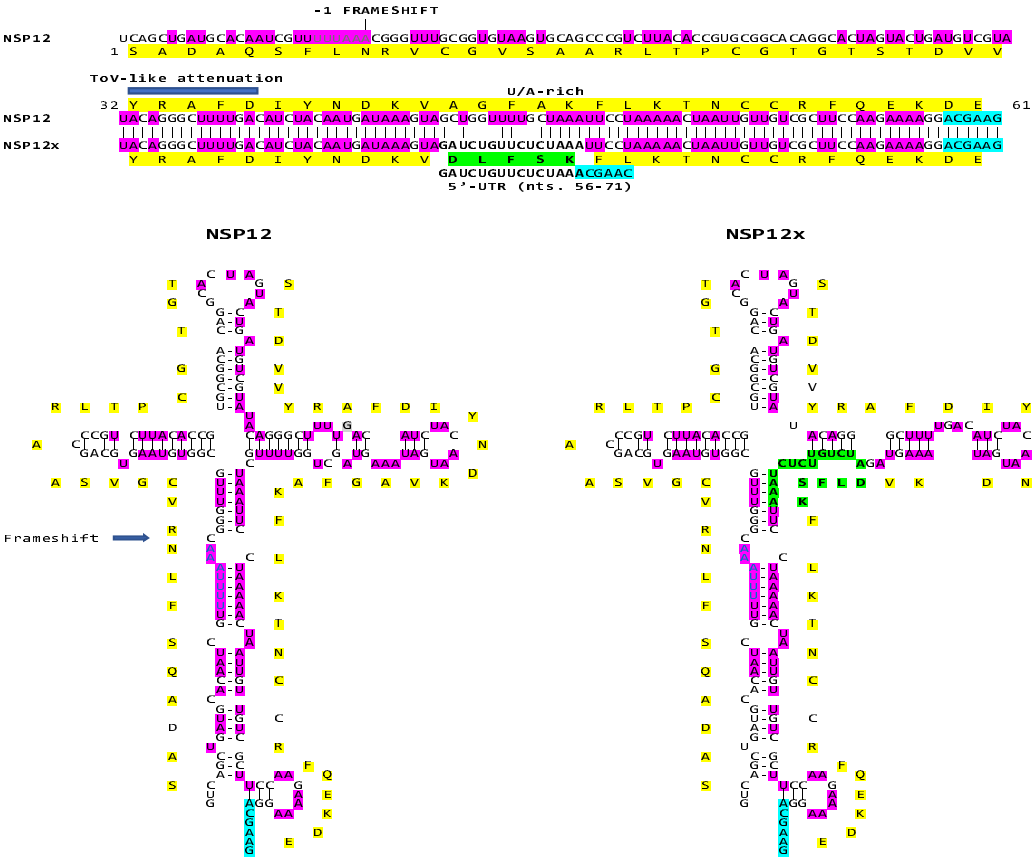

-1 FRAMESHIFT
NSP12
NSP12x
ToV-like attenuation
U/A-rich
GAUCUGUUCUCUAAACGAAC
5'-UTR (nts. 56-71)
Frameshift

**Figure 6.**

**A.**

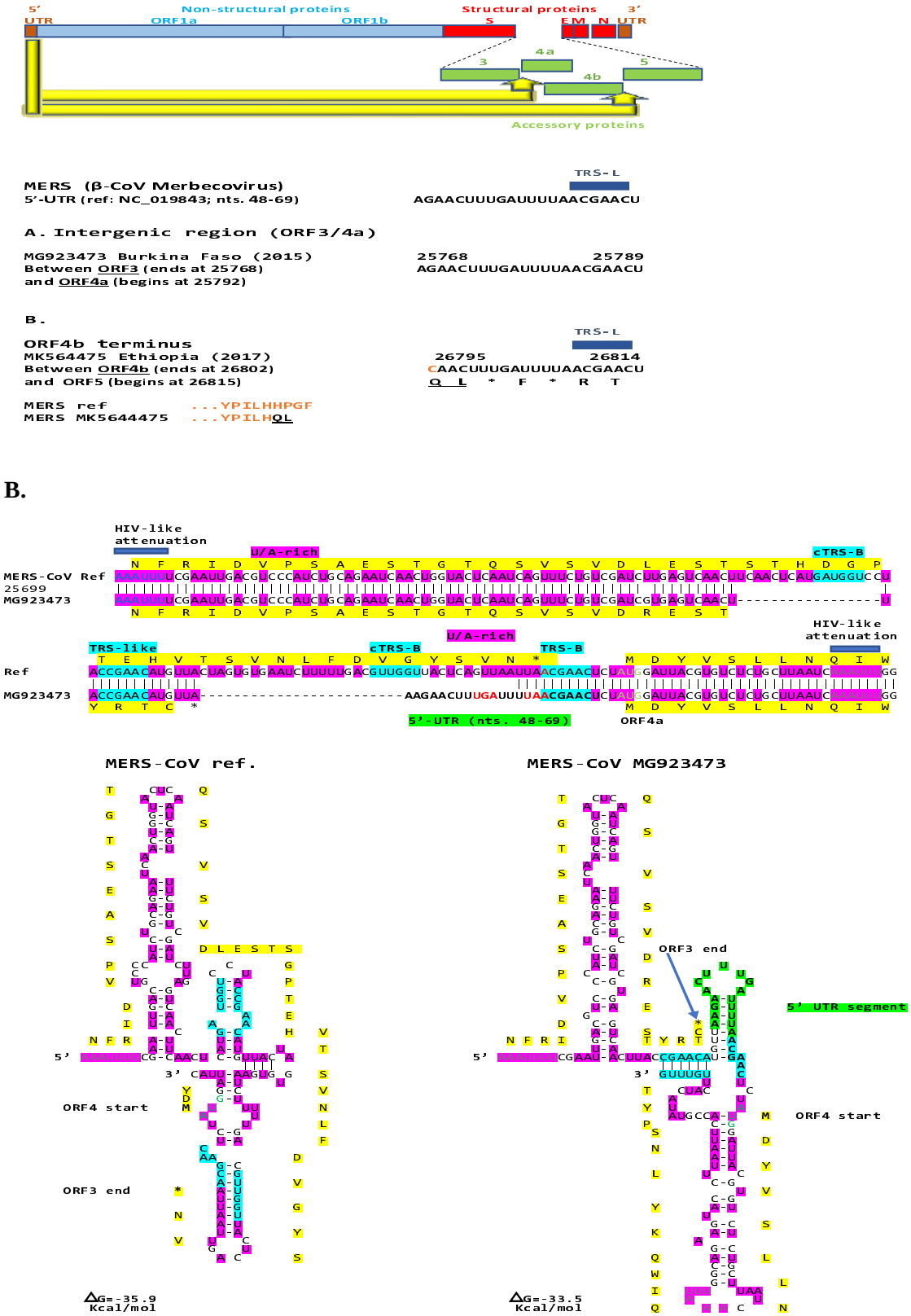
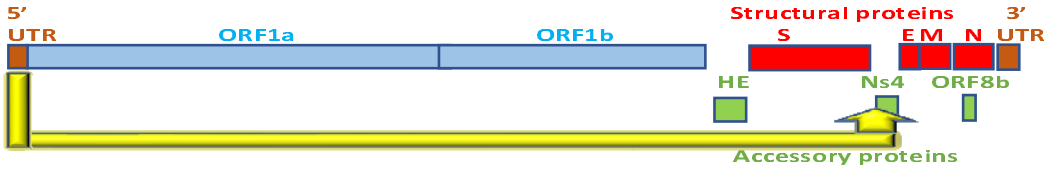

5' UTR
Non-structural proteins
ORF1a
ORF1b
Structural proteins
S
E M
N
3' UTR
3
4a
4b
5
Accessory proteins
MERS (β-CoV Merbecovirus)
5'-UTR (ref: NC_019843; nts. 48-69)
AGAACUUUGAUUUUAACGAACU
TRS-L
A. Intergenic region (ORF3/4a)
MG923473 Burkina Faso (2015)
Between ORF3 (ends at 25768)
and ORF4a (begins at 25792)
25768
25789
AGAACUUUGAUUUUAACGAACU
B.
ORF4b terminus
MK564475 Ethiopia (2017)
Between ORF4b (ends at 26802)
and ORF5 (begins at 26815)
26795
26814
CAACUUUGAUUUUAACGAACU
Q L * F * R T
MERS ref ...YPILHHPGF
MERS MK5644475 ...YPILHQL
HIV-like attenuation
U/A-rich
cTRS-B
MERS-CoV Ref
25699
MG923473
TRS-like
cTRS-B
TRS-B
Ref
MG923473
5'-UTR (nts. 48-69)
ORF4a
MERS-CoV ref.
MERS-CoV MG923473
ORF4 start
ORF3 end
5' UTR segment
ΔG=-35.9 Kcal/mol
ΔG=-33.5 Kcal/mol

**Figure 7.**

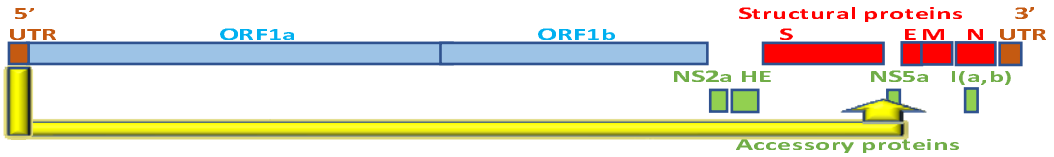
**A.**




Entire β-CoV hCoV-HKU-1 (Embecovirus) Intergenic region between S and Ns4

```
                                                                U/A-rich
                                                                TRS-B
hCoV-HKU-1 MH940245 (17 isolates)                               AUUUAAAUCUAAACUUUAUAU
                                                                |||||||||||||||||||
hCoV-HKU-1 AY597011 (31 isolates)                   AAUCUCUUGUCAGAUCUCAUUAAAUCUAAACUUUAUUU
                                                    |||||||||||||||||||||||||||||||||||||
                 5'-UTR AY597011 (nts. 42-74)       AUCUCUUGUCAGAUCUCAUUAAAUCUAAACUUU
```

**B.**



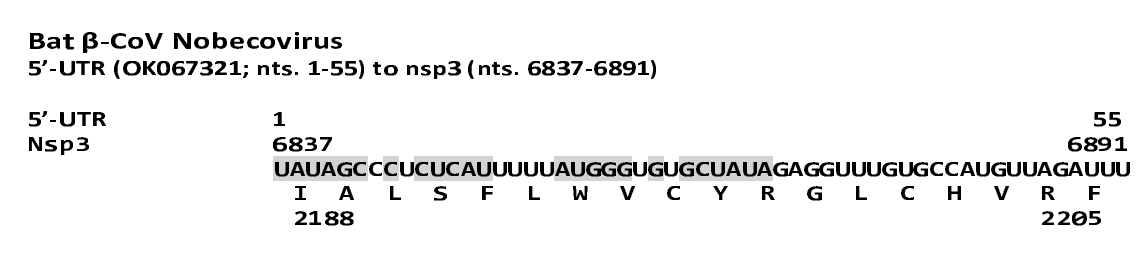

Entire β- CoV hCoV-OC43 (Embecovirus) Intergenic region between S and Ns5a

In all 245 isolates in GenBank

```
                                                                                                                ToV-like
                                                                                                                attenuation
                                U/A-rich                                                TRS-B          U/A-rich
hCoV-OC43 ref          GUUCGUCUUUGAUUCAUUGCAUUGACCUUUUGUUAGAUCUUUUUCUAAUCUAGCAUUUGUUA------------CCACGCCCUUUUA
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hCoV-OC43 KF923898     GUUCGUCUUUGAUUCAUUGCACUGAUCUUUUCUUAGAUCUUUUUGCAAUCUCGCAUUUAUUA------------CCACGCCCUUUUA
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hCoV-OC43 OK318944     GUUCGUCUUUGAUUCAUUGCACUGAUCUUUUGUUAGAUCUUUUUGCAAUCUAGCAUUUAUUAAA-UUCUA----CCACGCCCUUUUA
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hCoVOC43 NC_006213     GUUCGUCUUUGAUUCAUUGCACUGAUCUCUUGUUAGAUCUUUUUGCAAUCUAGCAUUUGUUAAAGUUCUUAAGGCCACGCCCUAUUA
                                           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
5'-UTR OC-43 KJ958218                 35  CACUGAUCUCUUGUUAGAUCUUUUUGUAAUCUA
                                           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
5'-UTR BCoV                           35  CACUGAUCUCUUGUUAGAUCUUUUCAUAAUCUAAACUUUAUAAAA--ACAU----CCACUCCCUGUAG
```

**Figure 8.**

**A.**


Bat β-CoV Nobecovirus
5'-UTR (OK067321; nts. 1-55) to nsp3 (nts. 6837-6891)
5'-UTR 1 55
Nsp3 6837 6891
UAUAGCCCUCUCAUUUUUAUGGGUGUGCUAUAGAGGUUUGUGCCAUGUUAGAUUU
I A L S F L W V C Y R G L C H V R F
2188 2205

**B.**

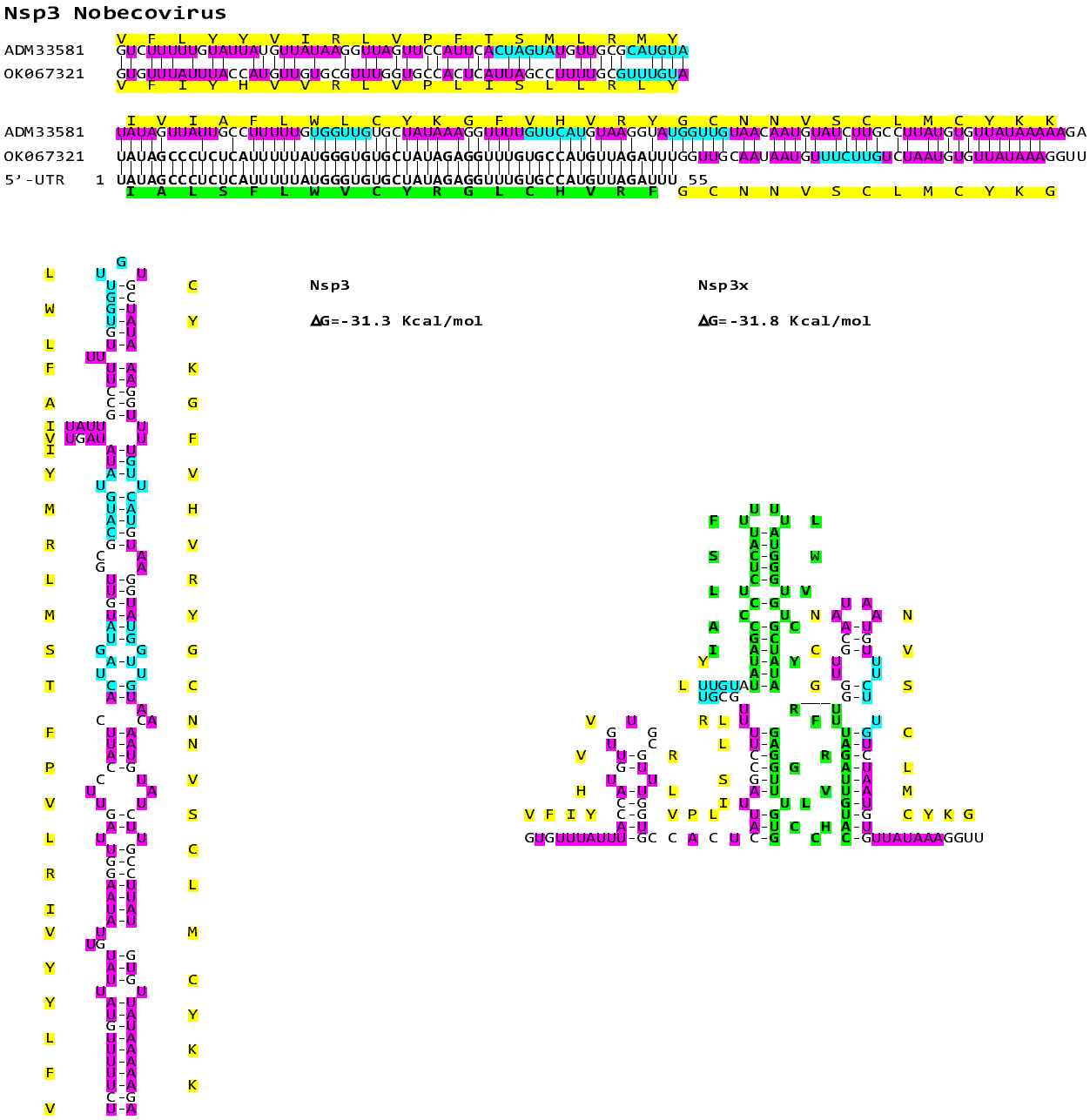

Nsp3 Nobecovirus
ADM33581
OK067321
5'-UTR 1
55
Nsp3
ΔG=-31.3 Kcal/mol
Nsp3x
ΔG=-31.8 Kcal/mol

**Figure 9.**

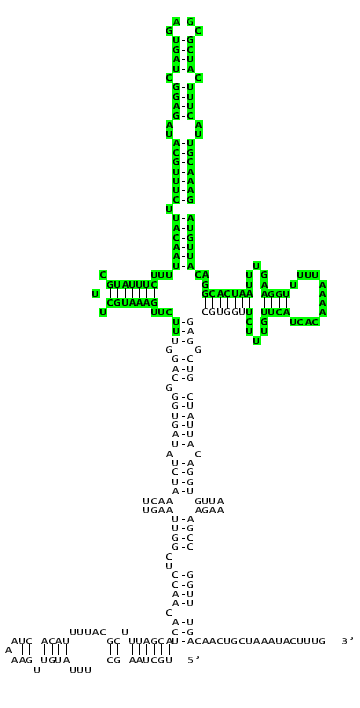
## Nsp2: Rodent alphacoronaviruses subgenus Luchacovirus

```
AcCoV-JC34 (KX964649; China, 10-2011) (nts. 1679-1760)
1679                                                                    1760
UGCUAAGCUUUAUGUUGAAAAUCACAUUUUACGCUUUAGCAUCACAACCUCGGUUAAGUUCAAAUCUAUAGUGGGCAGGU
 A  K  L  Y  V  E  N  H  I  L  R  F  S  I  T  T  S  V  K  F  K  S  I  V  G  R  F
Lucheng Rn rat CoV isolate Ruian 83 (MT820626; China 2014)
UGCCAAGCUCUAUGUUGAAAAUCACAUUUUGCGUUUUAGCAUUACUACUUCCACUAAGUUUAAGAACATTGTTGGACGGU
 A  K  L  Y  V  E  N  H  I  L  R  F  S  I  T  T  S  T  K  F  K  N  I  V  G  R  F
Fievel mouse CoV strain FiCoV/UMN2020 (OK655840; USA, 2018)
UGCUAGGCUUUAUGUUGAAAAUUUUAUCUUGAAGUUCAGUGUUACUACGGCUGUUAAAUUUAAGGAUGUGGUUUGCCGCU
 A  R  L  Y  V  E  N  F  I  L  K  F  S  V  T  T  A  V  K  F  K  D  V  V  C  R  F
```

```
AcCoV-JC34 (KX964649; China, 10-2011)
5'-UTR ([-]strand corresponding to nts. 1-119 in 432-long [+]strand; TRS: nts. 181-186)
119                                                                                                                                  1
UUCUUUAAAUGCUUCGUAUUUCUUUUAACAUUCUUUGCAUAGAGGCUAGUGAGCGCUACUUUCAUUGCAAAGAUGUUACAGGCACUAAUUUGAAGGUUUUUAAAACACUACUUGUUUCU
NSP2 (nts. 1761-1879)
1761                                                                                                                                1879
UUCUUGAAAUGCUUCGUAUUUCUUUUAACAUUCUUUGCAUAGAGGCUAGUGAGCGCUACUUUCAUUGCAAAGAUGUUACAGGCACUAAUUUGAAGGUUUUUAAAACACUACUUGUUUCU
  L  E  M  L  R  I  S  F  N  I  L  C  I  E  A  S  E  R  Y  F  H  C  K  D  V  T  G  T  N  L  K  V  F  K  T  L  L  V  S
Lucheng Rn rat CoV isolate Ruian 83 (MT820626; China, 2014)
UUCUUGAAAUGCUUCGAGUUUCCUUUAACAUUCUUUGUGUUGAUGCUAGCGAACGUUACUUUUGCUGCAAGGAUGUCACUGGUACAAACCUCAAGCUUUUCAAGACUCUGCUUGUCUCU
  L  E  M  L  R  V  S  F  N  I  L  C  V  D  A  S  E  R  Y  F  C  C  K  D  V  T  G  T  N  L  K  L  F  K  T  L  L  V  S
Fievel mouse CoV strain FiCoV/UMN2020 (OK655840; USA, 2018)
UUCUAGAGGUACUCAGGAGAUCCUUUGGCAUGCUCUGCGUUGAUGCCACUGAAUGUUGUUUCAAGUGCAGGGAUGUCACUGGUACUAACCUGAAACUCUUUAAGCGCCAGAUCGUUGCA
  L  E  V  L  R  R  S  F  G  M  L  C  V  D  A  T  E  C  C  F  K  C  R  D  V  T  G  T  N  L  K  L  F  K  R  Q  I  V  A
```

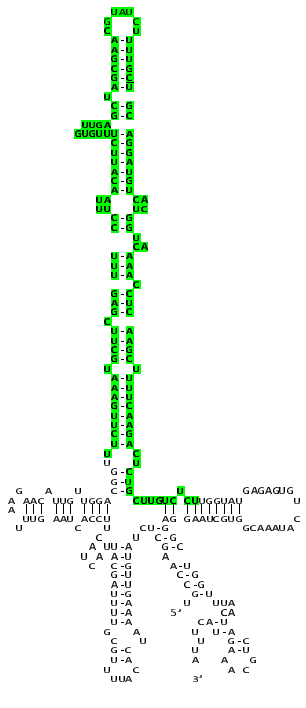
```
AcCoV-JC34 (KX964649; nts. 1880-1940)
1880                                                    1940
UGGUGCGAGGCUGCUAUUACAGGUGUUAAAGAAGCCGGUUUGACAACUGCUAAAUACUUUG
W  C  E  A  A  I  T  G  V  K  E  A  G  L  T  T  A  K  Y  F
Lucheng Rn rat CoV isolate Ruian 83 (MT820626)
UGGUAUGAGAGUGUCAUAAACGGUGCUAAGGAGGCUGGUUUAACUACUGCAAAGUACUUUA
W  Y  E  S  V  I  N  G  A  K  E  A  G  L  T  T  A  K  Y  F
Fievel mouse CoV strain FiCoV/UMN2020 (OK655840)
UCUUUUGAGUGCGCCAUCUCUGGUGUUAAGGAAGCUGGUCUUACCACCGCCAAGUACUUUU
S  F  E  C  A  I  S  G  V  K  E  A  G  L  T  T  A  K  Y  F
```

AcCoV-JC34 (KX964649)
*Apodemus*
China 2011



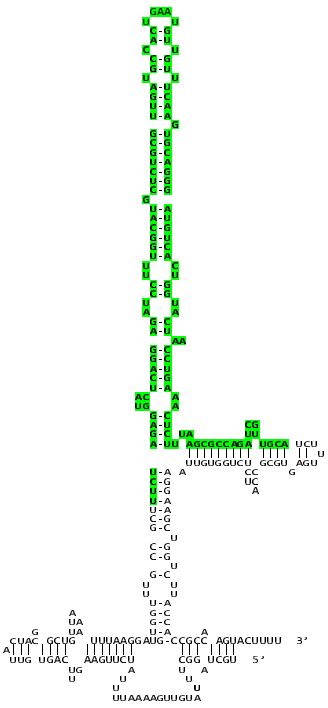
△G=-60.4 Kcal/mol

Lucheng Rn rat CoV isolate
Ruian 83 (MT820626)
China 2014



△G=-58.4 Kcal/mol

Fievel mouse CoV strain
FiCoV/UMN2020 (OK655840)
USA 2018



△G=-80.8 Kcal/mol

**Figure 10.**

**A.**

## Nucleocapsid amino-terminus: Bat alphacoronaviruses subgenus Nyctacovirus

```
Alphacoronavirus sp. Isolate WA2028 (MK472068; microbat; Australia, 2018)
5'-UTR ([-]strand corresponding to nts. 8-122 in 434-long [+]strand; TRS: nts. 206-211)
122                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 8
UCUAAACUAAACAAUGUCUGUUAAUUUUGCAGGCACUACUACUCAGCCACGUGGCCGUGUCCCUCUUUCGCUGUUCCAACCACUGCGAAACAACUCUACUCAACCUCUCCACAAG
Nts.25491-25605 including segment before N (with TRS-B) and amino-terminus of N (encoded by nts. 25454-26716)
25491                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 25605
UCUAAACUAAACAAUGUCUGUUAAUUUUGCAGGCACUACUACUCAGCCACGUGGCCGUGUCCCUCUUUCGCUGUUCCAACCACUGCGAAACAACUCUACUCAACCUCUCCACAAG
  *          M  S  V  N  F  A  G  T  T  T  Q  P  R  G  R  V  P  L  S  L  F  Q  P  L  R  N  N  S  T  Q  P  L  H  K

Alphacoronavirus sp. Isolate WA3301 (MK472069; microbat; Australia, 2018)
UCUAAACUAAACAAUGUCUGUUAAUUUUGCAGGCACUACUACUCAGCCACGUGGCCGUGUUCCUCUUUCGCUGUUCCAACCACUGCGAAACAACUCUACUCAGCCUCUCCACAAA
  *          M  S  V  N  F  A  G  T  T  T  Q  P  R  G  R  V  P  L  S  L  F  Q  P  L  R  N  N  S  T  Q  P  L  H  K

Alphacoronavirus sp. Isolate WAAlc1 (MK472071; microbat; Australia 2018)
UCUAAACUAAACAAUGUCUGUGAAUUUCUCAGACACUACUGCUCAGCCUCGUGGCCGUGUCCCUCUUUCGCUUUUCAACCGUUGCGAAACAAUUCUUCCCAGCCGAAAAUAUUC
                                CAA       C     AC
  *          M  S  V  N  F   S  D Q T T  A S  A P R  G  R  V  P  L  S  L  F  Q  P  L  R  N  N  S   S  Q  P  K  I  F

Tylonycteris bat coronavirus HKU3 (MK720944; bat; China, 09-09-2015)
UCUAAACUAAACAAUGUCUGUCAAUUUUGCAGGCGGAAGUUUACUAAGGUCCCCGGCGAGACCCGCACACGGAAACAGUCACAAAGUCGGGUAUGCAUUAUUACGAAGUGUUGGA
  *          M  S  V  N  F  A  G   G  S   L  L  R  S  P  A  R   P   A  H  G  N  S  H  K  V  G  Y   A  L  L  R  S  V  G

Alphacoronavirus Bat-CoV/P.kuhlii/Italy/206645-41/2011 (MH938448; bat; Italy,2011)
UCUAAACGAAAUGUCUUCCUCCAGGGGAAACGUCGGUUUUGACAAUGCAGCUAGGGGUCGUUCAGGGCGUGUACCACUUUCACUAUAUAUGCCCGUUAUCAACAACUCACCUAAG
  *        M  S   S   S  R  G  N  V   G   F  D  N  A  A  R  G  R  S  G  R  V  P  L  S  L  Y  M  P  V  I  N  N  S  P   K
```

**B.**

## ORF5b carboxyl-terminus: Bat alphacoronavirus subgenus Decacovirus

```
Rhinolophus bat coronavirus HKU32 strain TLC28A (MK720946; Rhinolophus sinicus; Hong Kong, 08-06-2015)[a]
5'-UTR (nts. 45-77 in 290-long [+]strand; TRS: nts. 67-72)
        45                                77
        AGACCUCGCGUCUCAUCCCCUCAACUAAACGAA
ORF5b (nts. 25782-26225) carboxyl-terminus; overlaps with beginning of E (nts. 26209-26433)
        26116                                26148                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                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                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    26225
ACUACACAGACCUCGCGUCUCAUCCCCUCAACUAAACGAACUCAUUAUAGUUUGUUUGUUGCAUUUACUAUAGUACCUGUAGUUUUGGUCUUCAUACACUAUGCUGACUUUAGUUAAU
T  T  Q  T  S  R  L  I  P  S  T  K  R  T  H  Y  S  L  F  V  A  F  T  I  V  P  V  V  L  V  F  I  H  Y  A  D  F  S  *
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                M  L  T  L  V  N
```

## ORF4b carboxyl-terminus: Bat alphacoronavirus subgenus Decacovirus

(annotated as unclassified coronavirus but 5'-UTR similar to that of isolate above)

```
Bat coronavirus isolate BtCoV/Rh/YN2012_Rs4259 (MG916903; China, 04-17-2013)[b]
5'-UTR (nts. 61-90 in 297-long [+]strand; TRS: nts. 74-79)
                61                        90
                GUCUCAUCCCCUCAACUAAACGAAAUUUUU
ORF4b (nts.25508-25954) carboxyl-terminus; overlaps with beginning of E (25935-26159)
                25848                        25877                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       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                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               25954
ACUAACCCUAGUAAAAGUCUCAUCCCCUCAACUAAACGACAUUAUUACUUCCUGCUACUUGCAUUUAUUCCACCUGCGUGGUUUGCCGUAUUAAUUAUCUACUAUGUUAACCCUAGUGGAU
T  N  P  S  K  S  L  I  P  S  T  K  R  H  Y  Y  F  L  L  L  A  F  I  P  P  A  W  F  A  V  L  I  I  Y  Y  V  N  P  S  G  *
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                M  L  T  L  V  N
```
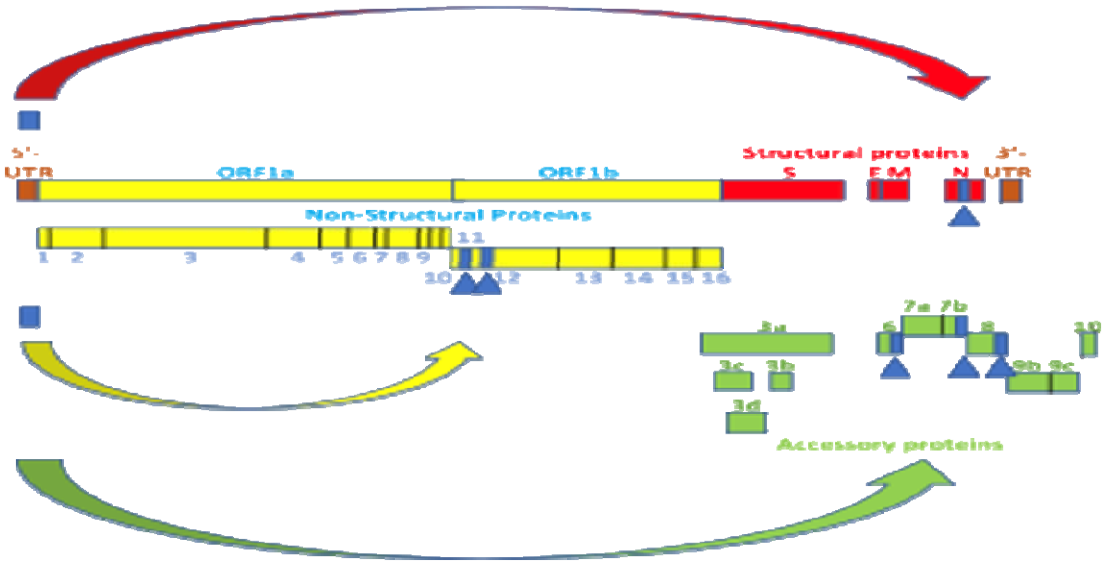
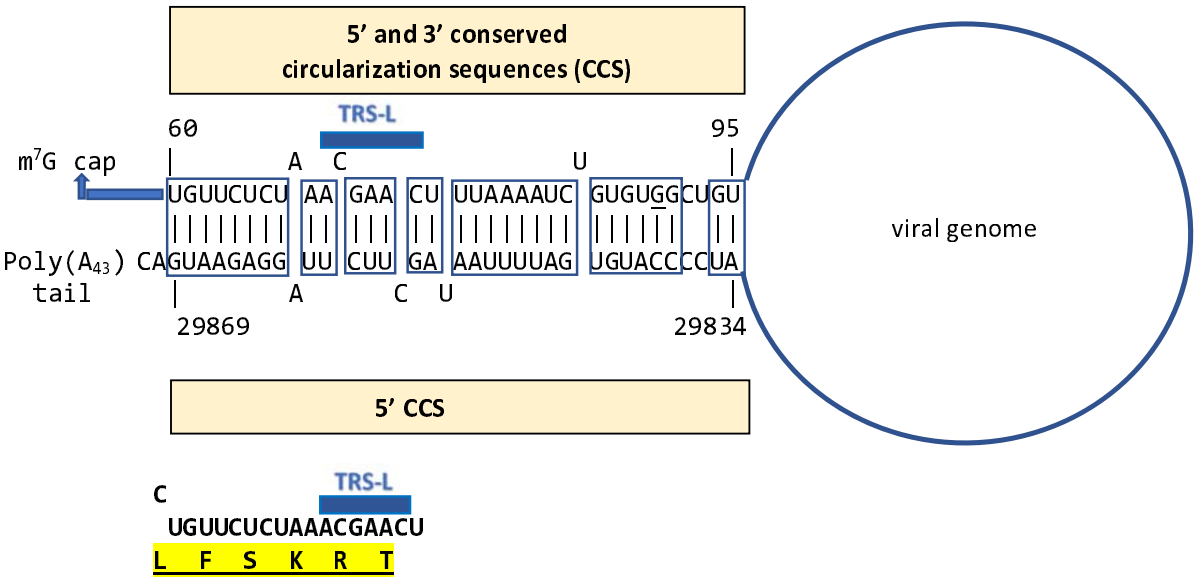

**Figure 11.**

**A.**


5'-
UTR
ORF1a
ORF1b
Structural proteins
S
E M
N
UTR
3'-
Non-Structural Proteins
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
3a
3c
3b
3d
6
7a 7b
8
10
9b 9c
Accessory proteins

**B.**


5' and 3' conserved
circularization sequences (CCS)
TRS-L
60
95
m7G cap
A C
U
UGUUCUCU AA GAA CU UUAAAAUC GUGUGGCUGU
Poly(A43) CAGUAAGAGG UU CUU GA AAUUUUAG UGUACCCCUA
tail
A C U
29869
29834
viral genome
5' CCS
TRS-L
C
UGUUCUCUAAACGAACU
L F S K R T