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Review

Alpha- and betacoronavirus *cis*-acting RNA elements

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Coronaviruses have exceptionally large RNA genomes and employ multiprotein replication/transcription complexes to orchestrate specific steps of viral RNA genome replication and expression. Most of these processes involve viral *cis*-acting RNA elements that are engaged in vital RNA–RNA and/or RNA–protein interactions. Over the past years, a large number of studies provided interesting new insight into the structures and, to a lesser extent, functions of specific RNA elements for representative coronaviruses, and there is evidence to suggest that (a majority of) these RNA elements are conserved across genetically divergent coronavirus genera. It is becoming increasingly clear that at least some of these elements do not function in isolation but operate through complex and highly dynamic RNA–RNA interactions. This article reviews structural and functional aspects of *cis*-acting RNA elements conserved in alpha- and betacoronavirus 5'- and 3'-terminal genome regions, focusing on their critical roles in viral RNA synthesis and gene expression.

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Introduction

The recent pandemic has sparked a surge of interest in the molecular biology of coronaviruses, particularly severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1], the causative agent of coronavirus disease 2019 (COVID-19) and member of the genus *Betacoronavirus* in the subfamily *Orthocoronavirinae*, which currently includes four genera termed *Alpha-*, *Beta-*, *Gamma-*, and *Deltacoronavirus* [2].

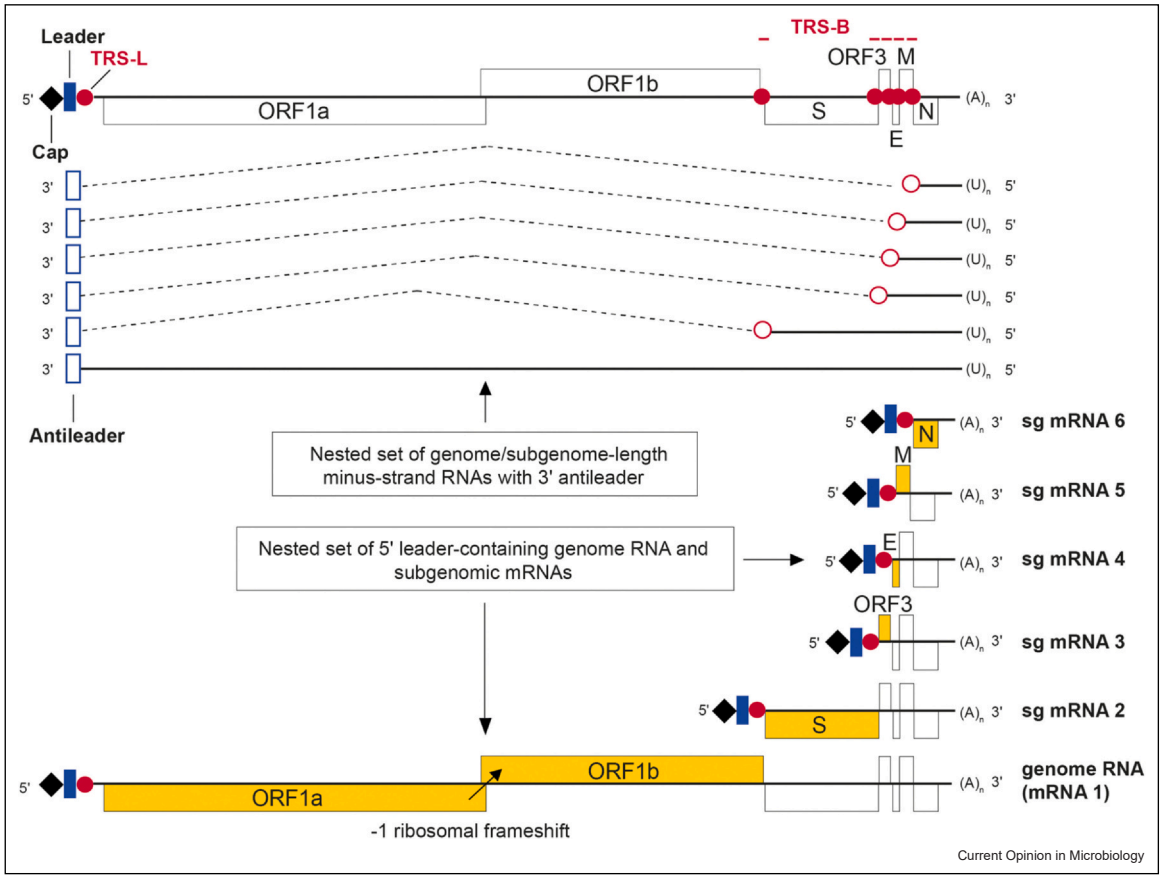
In coronavirus-infected cells, multiprotein replication/transcription complexes (RTCs) are formed that comprise virus-encoded nonstructural proteins (nsp) [3]. Individual nsps are produced from two ORF1a/1b-encoded polyprotein precursors (pp1a and pp1ab) by proteolytic processing involving two or three virus-encoded proteases [3,4]. Coronavirus RTCs are anchored to specialized intracellular membrane compartments, so-called replicative organelles [5,6]. RTCs (with presumably varying subunit composition) produce (i) multiple copies of the ~30-kb, 5'-capped and 3'-polyadenylated genome RNA ('replication') and (ii) a set of subgenomic (sg) mRNAs ('transcription') that direct the biosynthesis of viral structural and several accessory proteins [4,7]. Coronavirus single sg mRNAs contain a common 5' leader that is identical to the 5' end of the viral genome and harbors two stem-loop (SL) RNA structural elements termed SL1 and SL2. The complement of the 5' leader ('antileader') is attached to the 3' end of nascent minus-strand sgRNAs in a process called 'discontinuous extension of minus strands' [8]. The resulting antileader-containing sg minus-strand RNAs serve as templates for the production of 5' leader-containing sg mRNAs (Figure 1).

This article provides a brief overview of RNA structural elements identified in coronavirus 5'- and 3'-untranslated genome regions (UTR), focusing on those elements that have been shown to have critical roles in viral replication. In view of the scope of this article and the enormous number of studies published in recent years on RNA structural elements of SARS-CoV-2, it has not been possible to provide an exhaustive overview that includes all the excellent work published on SARS-CoV-2 and other betacoronavirus RNA structural elements and their interactors.

Conservation of RNA secondary structures in coronavirus 5'-terminal genome regions

Early studies on 5'-proximal RNA elements involved in viral RNA synthesis were performed for animal betacoronaviruses, such as bovine coronavirus (BCoV) and mouse hepatitis virus (MHV; reviewed in Ref. [9]). RNA

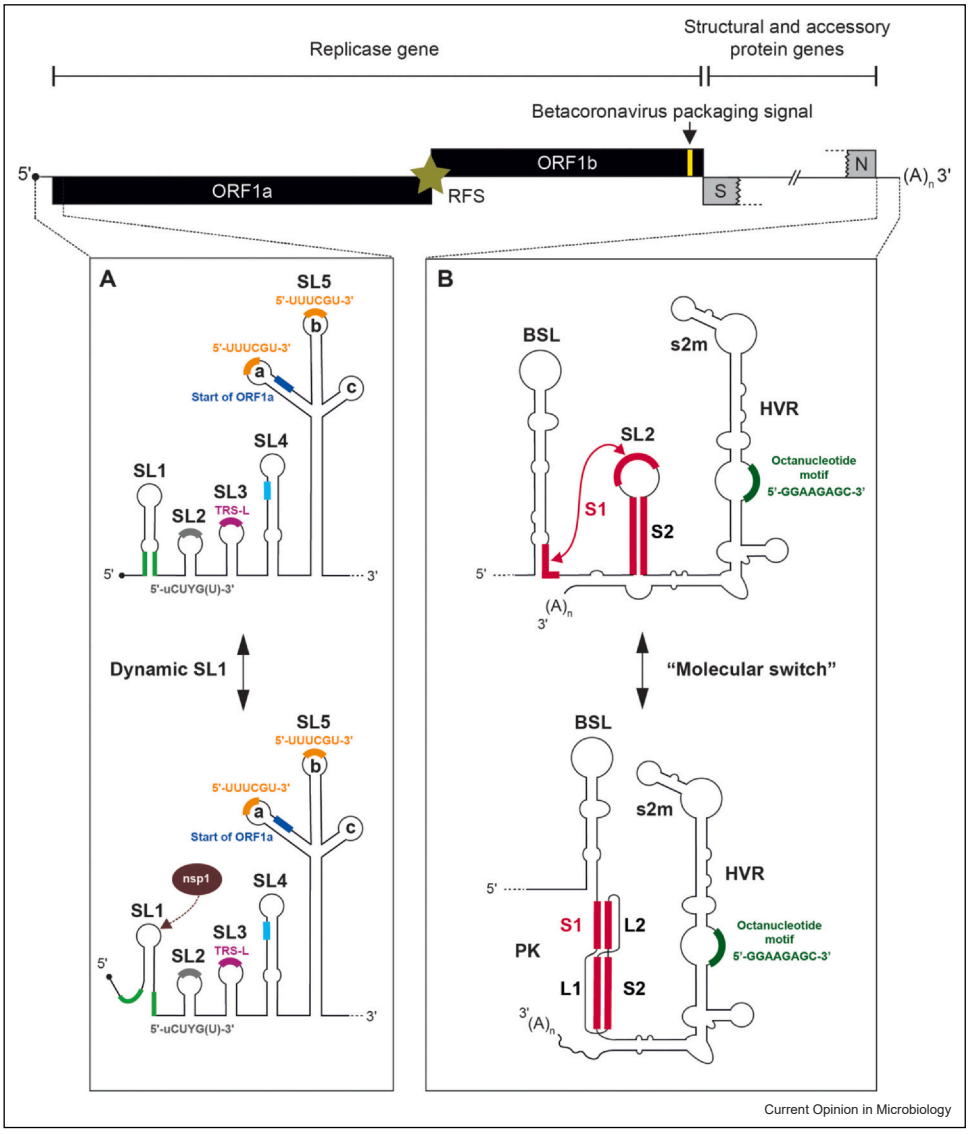
Figure 1



Overview of genomic and subgenomic RNAs produced in coronavirus-infected cells. Shown is the polycistronic genome structure of a representative coronavirus encoding the four major structural proteins (S, E, M, and N) and one accessory protein (ORF3). Production of new viral genome RNAs and a nested set of sg mRNAs from which the structural and accessory protein genes located in the 3'-terminal genome region are expressed involves the synthesis of a 'nested set' of coterminal minus-strand RNAs using the full-length plus-strand RNA genome as a template. Minus-strand RNAs contain, at their 3' end, the complement (antileader) of the ~65- to 95-nt leader sequence present at the 5' end of the genome. While the genome-length minus-strand RNA serves as template for the production of new viral genomic RNAs (genome replication), the subgenomic minus-strand RNAs serve as templates for the production of a 5'- and 3'-coterminal nested set of sg mRNAs (transcription). The production of sg minus-strand RNAs involves a 'discontinuous extension' mechanism during RNA synthesis in which base-pairing interactions between the sequence complement of a body TRS element (TRS-B) and the 5′ leader TRS element (TRS-L) guide the transfer of the viral RTC to the upstream position on the genome RNA. Following this transfer, RNA synthesis is completed by copying the 5' leader sequence. The resulting set of minus-strand RNAs (with different leader-body junction sites) is subsequently used to produce a set of 5' leader-containing sg mRNAs from which the structural and accessory proteins are translated (the 5' ORF translated from a given mRNA is indicated in yellow). The genomic RNA has an additional function by serving as an mRNA (mRNA 1) for the expression of the viral replicase gene (ORFs 1a and 1b). Expression of ORF1b involves a directed (–1) ribosomal frameshifting event occurring just upstream of the ORF1a stop codon. Recent studies identified, in coronavirus-infected cells, a large number of additional sgRNA species that lack canonical TRS-L/B junction sites. Although the biological significance of most of these sgRNAs remains to be characterized in detail, there is increasing evidence that the coronavirus transcriptome is more complex than previously thought [59–61]. 5' cap structures are indicated by tilted black squares; TRS-L and TRS-B elements: filled red circles, TRS-B complements: open red circles; 5' leader: filled blue box; antileader: open blue box. Note that only the TRS-L/B involved in joining the 5'-leader and mRNA body sequences of specific mRNAs (bottom) is indicated, while TRS-B elements located further downstream on the respective mRNA are not shown in this representation (see above for their positions in the viral genome). Also indicated are the 3' poly(A) tails of viral plus-strand RNAs and the 5' oligo(U) stretches of minus-strand RNAs.

structure probing and functional studies revealed five functionally relevant SL structures (SL1, SL2, SL3, SL4, and SL5) in most betacoronavirus 5' UTRs, while some (beta)coronaviruses, such as MHV, appear to lack an SL3 structure [10–12] (Figure 2). Subsequent studies suggested that alphacoronavirus 5' UTRs fold into four conserved SL1, SL2, SL4, and SL5 structures [11] (Figure 3). Recently, a large number of structural studies were performed on betacoronavirus RNA structures, which confirmed and extended the previously proposed conservation of 5'-proximal RNA structural elements and provided a wealth of additional information [13–18]. Collectively, the data obtained for representative alpha- and betacoronaviruses provide consistent evidence to

Figure 2



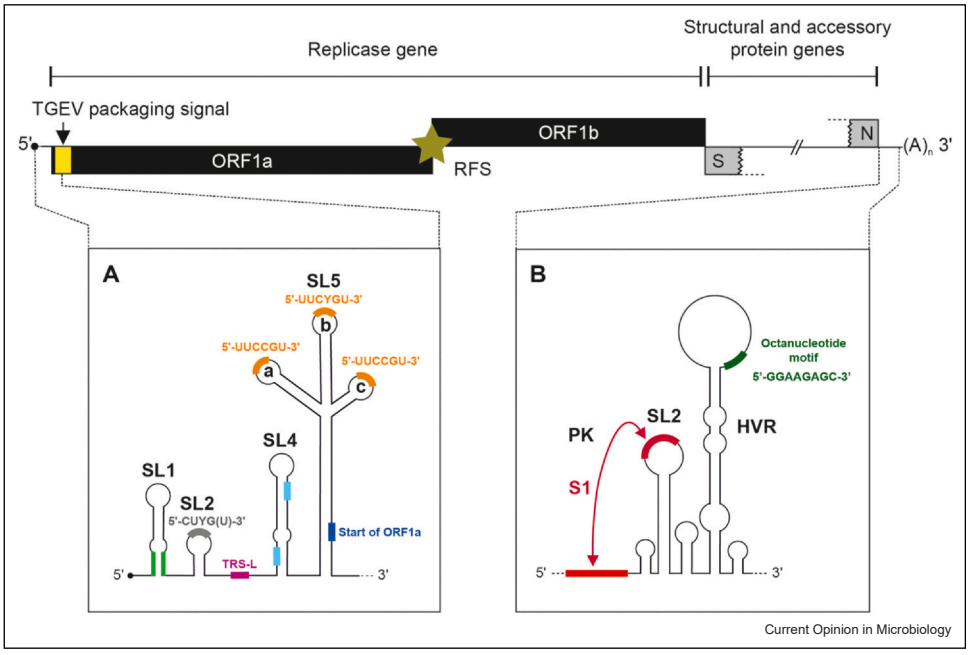
Conserved *cis*-acting RNA elements in the 5'- and 3'-terminal genome regions of betacoronaviruses. Shown is the coronavirus genome organization with the two large 5' ORFs, 1a and 1b, that together constitute the replicase gene, while details for the structural and accessory protein ORFs are not shown. Black circles at the RNA 5' ends indicate the 5' cap structure, $(A)_n$ indicates the 3' poly(A) tail. The –1 ribosomal frameshift signal (RFS) at the ORF1a/1b junction site is indicated by an asterisk. S, S gene; N, N gene. The arrow indicates the approximate positions of the PS determined for MHV and SARS-CoV-2. **(A)** Schematic representation of major RNA structural elements conserved in (most) betacoronavirus 5'-terminal genome regions (for details, see https://rfam.org/family/RF03117). The filled purple box indicates the TRS-L at the apical loop of SL3. The light blue box indicates the start codons of the uORF(s) upstream of ORF1a, and boxes in dark blue indicate the position of the ORF1a start codon. Orange boxes in SL5a and b indicate conserved RSM, 5'-UUYCGU-3'. Also shown is the dynamic structure of the lower part of SL1 (light green) and interactions of nsp1 with the upper part of SL1 (see text for details). **(B)** Schematic representation of RNA structural elements in betacoronavirus 3'-terminal genome regions (for details, see https://rfam.org/family/RF03122). Major conserved RNA structural elements are shown, together with base-pairing interactions required to form an H-type PK structure (indicated in red and by the double-headed arrow). Also shown is the position of the highly conserved octanucleotide sequence (green) located in a single-stranded region. L, loop; S, stem (for details, see https://rfam.org/family/RF00164).

suggest that the 5'-terminal ~320-nt genome regions fold into four or five major structures, SL1, SL2, (SL3), SL4, and SL5, which act as key *cis*-acting signals in viral RNA synthesis and/or have other important roles in the viral life cycle.

SL1

SL1 is conserved across all coronavirus genera [11,16,19]. It is structurally and functionally bipartite [13,17,18,20,21]. Destabilizing mutations in the upper

Figure 3



Conserved *cis*-acting RNA elements in alphacoronavirus 5'- and 3'-terminal genome regions. Shown is the coronavirus genome organization (for details, see legend in Figure 2). The approximate position of the PS determined for transmissible gastroenterits virus (TGEV) is indicated. **(a)** Representation of RNA structural elements in alphacoronavirus 5'-terminal genome regions (for details, see https://rfam.org/family/RF03116). The purple box indicates the TRS-L as single-stranded region. Conserved sequences of SL5 substructures are shown in orange. Light blue boxes indicate start codon(s) of uORF(s) located upstream of ORF1a, and the dark blue box indicates the position of the ORF1a start codon. **(b)** RNA structural elements conserved in alphacoronavirus 3'-terminal genome regions (for details, see https://rfam.org/family/RF03121). Base-pairing interactions predicted to be involved in the formation of a PK structure are indicated in red and by a double-headed arrow. Also shown is the position of the highly conserved octanucleotide sequence located in a single-stranded region. L, loop; S, stem; PK, pseudoknot (green).

and lower parts of the SL1 structure differentially affect viral replication, with the basal part being less sensitive to stem-destabilizing mutations than the upper part [20,21]. For some of the MHV SL1 mutants, evidence for a defect in minus-strand sgRNA synthesis was obtained, along with specific second-site suppressor mutation data indicating critical long-range interactions between the 5' and 3' UTR. Compared with MHV, the stability of the basal part of SL1 of human coronavirus 229E (HCoV-229E), an alphacoronavirus, proved to be more constrained [20,21]. In both beta- and alphacoronaviruses, disruption of the apical part of SL1 has a profound impact on the production of infectious virus progeny [20,21]. The available genetic evidence suggests significant structural constraints for the apical part of SL1, while the lower part has greater flexibility, which was proposed to promote dynamic long-range interactions between the 5' and 3' UTRs [20].

SL1 also plays an important role in the differential regulation of mRNA translation in cells infected with SARS-CoV-2 and several other betacoronaviruses. The element ensures that viral mRNAs (partially) escape the host translation shut-off mediated by nsp1 [22–26]. Structural studies showed that the SARS-CoV-2 nsp1 C-terminal domain (CTD) binds to and obstructs the mRNA entry channel of the 40S ribosomal subunit [25,26]. This interaction is conserved among a limited number of betacoronaviruses [27,28] but not in alphacoronaviruses, which lack the CTD and potentially employ other nsp1-mediated mechanisms to cause a translational shut-off in infected cells [24,28]. Interactions between the apical part of SARS-CoV-2 SL1 with nsp1 are thought to induce a conformational change in nsp1 that abolishes the entry block and thus allows the translation of viral mRNAs. Destabilization of the SL1 apical stem structure and/or changes of the spatial arrangement between the 5' cap and SL2 compromise this defense against nsp1-mediated translation inhibition [23,29]. The specific interaction of SARS-CoV-2 nsp1 with the ribosomal mRNA entry channel and its modification by SL1 provide an interesting example of a viral strategy that ensures efficient shut-off of host cell protein biosynthesis while largely retaining viral mRNA translation, with significant consequences that limit the cell's ability to activate efficient antiviral mechanisms [26]. The molecular mechanisms involving the remarkably divergent nsp1 proteins of different lineages of beta- and alphacoronaviruses remain to be characterized.

SL2

SL*2* is the most conserved RNA structural element of alpha- and betacoronaviruses [11,16,19]. It comprises an apical 5'–(U/C)UUG(U/C)–3' loop that is stacked on a 4–5 bp helical stem. For betacoronaviruses, the loop sequence was initially suggested to represent a U-turn motif and later defined as a uYNMG(U)a-like or uCUYG(U)a-like tetraloop [18,30–32]. Mutational studies destabilizing the stem structure in representative alpha- and betacoronaviruses revealed major defects in viral replication [21,30,31] and rapidly led to the selection of revertants, supporting key functions of SL*2* in viral RNA synthesis [21]. Nucleotide substitutions in the MHV SL*2* apical loop were confirmed to disrupt viral sgRNA synthesis [31], suggesting a co-operation of SL*2* with the neighboring transcription-regulating sequence (TRS) of the 5' leader (TRS-L) in sgRNA synthesis. Based on MHV mutagenesis data, SL*2* was suggested to serve as a scaffold that critically determines the folding of adjacent RNA elements (see below) [31]. The sequence of the apical loop is conserved across coronavirus genus boundaries [11,19] and substitution of SL*2* of HCoV-*229*E with that of betacoronaviruses yielded viable recombinant viruses [21]. The data support a critical role of the conserved apical loop of SL*2* in viral RNA synthesis, which remains to be elucidated in further studies.

SL3 and transcription-regulating sequence

The SL3 structure has been identified in a range of betacoronaviruses, such as BCoV, SARS-CoV, SARS-CoV-2, and HCoV-OC43 [13,14,17,19]. SL3 has a short stem and contains the TRS-L in its apical loop. Structure probing data obtained for MHV, HCoV-*229*E, and HCoV-NL63 suggest that the SL3/TRS-L structure is not universally conserved among coronaviruses [11,12,19,21], and there is evidence that short linker sequences located between the individual SLs in the 5' UTRs of coronaviruses determine whether or not an SL3 structure is formed [14,19]. Structure information obtained for HCoV-OC43 led to a model in which SL3 and SL4 coaxially stack to form a continuous helix, which fixes their relative orientations to maintain a consistent distance between the apical loops [14]. The coaxial stacking helps to orient the TRS-L element in the apical loop of SL3 to facilitate base-pairing interactions with downstream TRS (TRS-B) elements. The coaxially stacked SL3–4 helix also controls the orientation and spatial proximity of SL*2* relative to the TRS-L. This structure model validates earlier hypotheses, suggesting that the folding of SL*2* and its adjacent structures regulate viral sgRNA synthesis [31]. SARS-CoV-2 SL3 was also suggested to contribute to genome cyclization by participating in stable long-range RNA–RNA interactions to regulate coronavirus sgRNA synthesis [33]. Furthermore, the thermodynamically stable SL3–4 tertiary structure was proposed to repress the cap-dependent translation efficiency of viral mRNAs by acting as a steric block during ribosomal scanning [14]. Collectively, the available information suggests that short linker nucleotides between individual SLs, the topological arrangement of SL3 in relation to adjacent RNA elements, and structural flexibility play key roles in fine-tuning viral RNA synthesis, genome cyclization, and cap-dependent translation of viral mRNAs with distinct variations among different coronaviruses.

SL4

SL4 is a long hairpin structure that is located downstream of the TRS-L core sequence and conserved across all coronavirus genera [11,16,19]. SL4 structures comprise bipartite SLs, a and b, that are separated by a bulge [11,19]. Recent nuclear magnetic resonance (NMR) spectroscopy analyses revealed that the SARS-CoV-2 SL4 folds into an independent structure [14,18]. Earlier BCoV studies revealed that disruption of the SL4b structure abolishes RNA replication, but this defect can be rescued through compensatory mutations that restore the stem structure [34]. SL4 contains a short ORF (termed uORF) upstream of ORF1a. In MHV, the uORF in SL4b was found to be dispensable for viral replication [35]. Mutational analyses suggested that SL4, and particularly SL4b, primarily acts as a spacer element that probably has a specific role in sgRNA synthesis [36]. The HCoV-OC43 SL1–4 structure model provides strong support for this hypothesis [14]. Nucleotide substitutions that disrupt the coaxially stacked SL3/SL4 structure distort the relative orientation of the TRS-L, which is expected to affect interactions with cognate binding partners. The specific roles of SL4 in viral replication and transcription remain to be determined.

SL5

SL5 sequesters the ORF1a start codon and extends into the nsp1 coding sequence. SL5 adopts a multibranched RNA structure comprising three substructures called 5a, 5b, and 5c [13,19,37]. Interestingly, the apical loops of the SL5a-c substructures display conserved repetitive structural motifs (RSM), 5'-UUYCGU-3', that are conserved in many alpha- and betacoronaviruses, but not in betacoronaviruses of the subgenus *Embecovirus* such as MHV and BCoV, which appear to lack 5'-UUYCGU-3'-containing hairpins altogether [19,37]. Instead, these viruses possess multiple copies of AA-bulges within a long stem-bulge element (in the nsp15 coding sequence in ORF1b) that has been shown to function as a packaging signal (PS) [38,39]. The specific roles of these diverse RSMs in viral RNA synthesis and/or genome packaging (and, potentially, yet other mechanisms) remain to be elucidated in further studies.

Coronavirus 3'-terminal *cis*-acting elements

Despite marginal sequence identity, the structures and functions of RNA structural elements in betacoronavirus

3' UTRs appear to be functionally conserved as demonstrated by the successful rescue of replication-competent chimeric viruses with replacements of their cognate 3' UTRs with those of other betacoronaviruses but not alpha- or gammacoronaviruses [40–44], suggesting differences between coronavirus genera.

Cis-acting elements present in the 3'-terminal genome regions have been studied mainly in betacoronaviruses [9,13,33,40–43,45]. They include a bulged stem-loop (BSL) structure located just downstream of the N gene stop codon [40] and, further downstream, an RNA pseudoknot (PK) structure. Alphacoronaviruses lack a betacoronavirus-like BSL but were proposed to have other RNA structural elements that interact to form a PK structure [11]. The 3'-most RNA structural element is generally referred to as the multibranched hypervariable region (HVR). It includes a coronavirus-wide conserved octanucleotide sequence and a stem-loop II-like motif (s2m) [13,18,42]. In alphacoronaviruses, a similar stem-loop structure is predicted for the genome region downstream of the PK [11].

Bulged stem-loop and pseudoknot

The functional significance of the betacoronavirus BSL and PK structures has been studied extensively for MHV. Sequences involved in the formation of BSL and PK partly overlap. Formation of the first stem of the PK requires base-pairing interactions with the lower segment of the BSL [43,46], suggesting that the two structures are mutually exclusive [43]. Both structures proved to be essential for viral replication and were proposed to act as a 'molecular switch' that regulates specific steps of viral RNA synthesis [43]. Further MHV studies indicated direct interactions between loop 1 of the PK and the 3' end of the genome, and there is evidence for interactions of the PK region with two essential RTC subunits, nsp8 and nsp9 [43], suggesting specific functions for the PK in viral RNA synthesis. Surprisingly, none of the recent SHAPE and DMS-MaPseq analyses of the SARS-CoV-2 genome support the formation of a PK structure in the 3' UTR [13,17,18]. Further studies are required to determine whether the failure to detect this structure is due to the thermodynamic (in)stability proposed previously for the entire 'molecular switch' conformation or has another basis [47]. It would be interesting to develop an integrative model that combines the MHV mutagenesis data supporting an essential role for a 3'-proximal PK structure with the wealth of structural and functional information generated in recent studies.

Hypervariable region and stem-loop II-like motif

The region downstream of the PK, referred to as the HVR, contains two highly conserved elements: the octanucleotide sequence and the s2m domain [18,42,48]. Early studies showed that large portions or even the entire HVR can be deleted without causing major defects in MHV replication in cell culture, while MHV HVR mutants were highly attenuated *in vivo*, indicating a role in viral pathogenesis [42]. The octanucleotide sequence, 5'-GGAAGAGC-3', is conserved across all coronavirus genera, supporting its functional importance.

The conserved s2m motif was identified in coronaviruses and members of the families *Astroviridae*, *Caliciviridae*, and *Picornaviridae* [49,50]. Recently, a 4-nt palindrome (GUAC) in the terminal loop of the SARS-CoV-2 s2m element was suggested to be involved in homodimeric RNA–RNA kissing complex formation, which is further stabilized by the N protein–associated chaperone activity [51]. If the SARS-CoV-2 s2m structure was targeted by using antisense oligos (ASOs), viral replication in infected cells was reduced [52]. In contrast to this observation, a recombinant SARS-CoV-2 variant in which the s2m element was deleted was reported to replicate to wild-type levels *in vitro* and viral fitness *in vivo* was not affected, suggesting that the s2m structure is dispensable for SARS-CoV-2 replication [53]. Deletion of s2m in an infectious clone of infectious bronchitis virus (IBV), a gammacoronavirus, had a moderate effect on viral replication *in vitro* [54], which was largely compensated by a 36-nucleotide insertion in this genome region that was acquired during serial passaging in cell culture. Also, the conserved s2m structure was found to contribute to IBV pathogenesis *in vivo*. More work is needed to determine the precise function(s) of the s2m element and elucidate the basis for its conservation in selected viruses from different RNA virus families.

Conclusions and future challenges

Despite significant progress in the structural characterization of *cis*-acting RNA structures in betacoronavirus terminal genome regions, the precise functions of most of these elements in the viral replication cycle remain to be elucidated. Although the vast majority of studies were focused on just a few coronaviruses (primarily SARS-CoV-2 and few other betacoronaviruses), there is increasing evidence that a significant part of these structures is conserved (even across different genera) and may thus be targeted by broadly active antiviral approaches as shown recently by several studies. Thus, for example, ASOs targeting SL1 were reported to inhibit SARS-CoV-2 replication *in vitro* and *in vivo* [55,56]. Similarly, antisense circRNAs and amiloride-based compounds targeting the 5' UTR were successfully used to suppress viral replication [57,58].

In view of the major advances in the structural analysis of RNA structural elements of SARS-CoV-2 and a few

other coronaviruses over the past few years and the progress made in the production of genetically modified coronavirus variants by newly developed reverse-genetic systems, it seems reasonable to predict that the biological significance and specific functions of these RNA elements in viral replication will be determined in the near future.

CRediT authorship contribution statement

Ramakanth Madhugiri: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Hoang Viet Nguyen:** Writing – review & editing. **Heiko Slanina:** Writing – review & editing. **John Ziebuhr:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Data availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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