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# Viral and Cellular mRNA Translation in Coronavirus-Infected Cells

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## Abstract

Coronaviruses have large positive-strand RNA genomes that are 5' capped and 3' polyadenylated. The 5'-terminal two-thirds of the genome contain two open reading frames (ORFs), 1a and 1b, that together make up the viral replicase gene and encode two large polyproteins that are processed by viral proteases into 15–16 nonstructural proteins, most of them being involved in viral RNA synthesis. ORFs located in the 3'-terminal one-third of the genome encode structural and accessory proteins and are expressed from a set of 5' leader-containing subgenomic mRNAs that are synthesized by a process called discontinuous transcription. Coronavirus protein synthesis not only involves cap-dependent translation mechanisms but also employs regulatory mechanisms, such as ribosomal frameshifting. Coronavirus replication is known to affect cellular translation, involving activation of stress-induced signaling pathways, and employing viral proteins that affect cellular mRNA translation and RNA stability. This chapter describes our current understanding of the mechanisms involved in coronavirus mRNA translation and changes in host mRNA translation observed in coronavirus-infected cells.



## 1. INTRODUCTION

### 1.1 Overview of Translation Mechanism in Animal Cells

Being obligate intracellular parasites, viruses heavily depend on host cell structures and functions to complete their life cycle, and they also use the translational apparatus of the infected cell to express their proteins. In several cases, viruses have been shown to affect and/or modulate the status of the host translational machinery to achieve efficient viral protein synthesis and replication, while cellular mRNA translation is inhibited (Hilton et al., 1986; Narayanan et al., 2008a; Siddell et al., 1980, 1981a,b). In eukaryotic cells, translation occurs in the cytoplasm and essentially involves four steps: initiation, elongation, termination, and recycling (Kapp and Lorsch, 2004). The translational initiation step includes the recognition of an mRNA by the host translational machinery and assembly of the 80S complex, in which a methionyl initiator tRNA (Met-tRNA<sup>Met</sup>) binds at the peptidyl (P) site of the mRNA. In elongation, aminoacyl tRNAs enter the acceptor (A) site and, if the correct tRNA is bound, the ribosome catalyzes the formation of a peptide bond. After the tRNAs and mRNA are translocated such that the next codon is moved into the A site, the process is repeated. If a stop codon is encountered, the translation process is terminated, releasing the peptide from the ribosome. The recycling step involves

dissociation of the ribosome and release of mRNA and deacylated tRNA, thereby setting the stage for another round of translation initiation.

Translation initiation (rather than elongation or termination) is the key step in regulating protein synthesis events. Translational initiation requires at least nine eukaryotic initiation factors (eIFs) and comprises two steps: the formation of 48S initiation complexes with established codon–anticodon base pairing in the P-site of the 40S ribosomal subunits, and the joining of 60S subunits to 48S complexes to form the 80S complex. On capped mRNAs, 48S complexes are formed by the interaction of a 43S preinitiation complex (comprising a 40S subunit, the eIF2–GTP–Met–tRNA<sup>Met</sup> ternary complex, eIF3, eIF1, eIF1A, and probably eIF5) with the eIF4F complex (comprising eIF4E, a cap-binding protein, eIF4G, and eIF4A), which binds to the 5' cap region of the mRNA. By unwinding the mRNA's 5'-terminal secondary structure primarily by eIF4A in the eIF4F complex, the 43S complex then scans the 5' untranslated region (UTR) in the 5'–3' direction to the initiation codon. After initiation codon recognition, eIF2 triggers GTP hydrolysis, which is facilitated by eIF5 and eIF5B, leading to the displacement of eIFs, and the joining of a 60S subunit to form the 80S complex (Kapp and Lorsch, 2004).

Several studies showed coronavirus (CoV)-mediated control/alteration at translational initiation step (see Section 4). In contrast, little is known as to whether CoVs also affect the elongation, termination, or recycling steps in host protein synthesis. We also do not know whether synthesis of CoV-encoded proteins is regulated at the translation termination and/or recycling steps in infected cells, while the critical role of a –1 ribosomal frameshift event during translation elongation to produce the ORF1a/ORF1b-encoded replicase polyprotein (pp) 1ab is well established and has been extensively characterized (see Section 2.4).

In addition to cap-dependent translation initiation, several viruses, such as picornaviruses (which lack a 5'-end cap structure) (Dajjogo and Semler, 2011; Martinez-Salas et al., 2015), and some host mRNAs use cap-independent mechanisms for translation initiation. In contrast to cap-dependent translation, in which the 43S preinitiation complex binds to the 5'-terminal region of the mRNA through interaction between eIF3 in the 43S preinitiation complex and eIF4G in the eIF4F complex that is associated with the 5'-end of the mRNA, the 43S preinitiation complex (or the 40S ribosomal unit alone in some specific mRNA templates) directly binds to a specific region called internal ribosome entry site (IRES) of mRNAs that are translated by cap-independent translation mechanisms

(Kapp and Lorsch, 2004). The number and identities of translation initiation factors required for IRES-mediated translation initiation of specific mRNA species may vary significantly. For example, IRES-mediated translation of the picornavirus genome requires all translational initiation factors, except for eIF4E, 40S ribosome, and 60S ribosome subunits. However, other IRES elements, such as those present in the hepatitis C virus (HCV) genome or the cricket paralysis virus (CrPV) intergenic region, require a much smaller number of initiation factors compared to those required for cap-dependent translation. It is generally accepted that the vast majority of CoV proteins is synthesized by a cap-dependent translation mechanism and cap-independent translation initiation has only been reported for relatively few coronaviral mRNAs (see Section 2.7).

## 1.2 CoVs

CoVs are enveloped plus-strand RNA viruses that belong to the order *Nidovirales* in the subfamily *Coronavirinae* (family *Coronaviridae*) and are classified into four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (de Groot et al., 2011; Gorbalenya et al., 2004; Snijder et al., 2003; Woo et al., 2010, 2012). CoVs cause primarily respiratory and/or enteric diseases and are found in many animal species, including wild animals, domestic animals, and humans (Weiss and Navas-Martin, 2005). While most human CoVs (HCoV) cause relatively mild upper respiratory tract infections (common cold), two zoonotic viruses called severe acute respiratory syndrome (SARS) CoV and Middle East respiratory syndrome (MERS) CoV are associated with severe lower respiratory tract infections and are major public health threats. SARS-CoV, MERS-CoV, and some HCoVs, including HCoV-OC43 and HCoV-HKU1, belong to the genus *Betacoronavirus*, while other HCoVs, HCoV-229E, and HCoV-NL63, belong to the genus *Alphacoronavirus*. Animal CoVs from the genera *Alpha-* and *Betacoronavirus* are mainly associated with infections in mammals, while viruses in the genera *Gamma-* and *Deltacoronavirus* primarily (but not exclusively) infect birds. There is now compelling evidence to suggest that bats are the natural reservoir involved in the evolution and spread of many mammalian CoVs, including SARS-CoV and MERS-CoV (Lau et al., 2005; Li et al., 2005; Memish et al., 2013).

The CoV particles have a spherical shape with a diameter of roughly 100 nm (Davies and Macnaughton, 1979; Wege et al., 1979). They carry three major structural proteins (S, M, and E) in the envelope and contain

a helical nucleocapsid that is formed by the viral genomic RNA and the viral N protein. The viral S protein binds has receptor-binding and fusogenic functions (Heald-Sargent and Gallagher, 2012; Masters, 2006) and thus is essential for initiation of CoV infection.

### 1.3 Overview of CoV Genome Organization and Gene Expression Strategy

CoV genomes range between 27 and 32 kb, representing the largest RNA genome known to date. In common with typical mammalian mRNAs, the CoV genome has a 5'-terminal cap structure and a poly(A) sequence at the 3'-end (Masters, 2006) but, in contrast to most mammalian mRNAs, the CoV genome carries multiple open reading frames (ORFs) between the 5'- and 3'-terminal UTRs, both of which contain *cis*-acting signals involved in RNA replication (Brian and Baric, 2005; Masters, 2006). Genome regions upstream of these ORFs contain so-called transcription regulatory sequences (TRS) that are required for CoV transcription (Brian and Baric, 2005; Masters, 2006). All CoVs have two large ORFs, called ORF1a and ORF1b, that occupy the 5'-terminal two-thirds of the genome and are generally referred to as the viral replicase gene. Other ORFs located downstream of the replicase gene encode viral structural proteins and a varying number of accessory proteins, the latter being dispensable for virus replication in cell culture but involved in CoV pathogenicity (Liu et al., 2014; Narayanan et al., 2008b).

Following viral entry into the cell, the viral genome RNA undergoes translation to produce the viral proteins that are required for subsequent RNA replication and transcription. eIF4F and the 43S preinitiation complex access the 5'-end of the capped viral genome and the 43S preinitiation complex scans the 5' UTR. The 80S complex is assembled at the translation initiation codon and protein synthesis starts and proceeds until the first termination codon is encountered and the ribosome dissociates from its mRNA template, resulting in polyprotein (pp) 1a. Production of pp1ab (encoded by both ORF1a and ORF1b) requires a  $-1$  ribosomal frameshift before the translation stop codon is reached. This frameshift has been shown to occur in the overlap region between ORFs 1a and 1b. Viral proteins encoded downstream of ORFs 1a and 1b are not synthesized from the genome RNA but from a set of 5'-capped subgenomic mRNAs that carry the respective ORFs in their 5'-terminal regions. The two replicase polyproteins translated from ORFs 1a and 1b undergo proteolytic cleavage via viral-encoded proteinases encoded in ORF1a to generate 15–16 mature

nonstructural proteins, termed nsp1 to nsp16. All of these nsps, except for nsp1 (Hurst-Hess et al., 2015) and nsp2 (Graham et al., 2005), are considered essential for transcription and replication of CoV RNA (Newman et al., 2014). CoV RNA synthesis occurs at double membrane vesicles that are derived from endoplasmic reticulum (ER) membranes (reviewed in Newman et al., 2014) in the cytoplasm.

Besides its role as an mRNA for replicase polyprotein expression, the genome RNA is packaged into progeny virus particles (in contrast to subgenomic mRNAs that are not packaged efficiently). The number of subgenomic mRNAs differs among CoVs. These CoV mRNAs share their 3'-terminal regions, constituting a 3'-coterminal nested set of RNAs (reviewed in Masters, 2006). The 5'-end of CoV genomic RNA carries a ~70-nt-long leader sequence. The same leader sequence is also found at the 5'-end of all CoV mRNAs. Subgenomic minus-strand RNAs, each of which corresponds to each subgenomic mRNA species, also accumulate in infected cells. These subgenomic minus-strand RNAs carry complement of the leader sequence (antileader) at the 3'-end. It has been proposed that subgenomic minus-strands are synthesized from intracellular genome-length RNA, in a process called discontinuous extension and involving base pairing interactions between the 3'-end of the nascent minus-strand and the leader TRS. Subsequently, these antileader-containing subgenomic minus-strand RNAs are used as templates to produce 5' leader-containing mRNAs in which the 5' leader sequence is fused to the mRNA body at the TRS (Masters, 2006; Sawicki and Sawicki, 1990; Sawicki et al., 2007). Like the genomic RNA, the CoV subgenomic mRNAs, except for the smallest mRNA, are polycistronic and, with very few exceptions, only the 5'-terminal ORF of each of these subgenomic mRNAs is translated into protein.



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## **2. MECHANISMS AND CONTROL OF TRANSLATION OF CORONAVIRUS mRNAs**

### **2.1 Evidence for Cap-Dependent Translation of CoV mRNAs**

Because genomic and subgenomic CoV mRNAs have a 5' cap structure, most CoV mRNAs are thought to undergo cap-dependent translation using eIF4F. Cencic et al. reported several compounds that inhibit eIF4F activity by preventing eIF4E–eIF4G interaction (Cencic et al., 2011b). The same group demonstrated that a molecule (4E2RCat) that prevents the

interaction between eIF4E and eIF4G inhibits HCoV-229E replication (Cencic et al., 2011a), providing additional evidence to suggest that CoV mRNA translation depends on a 5' cap structure being present on viral mRNAs. The authors also found that a certain concentration of 4E2Rcat completely inhibited HCoV-229E replication, whereas it inhibited host protein synthesis by ~40%, indicating that HCoV-229E mRNAs show a higher dependency on eIF4F for ribosome recruitment compared to host mRNAs. To date, possible roles of eIF4F in viral mRNA translation have not reported for other CoVs.

## 2.2 Viral Enzymes Involved in Capping of CoV mRNAs

Formation of the cap structure of eukaryotic and eukaryotic viral mRNAs generally requires three successive enzymatic reactions (Furuichi et al., 1976). First, an RNA 5'-triphosphatase (TPase) removes the  $\gamma$ -phosphate group from the 5'-triphosphate end (pppN) of the nascent mRNA chain to generate the diphosphate 5'-ppN. Subsequently, an RNA guanylyltransferase transfers a GMP to the 5'-diphosphate end to produce the cap core structure (GpppN). Finally, an N7 methyltransferase (N7-MTase) methylates the attached GMP (cap) at the N7 position to produce a cap-0 structure (m7GpppN). Higher eukaryotes and viruses usually further methylate the cap-0 structure at the ribose 2'-O position of the first and second nucleotide of the mRNA by a ribose 2'-O-MTase to form cap-1 and cap-2 structures, respectively (Furuichi and Shatkin, 2000). Ribose 2'-O-methylation of viral RNA cap provides a mechanism for viruses to escape host immune recognition (Daffis et al., 2010; Züst et al., 2011).

The CoV genome encodes several RNA processing enzymes involved in RNA capping. The cap formation step by CoV has mainly been studied in SARS-CoV. In the first step of cap formation to generate diphosphate 5'-ppN, nsp13 may be involved because it has RNA 5'-TPase activity mediated by the NTPase active site of the nsp13-associated helicase domain (Ivanov and Ziebuhr, 2004; Ivanov et al., 2004). The next step is the formation of the cap core structure (GpppN) by an RNA guanylyltransferase. At present, it is not clear whether or not CoVs encode this enzyme; perhaps CoVs use cellular enzymes to perform this step. The third step is the methylation of the cap guanosine at the N7 position. This reaction is mediated by the C-terminal domain of CoV nsp14 (Chen et al., 2009) using S-adenosyl methionine (SAM) as a methyl group donor. Apparently, the enzyme is not specific for viral substrate RNAs (Bouvet et al., 2010). Conversion of cap-0 to cap-1



structures involves nsp16 that acts as a 2'-O-MTase (Bouvet et al., 2010; Decroly et al., 2008) and forms a complex with nsp10 (Chen et al., 2011) that appears to be required for efficient binding to SAM and the RNA substrate. Interestingly, SARS-CoV nsp10 plays an essential role in the specific binding of nsp16 to m7GpppA-capped RNA (first nucleotide is adenine). Considering that both the genomic and subgenomic mRNAs of SARS-CoV start with an adenine, this feature appears beneficial for SARS-CoV replication. The crystal structure of the heterodimer of nsp16/nsp10 with bound methyl donor SAM showed that nsp10 may stabilize the SAM-binding pocket and extend the RNA-binding groove of nsp16 (Chen et al., 2011).

### 2.3 Changes in the Poly(A) Tail Length During CoV Replication

CoV genomic and subgenomic mRNAs carry a poly(A) tail at their 3' ends. Hofmann and Brian postulated that viral RNA-dependent RNA polymerase or a cellular cytoplasmic poly(A) polymerase synthesizes the poly(A) tail of CoV mRNAs (Hofmann and Brian, 1991). Wu et al. reported that the length of the poly(A) tail of bovine CoV (BCoV) mRNAs in infected human rectal tumor-18 cells varies at various times postinfection (p.i.), ranging from ~45 nt immediately after virus entry to ~65 nt at 6–9 h p.i. and ~30 nt at 120–144 h p.i. (Wu et al., 2013). Differences in poly(A) length of viral mRNAs at different times p.i. was also observed in several other BCoV-infected cell lines and cells infected with different strains of infectious bronchitis virus (IBV) (Shien et al., 2014), indicating that changes in the poly(A) length during virus replication may be a common feature of CoVs. Factor and mechanisms involved in this process remain to be studied. Because the length of the poly(A) tail contributes to the efficiency of translation and replication of CoV defective interfering RNAs (Spagnolo and Hogue, 2000; Wu et al., 2013), the regulated changes in the length of the CoV poly(A) tail may affect efficiencies of viral translation and replication over the course of infection.

### 2.4 Ribosomal Frameshift in CoV Gene 1 Protein Expression

As mentioned earlier, two large polyproteins, one of which being translated from ORF1a and the other from ORFs 1a and 1b, are synthesized from the viral genome RNA. The synthesis of polyprotein 1ab involves a  $-1$  ribosomal frameshift during the translational elongation step and occurs in the

overlap region between ORFs 1a and 1b (Bekaert and Rousset, 2005; Brierley et al., 1987, 1989). Polyprotein 1ab is thus encoded by a (functionally) fused ORF produced from the two ORFs 1a and 1b. As most of the mature nsp proteins processed from these two large polyproteins are essential for CoV RNA synthesis, translation of polyprotein 1ab via  $-1$  ribosomal frameshifting is an essential step in CoV replication.

The signals required for  $-1$  ribosomal frameshifting in the ORF1a/1b overlap region were first identified in IBV (Brierley et al., 1987, 1989). Subsequently, putative ribosomal frameshift signals were also identified and characterized in other CoVs (Bekaert and Rousset, 2005). The  $-1$  ribosomal frameshifting signals are composed of a slippery sequence “UUUAAAC” followed by a “stimulatory” RNA secondary structure. The  $-1$  frameshifting occurs at this slippery sequence, where tRNAs are supposed to dissociate from the mRNA and then shift (by 1 nucleotide) to a codon in another reading frame, ORF1b (Plant and Dinman, 2008). The stimulatory structure is a complex RNA stem-loop structure RNA that varies among different CoVs. SARS-CoV’s stimulatory structure contains three stem loops (Baranov et al., 2005; Plant et al., 2005; Su et al., 2005), and their disruption affects frameshift efficiency (Plant and Dinman, 2006; Plant et al., 2005). Recently, Ishimaru et al. showed that a homodimeric RNA complex formed by the SARS-CoV’s stimulatory structure occurs within cells and that loop-to-loop kissing interactions involving stem3-loop2 modulate the  $-1$  ribosome frameshift efficiency (Ishimaru et al., 2013). These reports indicate that an optimal secondary RNA structure and RNA–RNA interaction within the ribosomal frameshifting signal are important for efficient  $-1$  ribosomal frameshifting.

Several studies showed that reduction of frameshifting efficiency affects virus infectivity and replication (Ishimaru et al., 2013; Plant et al., 2010, 2013). Plant et al. showed that the ratio of ORF1a- and ORF1b-encoded proteins plays a critical role in CoV replication efficiency (Plant et al., 2010). The authors proposed that CoVs have evolved to produce optimal levels of  $-1$  ribosomal frame shift efficiency for efficient virus propagation. CoV frameshift signals characterized previously have  $-1$  ribosomal frameshift efficiencies in the range of 20–45% (Baranov et al., 2005; Brierley et al., 1987; Herold and Siddell, 1993; Plant and Dinman, 2008). A recent study using ribosome profiling of mouse hepatitis virus (MHV)-infected cells suggests that the frameshift rate may be even slightly higher (Irigoyen et al., 2016).

## 2.5 Ribosomal Shunting Mechanism of Translation in CoVs

Ribosomal shunting is a translation initiation depending on cap-dependent discontinuous scanning, whereby ribosomes are loaded onto mRNA at the 5' cap structure and scanning is started for a short distance before bypassing the large internal leader region and initiating at a downstream start site (Firth and Brierley, 2012). For some strains of transmissible gastroenteritis virus (TGEV), mRNA having ORF 3b as the first ORF is not produced and ORF 3b is present as a nonoverlapping second ORF on mRNA 3. O'Connor et al. proposed a possible ribosomal shunting in the translation of ORF 3b in TGEV (O'Connor and Brian, 2000). The basis for this proposal was as follows: (i) if 3b protein was translated by a leaky scanning mechanism, a modification of ORF 3a to generate a favorable Kozak sequence would be expected to diminish protein synthesis from ORF3b compared to ORF3a; however, optimization of the Kozak context for ORF 3a did not affect translation efficiency of 3b; (ii) the translation of 3b was shown to be cap dependent; and (iii) deletion analysis failed to provide evidence for an IRES within the ORF 3a sequence. In several viral mRNAs, the presence of a specific donor structure with a large stem-loop with a 5'-adjacent, short ORF appears to be required for ribosome shunting (Hemmings-Mieszczak and Hohn, 1999; Pooggin et al., 1999), whereas TGEV ORF3b shunting does not depend on such a donor structure. Given that Sendai virus mRNA (Latorre et al., 1998), avian orthoreovirus mRNA (Racine and Duncan, 2010), and avihepadnavirus mRNA (Cao and Tavis, 2011) undergo ribosomal shunting, with no apparent requirement for a donor structure, it seems reasonable to suggest that translation of the TGEV 3b protein may involve a ribosomal shunting-driven translation mechanism in the absence of a specific donor structure.

## 2.6 Leaky Scanning Translation Mechanism of CoV Internal ORFs

An internal CoV ORF was initially found within the 5' half of the BCoV N gene ORF (Lapps et al., 1987). This internal ORF gene encodes the I protein and is likely translated by a leaky scanning mechanism in which ribosomes occasionally bypass the first AUG (the start codon for the N protein) and initiate translation from the AUG codon of the internal ORF encoding the I protein. The first AUG has a suboptimal Kozak context, while the downstream AUG (the start codon for the I protein) is in a more favorable Kozak context (Senanayake and Brian, 1997; Senanayake

et al., 1992). The N genes of MHV-A59, HCoV-HKU1, HCoV-OC43, SARS-CoV, and MERS-CoV also have an internal ORF (Armstrong et al., 1983; Kamahora et al., 1989; Rota et al., 2003; van Boheemen et al., 2012; Woo et al., 2005); in HCoV-OC43, the AUG start codon of the internal ORF is changed to AUC.

Because many CoVs carry the internal ORF in the N gene, it is possible that the encoded proteins may have (an) important biological function(s) for virus replication/survival, whereas our knowledge of their biological functions is limited. Fischer et al. reported that the MHV I protein is not essential for virus replication in cultured cells and its natural host (Fischer et al., 1997). However, a MHV mutant lacking the I gene formed smaller plaques than did wild-type MHV, indicating that I protein expression could give some minor growth advantage to the virus. Shi et al. reported that the SARS-CoV internal ORF protein, called the 9b protein, which is encoded within the N gene ORF, evades host innate immunity by targeting the mitochondrial-associated adaptor molecule, MAVS (Shi et al., 2014), while a previous study using a SARS-CoV mutant lacking viral accessory genes, including 6, 7a, 7b, 8a, 8b, and 9b, showed that deletion of these genes had little effect on the pathogenicity of the virus in a mouse model (Dediego et al., 2008). Further investigation is required to better understand the role of the SARS-CoV 9b protein in viral pathogenicity.

## 2.7 IRES-Mediated Translation in CoVs

Some CoV proteins are synthesized by IRES-mediated translation. Liu and Inglis reported that mRNA 3 of IBV was functionally tricistronic, having the capacity to encode three proteins (3a, 3b, and 3c) from three ORFs (Liu and Inglis, 1992). Their study showed that (i) a synthetic mRNA whose peculiar 5'-end structure prevents translation of the 5'-proximal ORFs (3a and 3b) directs efficient synthesis of 3c, (ii) translation of 3c is not affected by the absence/presence of the 5' cap analog as well as changes in the sequence contexts for initiation of ORF 3a and 3b translation, (iii) an mRNA in which the 3a/b/c coding region was placed downstream of the influenza A virus nucleocapsid protein gene directed the efficient synthesis of only 3c and nucleocapsid protein, and (iv) expression of the 3c ORF from this mRNA was abolished when the 3a and 3b coding region was deleted, the latter indicating that 3c translation initiation depends on upstream 3a/b sequences that serve as an IRES element. Further analyses showed that the sequence prior to the initiator AUG of 3c can form an RNA secondary structure comprised

of five RNA stem-loops that can be modeled into a compact superstructure formed by interactions of two predicted pseudoknot structures. The proposed structure shares structural features with IRES elements conserved in picornavirus genome RNAs, and a base pairing model between mRNA 3 and 18S ribosomal RNA (rRNA) was suggested (Le et al., 1992, 1993). IRES-mediated translation was also reported in MHV. The unique region of MHV mRNA 5 has two ORFs, ORF 5a, and ORF 5b. Thiel and Siddell reported that a synthetic mRNA containing both ORFs is functionally bicistronic and expression of ORF 5b, but not ORF 5a, is maintained in a tricistronic mRNA containing an additional 5'-proximal ORF. These data suggested that initiation of protein synthesis from ORF 5b may be mediated by an internal entry of ribosomes (Thiel and Siddell, 1994). Further studies by Jendrach et al. identified the IRES element of mRNA 5, which contains  $\leq 280$  nucleotides including the ORF 5b initiation codon (Jendrach et al., 1999). The authors also showed that the IRES element of mRNA 5 interacts specifically with protein factors present in an L-cell lysate (Jendrach et al., 1999).

## 2.8 Presence of Upstream ORF in CoV Genomic RNAs

Short AUG-initiated upstream ORFs (uORF) located in the 5' UTR of eukaryotic mRNAs are generally translated by 5' cap-dependent ribosomal scanning, resulting in translation repression of the major downstream ORF expressed from this mRNA (Calvo et al., 2009; Somers et al., 2013). In CoVs, a uORF is found within the 5' UTR of the genomic RNA, just downstream of the genomic leader sequence. Hence CoV genomic RNA, but not sub-genomic mRNAs, carry a uORF in their 5'-terminal region(s) (Wu et al., 2014), with few exceptions (Hofmann et al., 1993). Wu et al. reported that the uORF encoding a potential polypeptide of 3 to 13 amino acids is found within the 5' UTR of more than 75% of CoV genomes based on 38 reference strains (Wu et al., 2014), suggesting a functional significance for this sequence. The authors further showed by using an in vitro translation system that the MHV uORF is involved in a decreased rate of translation from the ORF1a start codon; wild-type MHV and mutants that lack the uORF showed similar growth properties in cell culture and, within 10 passages in cell culture, the uORF-disrupting mutations reverted back to the wild-type sequences or generated a new uORF sequence (Wu et al., 2014). These results suggest that the uORF represses ORF1a translation, yet plays a beneficial, but nonessential role in CoV replication in cell culture.



### 3. HOST AND VIRAL FACTORS THAT REGULATE CORONAVIRUS mRNA TRANSLATION

#### 3.1 Factors That Bind to Viral UTRs

The 5' and 3' UTRs of CoV genomic RNA carry *cis*-acting elements that are important for viral RNA synthesis (Chen and Olsthoorn, 2010). A number of host factors have been identified to interact *in vitro* with *cis*-acting elements of the 5' and 3' UTRs; these include heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and Q (Choi et al., 2004; Li et al., 1997), polypyrimidine tract-binding protein (PTB) (Li et al., 1999), mitochondrial aconitase (Nanda and Leibowitz, 2001), poly(A)-binding protein (PABP) (Spagnolo and Hogue, 2000), the glutamyl-prolyl-tRNA synthetase (Galan et al., 2009), the arginyl-tRNA synthetase (Galan et al., 2009), and the p100 transcriptional coactivator (Galan et al., 2009). Among these, hnRNP A1, hnRNP Q, and PTB have been shown to have a role in MHV RNA synthesis (Choi et al., 2002; Li et al., 1999; Shen and Masters, 2001; Shi et al., 2000). Likewise, PABP, hnRNP Q and EPRS proteins have been shown to play a positive role in TGEV infection (Galan et al., 2009). Currently, it is unclear whether any of these host proteins affect translation of viral proteins.

Senanayanke and Brian reported that replacement of the natural 77-nt 5' UTR on synthetic transcripts of BCoV mRNA 7 (Senanayake and Brian, 1999), which encodes the N and I proteins, with the 210-nt 5' UTR from BCoV mRNA 1 caused approximately twofold-less N protein in an *in vitro* translation system as well as in uninfected cells using a T7 RNA polymerase-driven transient transfection system. In infected cells, this difference became 12-fold as the result of both a stimulated translation from the 77-nt 5' UTR and a repression of translation from the 210-nt 5' UTR, demonstrating a differential 5' UTR-directed regulation of translation in CoV-infected cells and suggesting that this regulation involves viral or virus-induced cellular factors that interact with *cis*-acting elements in the 5' UTR.

#### 3.2 N Protein-Mediated Enhancement of Viral Translation

Tahara et al. reported that capped chimeric mRNAs, in which the MHV 5'-leader sequence was positioned upstream of the human alpha-globin coding region, were translated three- to fourfold more efficiently in cell-free extracts derived from MHV-infected cells compared to extracts obtained from uninfected cells. In contrast, nonviral mRNAs were found to be

translated equally efficiently in cell-free extracts derived from infected and uninfected cells (Tahara et al., 1994). The same group subsequently demonstrated that the MHV N protein (i) stimulates translation of a reporter mRNA carrying the MHV 5'-UTR (Tahara et al., 1998) and (ii) binds with high affinity to the leader sequence and TRS (Nelson et al., 2000), suggesting that CoV N proteins may act as *trans*-acting factors that enhance the translation efficiency of CoV mRNAs.



## 4. CORONAVIRUS-MEDIATED CONTROL OF HOST TRANSLATION

CoV replication in cultured cells is often linked to an inhibition of host protein synthesis (Hilton et al., 1986; Narayanan et al., 2008a), with some exceptions (Cencic et al., 2011a). MHV infection drastically inhibits host protein synthesis between 3 and 6 h p.i., while viral protein synthesis increases from 3 h p.i. and peaks at 6 h p.i. (Siddell et al., 1980, 1981a,b). An increased number of free 80S ribosomes and a shift to lighter polysomes during MHV infection suggest a potential suppression at the translation initiation phase (Anderson and Kedersha, 2009; Siddell et al., 1981b).

Banerjee et al. reported that MHV infection induces degradation of 28S rRNAs as early as 4 h p.i. and almost all 28S rRNAs are degraded by 24 h p.i., while 18S rRNA remains stable (Banerjee et al., 2000). Although the biological significance of MHV-induced 28S rRNA degradation is not entirely clear, it may contribute to the virus-induced translational suppression because the 28S rRNA is an integral component of the 60S ribosomal subunit. In addition to 28S rRNA, MHV infection was reported to stimulate degradation of specific host mRNAs (Kyuwa et al., 1994), thereby further limiting host protein expression.

### 4.1 CoV Proteins That Suppress Translation

One of the CoV proteins that have a role in inhibiting host translation was found to be nsp1, the most N-terminal pp1a/pp1ab-derived processing product. CoV nsp1 equivalents appear to employ divergent strategies to suppress host gene expression. For instance, SARS-CoV nsp1 inhibits mRNA translation and promotes host mRNA degradation by binding to the 40S ribosomal subunits (Kamitani et al., 2009), while nsp1 of MERS-CoV inhibits host mRNA translation and induces host mRNA cleavage without binding to the 40S ribosomal subunits (Lokugamage et al., 2015). Nsp1 of TGEV uses a yet another, currently unknown mechanism to suppress host

protein synthesis without binding to the 40S ribosomal subunits or inducing mRNA degradation (Huang et al., 2011a).

SARS-CoV nsp1 inhibits cap-dependent and IRES-driven translation by suppressing multiple steps of translation initiation (Lokugamage et al., 2012). SARS-CoV nsp1 inhibits both 48S and 80S initiation complex formation with a stronger inhibition of the latter on cap-dependent and encephalomyocarditis virus (EMCV)-IRES-driven mRNA templates. SARS-CoV nsp1 inhibits different steps of initiation on mRNA templates that use the IRES-40S binary complex to initiate translation, such as CrPV and HCV-IRES-driven mRNAs. Nsp1 inhibits binary complex formation on CrPV-IRES and 48S complex formation on HCV-IRES (Lokugamage et al., 2012). In addition to the translation suppression function, SARS-CoV nsp1 promotes host mRNA degradation by inducing endonucleolytic RNA cleavage in the 5'-UTR of host mRNAs (Huang et al., 2011b). The internally cleaved mRNAs are then degraded by the cellular Xrn1-mediated 5'-3' exonucleolytic mRNA degradation pathway (Gaglia et al., 2012; Huang et al., 2011b). SARS-CoV nsp1-induced degradation of host mRNAs also contributes to inhibition of host gene expression in infected cells (Narayanan et al., 2008a). While SARS-CoV nsp1 induces endonucleolytic cleavage of host mRNAs, viral mRNAs appear to be resistant to these cleavage mechanisms (Huang et al., 2011b; Kamitani et al., 2009; Lokugamage et al., 2012), most likely because the 5' leader sequence present on these RNAs protects them from nsp1-induced endonucleolytic cleavage. SARS-CoV nsp1 was also shown to inhibit translation of viral mRNAs in cell-free extracts (Huang et al., 2011b) as well as infected cells (Narayanan et al., 2014). In contrast, Tanaka et al. postulated that specific interaction of SARS-CoV nsp1 with the stem-loop I structure in the 5'-UTR of the SARS-CoV genome facilitates efficient viral gene expression in infected cells (Tanaka et al., 2012).

Unlike SARS-CoV nsp1, which is a cytoplasmic protein (Kamitani et al., 2006), MERS-CoV nsp1 is localized in both the cytoplasm and the nucleus of cells transfected with suitable plasmid construct or virus-infected cells (Lokugamage et al., 2015). Interestingly, MERS-CoV nsp1 selectively inhibits translation and induces degradation of mRNAs that are transcribed in the nucleus and transported to the cytoplasm, whereas it does not suppress the expression of exogenous mRNAs directly introduced into the cytoplasm or viral mRNAs produced in the cytoplasm of infected cells (Lokugamage et al., 2015). As MERS-CoV mRNAs are of cytoplasmic origin, their expression remains unaffected by MERS-CoV-encoded nsp1 in infected cells (Lokugamage et al., 2015).



Xiao et al. reported that the S proteins of SARS-CoV and IBV interact with eIF3f, one of the subunits of eIF3, leading to the inhibition of host translation at a late point in the infection (Xiao et al., 2008). The authors suggested that the interaction between CoV S protein and eIF3f may play a functional role in controlling the expression of specific host genes, especially those that are induced by the viral infection.

Using a yeast two-hybrid human protein library screen, Zhou et al. (2008) found that the SARS-CoV N protein binds to eukaryotic translation elongation factor 1 alpha (eEF1 $\alpha$ ), an integral part of the translational machinery; GTP-bound EF1alpha plays a key role in recruiting aminoacyl-tRNA to the A site of the 80S translation initiation complex during translation. The authors showed that expression of SARS-CoV N protein induces the aggregation of eEF1 $\alpha$ , resulting in the inhibition of host protein synthesis (Zhou et al., 2008).

Another CoV protein that affects host translation is the SARS-CoV 7a protein, a multifunctional protein that inhibits host translation, induces apoptosis, activates the p38 mitogen-activated protein kinase (Kopecky-Bromberg et al., 2006), and arrests the cell cycle at the G0/G1 phase (Yuan et al., 2006). Accumulating evidence suggests that the 7a protein inhibits cellular gene expression at the level of translation (Kopecky-Bromberg et al., 2006), whereas the precise mechanism of translation inhibition by the 7a protein remains to be determined.

## 4.2 CoV-Mediated Induction of ER Stress and Unfolded Protein Response

In eukaryotic cells, the ER plays a central role in the synthesis of secretory or transmembrane proteins, including folding of these proteins into their native conformation and mediating a range of posttranslational modifications. The ER maintains a homeostasis suitable to regulate the processing and prevent the aggregation of these proteins. If the accumulation of nascent, unfolded polypeptides exceeds the folding and processing capacity of the ER, the homeostasis is perturbed, resulting in ER stress and triggering the activation of the unfolded protein response (UPR). The latter contributes to restoring the normal function of ER or initiates apoptosis if the ER stress remains unchanged. UPR signaling activates three main branches: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein-1 (IRE1). Once activated these pathways send signals to inhibit translation, degrade misfolded proteins through ER-associated degradation, express ER molecular chaperones, and expand the ER

membrane to decrease the load of proteins and increase the protein-folding capacity in the ER. ER stress occurs under various conditions, such as nutrient deprivation, developmental processes, genetic changes, and invasion by pathogens, such as viruses and intracellular bacteria. Generally, in virus-infected cells, a copious amount of viral proteins is produced, often perturbing ER homeostasis and eventually causing ER stress (Schroder and Kaufman, 2005; Zhang and Wang, 2012).

Mounting evidence suggests that CoV replication causes ER stress that triggers UPR activation, thus possibly regulating host antiviral responses. Several studies have observed the induction of ER protein chaperones, such as the immunoglobulin heavy chain-binding protein (BiP), also known as glucose-regulated protein 94 (GRP94), which are considered indicators of ER stress, in SARS-CoV-infected cells (Chan et al., 2006; Yeung et al., 2008). In one of these studies, induction of luciferase reporter gene expression from BiP- or GRP94-driven promoters was observed in SARS-CoV-infected cells (Chan et al., 2006). Also other CoVs, such as MHV and IBV, have been shown to cause ER stress in infected cells (Fung and Liu, 2014; Versteeg et al., 2007). Cells overexpressing the S protein of SARS-CoV, MHV or HCoV-HKU1 also induce ER stress, suggesting that the S protein could be the main modulator of ER stress in CoV infections (Versteeg et al., 2007). Due to its large molecular weight and extensive glycosylation, the S protein requires ER protein chaperones for proper folding and maturation. ER stress has also been observed in cells that overexpress specific CoV accessory proteins, such as the 3a, 6, and 8ab proteins of SARS-CoV (Minakshi et al., 2009; Sung et al., 2009; Ye et al., 2008). In addition to the contribution of viral proteins in inducing ER stress, an increased overall ER burden, the formation of double membrane vesicle, and the depletion of ER lipids may contribute to ER stress, thereby affecting cellular mRNA expression in CoV-infected cells.

### 4.3 Status of Stress Granules and Processing Bodies in CoV Replication

In response to cellular stress caused by either environmental stimuli or viral infections, cells may reduce or even shut off their protein synthesis. Induction of translational shutoff also occurs by increased phosphorylation of eIF2 $\alpha$  (de Haro et al., 1996), resulting in dissociation of polysomes which, in turn, leads to stalled 43S and 48S preinitiation complexes and non-translating mRNAs that may be incorporated into stress granules (SGs), one of the two major cytoplasmic RNA granules. SGs are reversible foci

and temporary store houses for these complexes. When the stress conditions are over, stored initiation complexes are rapidly released to resume translation. Under normal conditions, processing bodies (P-bodies), another type of cytoplasmic RNA granule, are present in cells. Typically, P-bodies increase in number and size under host shutoff conditions. Both SGs and P-bodies contain translationally incompetent mRNAs, while P-bodies contain exonucleases, deadenylases, and enzymes for decapping (Anderson and Kedersha, 2009; Beckham and Parker, 2008; Buchan and Parker, 2009). Although P-bodies are enriched with RNA decay machinery proteins, it is controversial as to whether RNA decay occurs within P-bodies. Although virus infections activate cellular stress responses, some viruses, such as poliovirus and CrPV, do not induce SGs in the course of infection. Other viruses, including the alphavirus Semliki Forest virus, rotavirus, dengue virus, and West Nile virus, may actively inhibit SG formation (Emara and Brinton, 2007; Khong and Jan, 2011; McInerney et al., 2005; Montero et al., 2008).

Increased formation of SGs and P-bodies has been shown to occur during replication of some CoVs. Both TGEV and MHV form SGs as the infection progresses (Raaben et al., 2007; Sola et al., 2011). During MHV infection, formation of SGs and P-bodies were observed concomitantly with increased eIF2 $\alpha$  phosphorylation as early as 6 h p.i. when host translational shutoff and mRNA decay are apparent. These data led Raaben et al. to propose that MHV replication induces host translational shutoff by triggering an integrated stress response (Raaben et al., 2007). The authors further demonstrated that, when host translational shutoff was experimentally impaired, replication of MHV was not negatively affected but rather enhanced (Raaben et al., 2007). The appearance of SGs was also observed in TGEV infection, and these granules were not disassembled at later stages of the infection (Sola et al., 2011). However, when SG components were depleted, an increased replication of TGEV was observed, suggesting that SGs may restrict CoV replication.

#### 4.4 Activation of PKR, PERK, and eIF2 $\alpha$ Phosphorylation

Viral infections induce ER stress, thereby triggering phosphorylation of the serine residue at position 51 of eIF2 $\alpha$ . Phosphorylation of eIF2 $\alpha$  leads to translation inhibition. Two of the four protein kinases that are known to phosphorylate eIF2 $\alpha$  at Ser51 are protein kinase RNA-activated (PKR) and PERK. Double-stranded RNA and ER stress activate PKR and PERK, respectively. Several studies examined the status of eIF2 $\alpha$  phosphorylation

and PKR/PERK activation in CoV-infected cells. Zorzitto et al. reported minimal transcriptional activation of PKR in MHV-1-infected cells at later time points (Zorzitto et al., 2006). Ye et al. found that PKR and eIF2 $\alpha$  are not phosphorylated in MHV-A59-infected cells (Ye et al., 2007). In contrast, Bechill et al. observed significant phosphorylation of eIF2 $\alpha$  in MHV-A59-infected cells and efficient translation of MHV mRNAs in the presence of phosphorylated eIF2 $\alpha$  (Bechill et al., 2008). This discrepancy in the status of eIF2 $\alpha$  phosphorylation and PKR activation in these studies could be due to the use of different cell types and virus strains. Krahling et al. reported that SARS-CoV infection not only activated PKR and PERK but also phosphorylated eIF2 $\alpha$  at 8 and 24 h p.i. (Krahling et al., 2009), whereas activation of PKR and PERK or eIF2 $\alpha$  phosphorylation did not impair SARS-CoV replication. Based on these data, the authors hypothesized that SARS-CoV has evolved a mechanism to overcome the inhibitory effects of phosphorylated eIF2 $\alpha$  on viral mRNA translation. Wang et al. reported that that phosphorylation of eIF2 $\alpha$  was severely suppressed in human and animal cells infected with IBV (Wang et al., 2009). The level of phosphorylated PKR was found to be greatly reduced in IBV-infected cells, and nsp2 was a weak PKR antagonist. Also GADD34, a component of the protein phosphatase 1 (PP1) complex that dephosphorylates eIF2 $\alpha$ , was significantly induced in IBV-infected cells. Inhibition of the PP1 activity and over-expression of wild-type and mutant GADD34, eIF2 $\alpha$ , and PKR provided evidence to suggest that these virus-modulated pathways play a synergistic role in facilitating IBV replication. It was also postulated that IBV may employ a combination of two mechanisms, i.e., blocking PKR activation and inducing GADD34 expression, to maintain de novo protein synthesis in infected cells to enhance viral replication (Wang et al., 2009).



## 5. CONCLUDING REMARKS

This chapter summarizes experimental studies of mechanisms that drive and control the translation of viral and host cell mRNAs in CoV-infected cells. There is now a large body of information to suggest that (i) CoV evolved a range of mechanisms to control viral gene expression at the posttranscriptional level and (ii) CoV replication results in cell stress, leading to the activation of stress-induced signaling pathways that suppress host translation. Also, recent studies have uncovered that a number of CoV-encoded proteins, such as nsp1, suppress host translation without severely affecting viral gene expression. Although studies of the

posttranscriptional regulation of CoV gene expression have been making significant progress, our understanding of the translation mechanisms used by specific CoV mRNAs requires further investigation.

A major question to be addressed in more detail pertains to the mechanism that is used to ensure efficient CoV mRNA translation in cells in which host protein synthesis is suppressed. Replication of some CoVs, such as SARS-CoV, induces host mRNA decay (Lokugamage et al., 2015; Narayanan et al., 2008a). While the reduction of host mRNA levels is one of the factors involved in suppressing host translation in CoV-infected cells, it is unlikely to be the sole reason for the inhibition of host protein synthesis (Narayanan et al., 2008a). CoV mRNAs and host mRNAs share important structural features (such as the 5' cap structure and 3' poly(A) tail), suggesting that the translation of CoV and host mRNAs may be equally sensitive to the inhibition of factors that control specific translation steps, including eIF2 $\alpha$  and its phosphorylated form. A possible mechanism for efficient CoV translation under conditions in which host translation is suppressed may be related to the extreme abundance of CoV mRNAs that, at least in part, may counterbalance effects of translational suppression. Alternatively, CoV mRNAs and host mRNAs may use a slightly different repertoire of translation factors. It has been reported that alphaviruses can use eIF2A, instead of eIF2 $\alpha$ , in viral translation (Ventoso et al., 2006). If CoV mRNAs have similar requirements, then eIF2 $\alpha$  phosphorylation may not or less profoundly affect the translation of CoV mRNAs.

Because CoV mRNAs are capped at the 5'-end and polyadenylated at the 3'-end, it has been postulated that most of the CoV mRNAs undergo cap-dependent translation. However, experimental data that support this hypothesis have only been provided for HCoV-229E-infected cells (Cencic et al., 2011b). Unlike many other CoVs, HCoV-229E replication does not inhibit translation of host proteins (Cencic et al., 2011b). Do mRNAs of other CoVs also undergo cap-dependent translation? Recent studies showed that m<sup>6</sup>A-modification in the 5' UTR of mammalian mRNA may stimulate cap-independent translation (Meyer et al., 2015; Zhou et al., 2015). If the 5' UTR of some CoV mRNAs are subject to m<sup>6</sup>A-modification in infected cells, these CoV mRNAs might use a cap-independent mechanism of translation.

Previous studies suggested that the CoV leader sequence that is attached to the 5'-end of all CoV mRNAs affects translation and stability of CoV mRNAs. For example, the MHV leader sequence was shown to promote translation in cell-free extracts from MHV-infected cells (Tahara et al.,

1994); the N protein was reported to bind to the MHV leader sequence and to facilitate translation (Nelson et al., 2000; Tahara et al., 1998); and the SARS-CoV leader sequence was shown to mediate viral mRNA escape from SARS-CoV nsp1-induced endonucleolytic RNA cleavage (Huang et al., 2011b). However, our understanding of the biological functions of the leader sequence of other CoVs at the posttranscriptional level remains limited. Also, it is possible that leader sequences of different CoVs have evolved to meet differential requirements imposed by specific (and different) target cells.

Mechanisms that control cellular gene expression at the posttranscriptional level remains a major research field, generating surprising and exiting new discoveries at an amazing speed and, undoubtedly, will influence our future studies of the posttranscriptional regulation of CoV gene expression.

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