

## Chapter 2

# Coronavirus Genome Replication

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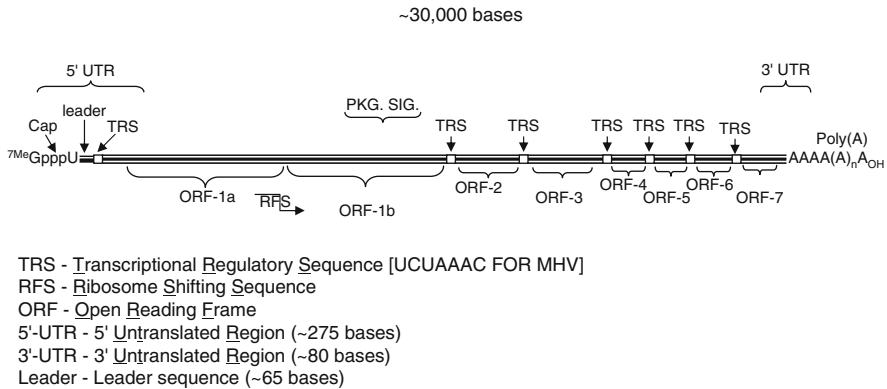
Viruses belonging to the family Coronaviridae are unique among RNA viruses because of the unusually large size of their genome, which is of messenger- or positive- or plus-sense. It is ~30,000 bases or 2–3 times larger than the genomes of most other RNA viruses. Coronaviruses belong to the order Nidovirales, the other three families being the Arteriviridae, Toroviridae and Roniviridae. (For a review of classification and evolutionary relatedness of Nidovirales see Gorbalenya et al. 2006.) This grouping is based on the arrangement and relatedness of open reading frames within their genomes and on the presence in infected cells of multiple subgenomic mRNAs that form a 3'-co-terminal, nested set with the genome. Among the Nidovirales, coronaviruses (and toroviruses) are unique in their possession of a helical nucleocapsid, which is unusual for plus-stranded but not minus-stranded RNA viruses; plus-stranded RNA-containing plant viruses in the Closteroviridae and in the Tobamovirus genus also possess helical capsids. Coronaviruses are very successful and have infected many species of animals, including bats, birds (poultry) and mammals, such as humans and livestock. Coronavirus species are classified into three groups, which were based originally on cross-reacting antibodies and more recently on nucleotide sequence relatedness (Gonzalez et al. 2003). There have been several reviews of coronaviruses published recently and the reader is referred to them for more extensive references (Enjuanes et al. 2006; Masters 2006; Pasternak et al. 2006; Sawicki and Sawicki 2005; Sawicki et al. 2007; Ziebuhr 2005).

The genome of coronaviruses is depicted in Fig. 2.1. Its length varies from ~27.5 to ~31 kb among the various species of coronaviruses. The 5'-end is capped although the exact structure of the capped 5'-end has not been determined. The 3'-end is polyadenylated and the genome, as well as subgenomic mRNAs, can be isolated by oligo (dT) chromatography. At the 5'-end there is an untranslated region (5'-UTR) of ~200–500 nucleotides (nts) before the initiation codon for the open reading frame (ORF) that is translated from the genome (ORF1).

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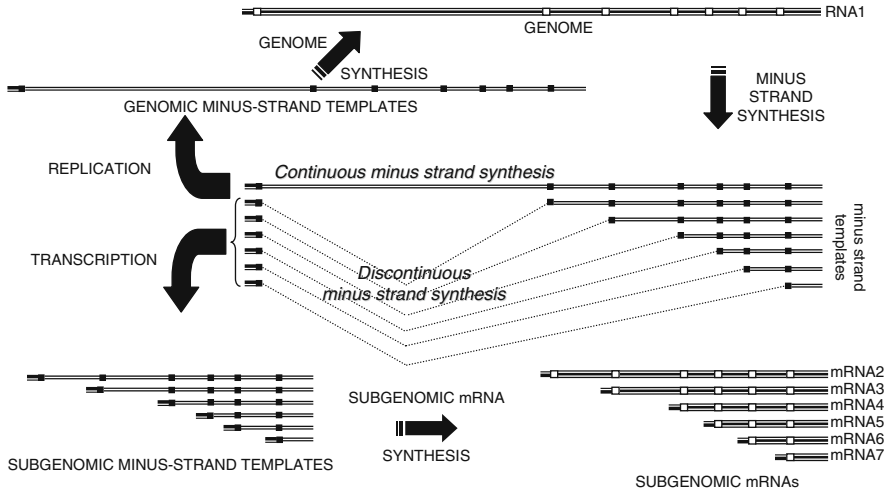
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**Fig. 2.1** Coronavirus genome.

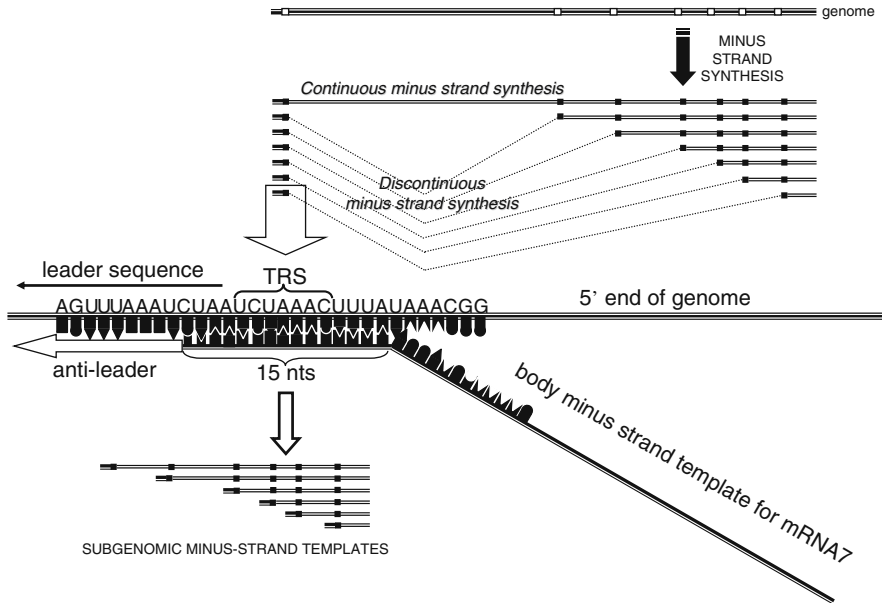
At the 3'-end there is an untranslated region (3'-UTR) of ~250–500 nts after the end of the last ORF and before the poly(A). ORF1 is divided into two large open reading frames (ORF1a and ORF1b); the end of ORF1a overlaps the beginning of ORF1b. A ribosome frame-shifting sequence (RFS) at the end of ORF1a causes the genome to be translated into two unusually long polyproteins, pp1a and pp1ab (see below). After ORF1 there is a series of multiple ORFs, depending on the virus, which are each preceded by a short repeated sequence called the transcription regulating sequence (TRS) immediately upstream of the initiating AUG for that ORF. A TRS is also found about 65 nts from the 5'-end of the genome. The sequence at the 5' end of the genome, up to this first TRS, is called the leader sequence (Fig. 2.1). The organization of multiple genes was first observed with IBV when its genome was sequenced, which was a feat of manual sequencing skill (Bournsnel et al. 1987). After MHV and other coronaviruses were sequenced and shown to have a similar size and organization, equine arteritis virus (EAV, the type member of the Arteriviridae) was sequenced and found to have a similar organization of genes but with half the number of bases as coronaviruses (den Boon et al. 1991). Another distinguishing feature between coronaviruses and arteriviruses is that while coronaviruses have helical nucleocapsids, arteriviruses have the more usual, for plus-stranded RNA viruses, icosahedral-type nucleocapsids. With group 2a coronaviruses, a packaging signal in ORF1b (Chen et al. 2007a) permits the genome, but not the subgenomic mRNA, to be assembled into virions. Some species of coronaviruses package varying amounts of subgenomic mRNAs into virions or membranous structures that have the same density of virions.

The genome replication strategy of coronaviruses, which was originally proposed in 1996 (Sawicki and Sawicki 1995), is depicted in Fig. 2.2. The ORF1 in the genome is translated to form the replicase which can then copy the genome continuously from one end to the other to produce a complementary copy of the genome, i.e., the genomic minus-strand template, that serves in turn to be copied into more



**Fig. 2.2** Coronavirus genome replication.

genomes, i.e., genome replication. In addition to making genomic minus-strand templates, the replicase appears to recognize sites at or surrounding the internal TRS, and after copying that internal TRS it then moves discontinuously, or translocates, to the 5'-end of the genome, thereby bypassing a large section of the intervening sequence between any one of the TRS elements and the leader sequence at the 5'-end of the genome. It then continues elongation by copying the leader sequence. Because of this discontinuous event, subgenomic minus-strand templates are produced that also contain a sequence complementary to the leader sequence, i.e., the anti-leader, at the 3'-ends of both genomic and subgenomic minus-strand templates. The subgenomic minus-strand templates, as well as the genomic minus-strand template, would be recognized by the viral transcriptase and copied into subgenomic mRNAs or genomes, respectively. I will refer to the activity of the replication/transcription complex (RTC) that recognizes the genome and synthesizes minus strands as the replicase and the activity of the RTC that recognizes the minus strands (genome-sized as well as subgenome-sized) and synthesizes plus strands as the transcriptase. As discussed below, these are two distinguishable activities of the RTC: The replicase recognizes only the genome as a template and copies it into both genomic and subgenomic minus strands and the transcriptase recognizes both the genomic minus-strand templates and the subgenomic minus-strand templates and copies them into genomes and subgenomic mRNAs, respectively. Because only the genome acts as a template for the production of subgenomic minus-strand templates, a replication signal would be only present on the genome but missing from the subgenomic mRNAs. In contrast, both the genomic and the subgenomic minus strands appear to contain a transcription signal that determines their capacity to serve as templates for plus-strand synthesis.



**Fig. 2.3** 3'-Discontinuous extension of subgenomic minus-strand templates.

Figure 2.3 depicts the key event in the discontinuous synthesis of subgenomic minus-strand templates. The replicase is thought to pause after copying the TRS element and then move with the nascent subgenomic minus strand, which has an anti-TRS at its 3'-end, to the TRS at the end of the leader where it serves to prime and resume elongation before terminating and completing the synthesis of a minus-strand template. Thus, termination of minus-strand synthesis would be the same for genomic as well as subgenomic minus-strand templates. This has been termed facilitated recombination (Brian and Spaan 1997) and creates a subgenomic minus-strand template where the body of the minus strand is joined to the anti-leader at the TRS (actually the complement of the TRS), which results in the subgenomic minus-strand templates all having the same 3'-end as the genomic minus-strand templates. Because they all possess identical 3'- and 5'-ends, all of the minus-strand templates would be equally recognized by the transcriptase. Thus, for coronaviruses to replicate their genome, they need only two activities: One, the replicase that recognizes the genome as a template to make both genomic and subgenomic minus-strand templates and a second, the transcriptase, that recognizes both the genomic and the subgenomic minus-strand templates for the transcription of the viral plus strands. Furthermore, both the genome and all the subgenomic mRNAs have the same 5'-end, which would give each the same ribosome recognition signal. With such a scheme, not only the relative abundance of the different plus strands, but also the relative abundance of the different viral proteins would be determined solely at the level of the minus-strand synthesis. Thus, the crucial determinant or key

event in coronavirus genome replication is how the virus determines how much of a particular minus-strand template to produce, i.e., its relative abundance. Each minus-strand template then would be equally susceptible to being copied into a plus-strand RNA because each has the same 5'- and 3'-ends. Furthermore, each plus strand would be equally susceptible to interacting with ribosomes because they all have the same 5'-end sequence and all are polyadenylated, although the genome might be more or less efficiently translated compared to the subgenomic mRNAs because it has a longer 5'-UTR. The initiating AUG on the subgenomic mRNA is very close to the TRS, while on the genome there are ~250 nucleotides between the TRS and the initiating AUG for ORF1. Thus, coronaviruses appeared to have evolved a genome replication strategy that simplifies the problem of coordinating mRNA and protein abundance (gene expression) by focusing on controlling minus-strand template abundance. Thus, the answer to the question “Why do coronaviruses, and also arteriviruses, but not toroviruses or roniviruses possess a leader?” is that they regulate the expression of their genes by controlling minus-strand template abundance. Their regulation of minus-strand template abundance must be considered as a mechanism driving their capacity to have larger RNA genomes and/or many more genes than most other RNA viruses and as responsible for their species diversity.

The genome replication strategy of coronaviruses presented in Fig. 2.2 is based on

- (1) Subgenomic mRNA constitutes a 3'-nested set with the genome and they all contain a leader sequence at their 5'-ends; and the leader sequence occurs only once in the genome, also at its 5'-end (Lai et al. 1983; Spaan et al. 1983);
- (2) Splicing, i.e., fusion of the 5'- and 3'-sequences of the genome and deletion of the intervening sequences, does not occur (Jacobs et al. 1981; Stern and Sefton 1982);
- (3) Subgenomic, in addition to genomic, minus-strand templates are present in infected cells at similar ratios as their corresponding plus strands (Sethna et al. 1989). This corrected the earlier reports that found only genomic minus-strand templates in infected cells (Baric et al. 1983; Lai et al. 1982).
- (4) Subgenomic minus-strand templates are present in replication intermediates (RIs) that are actively engaged in plus-strand synthesis (Sawicki and Sawicki 1990),
- (5) Replicative form (RF) RNA, i.e., the RNase resistant double-stranded core, with subgenomic minus strands do not arise from replication intermediates (RIs) whose templates were genomic minus strands (Sawicki et al. 2001; Sawicki and Sawicki 1990);
- (6) The subgenomic minus strands contained the same anti-leader sequence at their 3'-ends as did the genomic minus strands (Sawicki and Sawicki 1995; Sethna et al. 1991);
- (7) Subgenomic mRNA (Brian et al. 1994) or defective interfering (DI) RNA containing only the leader and the TRS at their 5'-end cannot replicate in the presence of helper virus (Makino et al. 1991) but can if they contain at

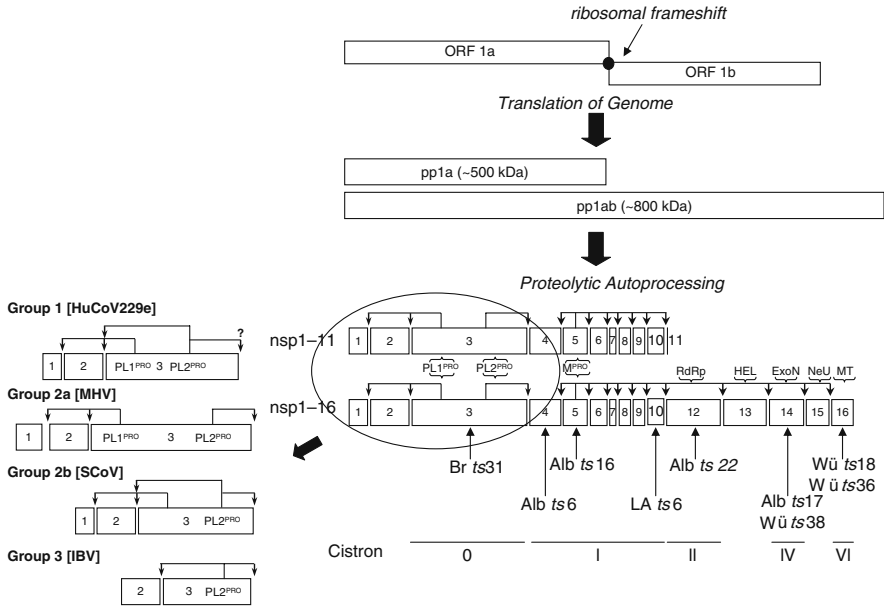
least ~250 nts of 5'-end of the genome (Brian et al. 1994; Makino et al. 1991; Masters et al. 1994);

- (8) RIs containing subgenomic minus-strand templates exist in infected cells and treatment with RNase generate the appropriate RF RNA (Sawicki and Sawicki 1990).

The reader is directed to (Sawicki and Sawicki 2005) for a more detailed account of the history of coronavirus transcription and the other two models proposed for generating subgenomic mRNA by coronaviruses. Eric Snijder and his students and colleagues adopted the discontinuous transcription model (den Boon et al. 1996; van Dinten et al. 1997) to explain EAV genome replication and devised elegant experiments using the infectious clone of EAV and site specific mutations to validate the proposal that it was during minus-strand synthesis that the discontinuous event occurs, whereby nascent minus strands pause at the TRS, relocate and recognize the TRS at the 5'-end of the genome and then act as a primer and complete elongation of the subgenomic minus strands (see Pasternak et al. 2001 for details).

In order to understand how coronaviruses replicate their genome, several questions must be answered: What viral proteins are required for coronavirus genome replication and how exactly do they function? What are the template requirements that specifically permit the viral replicase to recognize the coronavirus genome and copy it into minus-strand templates for genome and subgenomic mRNA? What are the template requirements that specifically permit the transcriptase to recognize the minus-strand templates and copy them into genome and subgenomic mRNA? And, what does the host supply for the replication of the coronavirus genome?

Coronaviruses are typical plus-stranded RNA virus. They do not package a RNA-dependent RNA polymerase in their virions and do not bring this enzyme into the infecting cell. Therefore, they must synthesize such a polymerase by translating its core components from the genome. Figure 2.4 depicts the translational products of ORF1. Two things are striking about the initial polyproteins (pp1a and pp1ab) that are formed. First is their unusually large size (~7,100 amino acids or ~800 KDa) and second is the large number of potential protein products, i.e., 15–16 (called nsp for *nonstructural proteins* and numbered according to their order from the N-terminus to the C-terminus of pp1a and pp1ab), that would be formed after proteolytic processing by either the papain-like cysteine proteases (PL<sup>PRO</sup>) or the poliovirus 3C-like or coronavirus “main” protease (M<sup>PRO</sup>) included within pp1a and pp1ab. Sequence analysis of the nonstructural proteins (nsps) predicts that they are associated with at least eight enzymatic activities (Snijder et al. 2003). Bartlam et al. (2007) review the structural proteomics approach to determining the structure–function relationship of the nsp of SARS-CoV, many of which have been crystallized (Cheng et al. 2005; Egloff et al. 2004; Joseph et al. 2006, 2007; Ricagno et al. 2006; Su et al. 2006; Sutton et al. 2004; Yang et al. 2003; Zhai et al. 2005). Some of these activities, e.g., proteinases, RNA-dependent RNA polymerase (RdRp) and helicase (HEL), are common to RNA viruses but others appear to be unique to coronaviruses. Recently, nsp8 was shown to be a second RdRp in addition to nsp12 but one that is less processive and causes the synthesis of complementary



**Fig. 2.4** Synthesis and processing of the pp1a and pp1ab polyproteins produced from ORF1 and the location of temperature-sensitive mutants of MHV that do not make viral RNA at 40C and their groupings into cistrons.

oligonucleotides of ~6 residues in a reaction whose fidelity is relatively low. Distant structural homology between the C-terminal domain of nsp8 and the catalytic palm subdomain of RdRps of RNA viruses suggests a common origin of the two coronavirus RdRps, which however may have evolved different sets of catalytic residues (Imbert et al. 2006). Clearly, most of the enzymatic functions associated with coronavirus nsps are concerned with viral RNA synthesis but it should also be noted that some of these activities might have relevance to cellular processes. For example, nsp3 in addition to containing PL<sup>PRO</sup> has been shown to express a deubiquitinating activity and is capable of de-ISGylating protein conjugates (Barretto et al. 2005; Chen et al. 2007b; Ratia et al. 2006), perhaps to subvert cellular processes and facilitate viral replication. Also, the adenosine diphosphate-ribose 1''-phosphatase (ADRP) activity of nsp3, which is not required for coronavirus genome replication (Egloff et al. 2006), may act to influence the levels of cellular ADP-ribose, a key regulatory molecule. Also nsp1, which is probably not essential for genome replication (Graham and Denison 2006; Ziebuhr et al. 2007), is proposed to cause degradation of host mRNA in SARS-CoV infected cells (Kamitani et al. 2006). Thus, it is important to discern those activities or functions that are required to produce viral RNA from those that influence the infected cells to allow viral RNA synthesis and/or to prevent an anti-viral response from foiling genome replication.

If all of the coronavirus proteins were to be assembled into a replicase, it would rival the size and complexity of eukaryotic transcription complexes. Do all of these

proteins actually function directly in coronavirus genome replication? Based on sequence analysis, the part of ORF1 starting with PL2<sup>PRO</sup> at the carboxyl half of nsp3 to the end of nsp16 is highly conserved among coronaviruses, while the sequence from nsp1 to the middle of nsp3 is not highly conserved. Group 3 coronaviruses (Fig. 2.4) exemplified by IBV do not encode an nsp1. Also, reverse genetic experiments showed that nsp1 and nsp2 are not essential for MHV and SARS-CoV genome replication (Deming et al. 2006; Graham et al. 2005; Zust et al. 2007) although recently an RNA stem-loop within nsp1 of group 2a coronavirus might be required for the genome to serve as a template for minus-strand synthesis (Brown et al. 2007). Using classical (forward) genetics or complementation analysis of temperature sensitive (*ts*) mutants (Sawicki et al. 2005; Helen Stokes and Stuart Siddell, personal communication) *ts* mutants that cannot synthesize viral RNA at 39–40°C (the non-permissive temperature) could be grouped into at least five complementation groups or cistrons 0, I, II, IV and VI. These cistrons were mapped to nsp3, nsp4-10, nsp12, nsp14 and nsp16, respectively. The *ts* mutants tested with causal mutations in nsp4, nsp5 and nsp10 all were found to belong to the same complementation group, i.e., cistron I, suggesting that they are cis-acting. This means that either the polyprotein nsp4/5/6/7/8/9/10 +/- 11 functions in genome replication as the unprocessed polyprotein or nsp4, 5, 6, 7, 8, 9 and 10/11 associate with one another before they are proteolytically processed into individual proteins and thus are not individually diffusible (*trans-active*). Recently, a single nucleotide mutation that caused an arginine to proline substitution in nsp13 (HEL) was found to be lethal for IBV (Fang et al. 2007). Interestingly, this mutation produces the same phenotype of blocking subgenomic mRNA synthesis but allowing genomic RNA synthesis as was found by van Dinten et al. (1997, 2000) for a point mutation in the helicase of EAV. Therefore, it is reasonable to predict that *ts* mutants will be found that have a causal mutation in nsp13 and this may give another cistron, although it is possible that nsp13 will function together with nsp12 or nsp14 and be assigned to cistron II or cistron IV, respectively. A recent report (Eckerle et al. 2006) claimed that the putative active site residues of nsp14 could not be substituted without loss of replication in culture, supporting its essential role. However, whatever functions nsp14 serves appear to be retained by uncleaved or partially processed nsp14, since abolition of either the amino-terminal or carboxy-terminal cleavage site allowed recovery of viable virus. No *ts* mutants with an RNA-negative phenotype and a causal mutation in nsp15 have been found, although single amino acid substitution of its homologue in EAV did result in loss of viral replication (Ivanov et al. 2004), and it would appear that nsp15 probably functions in genome replication, although it might also map to cistron IV or cistron VI. Thus, there might be only five cistrons that encompass replication/transcription functions of pp1a and pp1ab, a result that would argue that certain partially cleaved nonstructural polyproteins are functional in the RTC. At this time it is premature to propose a model for how the viral proteins that are required for coronavirus genome assemble and function in genome replication.

In addition to the nsps that function in viral RNA synthesis, the nucleocapsid protein (N) has been implicated in virus RNA synthesis (Almazan et al. 2004; Bost



et al. 2000; Chang and Brian 1996; Shi and Lai 2005; van der Meer et al. 1999) and its expression rescues recombinant coronaviruses from cells transfected with infectious RNA (Almazan et al. 2000; Casais et al. 2001; Coley et al. 2005; Yount et al. 2000, 2003, 2002). According to our model (Sawicki et al. 2007), the subgenomic mRNA expressing N would form almost immediately after the initiation of viral RNA synthesis, in addition to it being present in the infected cell because it would be brought in with the infecting virus. Therefore, it likely does not serve as a replication–transcription switch. It could act as an RNA chaperone, as proposed recently for the N protein of hantaviruses (Mir and Panganiban 2006) and facilitate folding of the genome RNA to permit its copying for the production of a genome-length minus strand. In the case of coronaviruses, such activity could be relevant to, for example, the initiation of minus-strand synthesis or, perhaps, during template switching at the TRS element during discontinuous synthesis. Second, it has not escaped our notice that coronaviruses possess helical nucleocapsids. Thus, similar to many minus-strand virus strategies, its role may be to produce a template that is “configured” to balance the ratio of RTCs engaged in the synthesis of templates either for genome or for subgenomic mRNA. Supporting such a possibility is the observation that replication and transcription from the EAV genome, a virus that has an icosahedral nucleocapsid structure, does not appear to involve N protein function (Molenkamp et al. 2000).

A number of host proteins have been reported to interact with viral RNA (Shi and Lai 2005) but it is unclear what roles these would play in the replication of coronavirus genomes especially since recently it was shown that the region to which these proteins bind can be deleted without preventing the virus from replicating (Goebel et al. 2007). The RTC is associated with double-membrane structures located between the endoplasmic reticulum and the Golgi compartment (Brockway et al. 2003; Gosert et al. 2002; Prentice et al. 2004a,b; Snijder et al. 2006). Transmembrane domains in nsp3, nsp4 and nsp6 are believed to act to anchor the RTC to membranes.

What are the template requirements for the formation of the RTC and for it to make minus-strand templates and genomic and subgenomic mRNA? Using a model analogous to that for picornavirus replication–transcription (Bedard and Semler 2004), the 3'- and 5'-ends of the coronavirus genome may interact, either directly (RNA to RNA) or indirectly (protein to RNA or protein to protein), to form the promoter for minus-strand synthesis. Only genomes containing a 5'-element downstream of the leader would be able to engage the 3'-end to serve as templates for minus-strand synthesis. The subgenome-length mRNAs would be missing the 5'-element (although they would all contain the 3'-element) and this provides an explanation for why they are not able to replicate (Sawicki et al. 2007). Using defective interfering (DI) RNA, it has been proposed that stem-loop (SL) structure within the coding region of nsp1 was required for the replication of the DI-RNA (Brown et al. 2007). Four other SL structures (SLI-IV) located in the 5'-untranslated region (5'-UTR) of the coronavirus genome are implicated in replication and transcription (Brian and Baric 2005). A region of the 5'-UTR, including the 3'-end

of the leader, has been postulated to function in joining of the body to the leader during minus-strand synthesis (Wu and Brain 2007; Wu et al. 2006).

Two regions of the 3'-untranslated region (3'-UTR) contain *cis*-acting regulatory elements that play a role in coronavirus RNA synthesis (Brian and Baric 2005). The first region of ~150 nucleotides adjoins the poly(A) stretch and is predicted to form a number of different stem-loop structures. It also contains 55 3'-terminal nucleotides next to the poly(A) that acts as a "minimal promoter" for MHV minus-strand synthesis in a DI-RNA (Lin et al. 1994). The second region contains two stem-loop structures, known as the bulged-stem-loop (BSL) and the hairpin-type pseudoknot (PK). The PK structure involves nucleotides at the base of the BSL structure, which means that the structures are mutually exclusive. It has been proposed that this may represent a form of "molecular switch", related in some, as yet unknown way, to different modes of RNA synthesis (Goebel et al. 2004).

In order for coronavirus to replicate their genome, coronaviruses must create two kinds of machines to synthesize RNA. One recognizes the genome as a template and synthesizes minus strands using both continuous synthesis to make templates for genome synthesis and discontinuous synthesis to make templates for subgenomic mRNA synthesis. The other macromolecular machine makes viral genomes and subgenomic mRNA using the minus-strand templates and continuous transcription. Besides having to recognize different templates and to use or not use discontinuous RNA synthesis, what other differences are there? One is that whereas minus-strand synthesis requires newly made proteins, i.e., minus-strand synthesis is inhibited almost immediately by inhibiting protein synthesis with cycloheximide, plus-strand synthesis continues, in the absence of protein synthesis, for at least 1 hour before decaying (Sawicki and Sawicki 1986). This suggests that only newly made, i.e., nascent, viral proteins function in minus-strand synthesis (replicase) and they are "converted" to plus-strand activity (transcriptase) by the mature RTC. It is possible that there are two independent pathways, one leading to formation of a replicase and one leading to the formation of a transcriptase. Use of temperature-sensitive (*ts*) mutants (Sawicki et al. 2005) supports this notion. Shifting certain *ts* mutants from the permissive (33°C) to the non-permissive (39°C), after minus- and plus-strand synthesis had commenced at 33°C, caused minus-strand synthesis to cease almost immediately while plus strand continued for 1 hour and then declined slowly. Temperature shift caused other mutants to stop plus-strand synthesis.

This is an exciting time both for coronavirologists (and Nidovirologists) who are using forward and reverse genetic and biochemical approaches to unravel the novel discontinuous mechanism of subgenomic minus-strand synthesis and for crystallographers who are probing the domain arrangements and structures of the viral nonstructural proteins, many of which are being found to possess novel folds. Not reviewed in this article are the additional issues of current and future interest that include the mechanism that allows such large RNA genome to avoid error catastrophe and the evolutionary implications of such mechanisms for viral-host interactions in their natural hosts, which include birds and mammals.

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