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MINIREVIEW

A Contemporary View of Coronavirus Transcription▽

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Coronaviruses are a family of enveloped, plus-stranded RNA viruses with helical nucleocapsids and extraordinarily large genomes. The hallmark of coronavirus transcription is the production of multiple subgenomic mRNAs that contain sequences corresponding to both ends of the genome. (Transcription is defined as the process whereby subgenome-sized mRNAs are produced, and replication is the process whereby genome-sized RNA, which also functions as mRNA, is produced.) Thus, the generation of subgenomic mRNAs involves a process of discontinuous transcription. The aim of this minireview is to describe our current understanding of coronavirus replication and transcription. For more detailed information, the reader is directed to other recent reviews (25, 28, 44a, 49, 63, 74, 94).

The coronavirus genomic RNA of approximately 30,000 nucleotides encodes structural proteins of the virus, nonstructural proteins that have a critical role in viral RNA synthesis (which we will refer to as replicase-transcriptase proteins), and nonstructural proteins that are nonessential for virus replication in cell culture but appear to confer a selective advantage in vivo (which we will refer to as niche-specific proteins). At least one niche-specific protein, nonstructural protein 2 (nsp2), and one structural protein, the nucleocapsid protein (N), are involved in viral RNA synthesis (1, 30, 68).

The expression of the coronavirus replicase-transcriptase protein genes is mediated by the translation of the genomic RNA. The replicase-transcriptase proteins are encoded in open-reading frame 1a (ORF1a) and ORF1b and are synthesized initially as two large polyproteins, pp1a and pp1ab. The synthesis of pp1ab involves programmed ribosomal frame shifting during translation of ORF1a (48). During or after synthesis, these polyproteins are cleaved by virus-encoded proteinases with papain-like (PL^{pro}) and chymotrypsin-like folds into 16 proteins; nsp1 to nsp11 are encoded in ORF1a, and nsp12 to nsp16 are encoded in ORF1b (Fig. 1) (96). The replicase-transcriptase proteins, together with other viral proteins and, possibly, cellular proteins, assemble into membrane-bound replication-transcription complexes (RTC). (We will use the term RTC to describe complexes copying or producing genome- or subgenome-length RNA.) These complexes accumulate at perinuclear regions and are associated with double-membrane vesicles (15, 76). Hydrophobic transmembrane domains are present in nsp3, nsp4, and nsp6 and likely serve to anchor the nascent pp1a/pp1ab polyproteins to membranes during the first step of RTC formation.

Subcellular fractionation, electron microscopic in situ hybridization, and immunofluorescence studies suggest that most, if not all, coronavirus nsp proteins are recruited to RTCs synthesizing both genome- and subgenome-length RNA (29, 32, 52, 76). At later times of infection, it appears that the nsp proteins encoded in ORF1a remain tightly bound to the RTC, while proteins encoded in ORF1b detach and diffuse to the cytosol (85). In addition to the nsp proteins, and consistent with it playing a role in RNA synthesis, the RTC contains the viral N protein (13, 85). A number of cellular proteins have been shown to interact with coronaviral RNA. These include heterogeneous nuclear ribonucleoprotein A1, polypyrimidine tract binding protein, poly(A)-binding protein, and mitochondrial aconitase (73), although there is no evidence that these proteins specifically colocalize with RTCs.

Sequence analysis of the nsp1 to -16 proteins predicts that they have at least eight enzymatic activities (75). Some of these activities, e.g., proteinase, RNA-dependent RNA polymerase (RdRp), and 5′-to-3′ helicase (HEL) activities, are common to RNA viruses, but others appear to be unique to coronaviruses or viruses closely related to them. Clearly, while most of these enzymatic functions are concerned with viral RNA synthesis, some may also have relevance to cellular processes. For example, nsp3, which contains the PL^{pro} activity, is a deubiquitinating enzyme and is capable of reversing the conjugation of protein with ISG15 (interferon-stimulated gene 15), which may subvert cellular processes to facilitate viral replication (7, 42). Also, the ADP-ribose 1′′-phosphatase (ADRP) activity of nsp3 may act to influence levels of ADP-ribose, a key regulatory molecule in the cell.

CORONAVIRUS RNA REPLICATION AND TRANSCRIPTION

Model of discontinuous extension during subgenome-length minus-strand synthesis. We have proposed a model of discontinuous extension during subgenome-length minus-strand synthesis to explain the generation of coronavirus mRNAs (65) (Fig. 2). Each subgenome-length mRNA contains a 5′ leader sequence corresponding to the 5′ end of the genome. This 5′ leader is joined to a mRNA "body," which represents sequences from the 3′-poly(A) stretch to a position that is upstream of each genomic ORF encoding a structural or niche-

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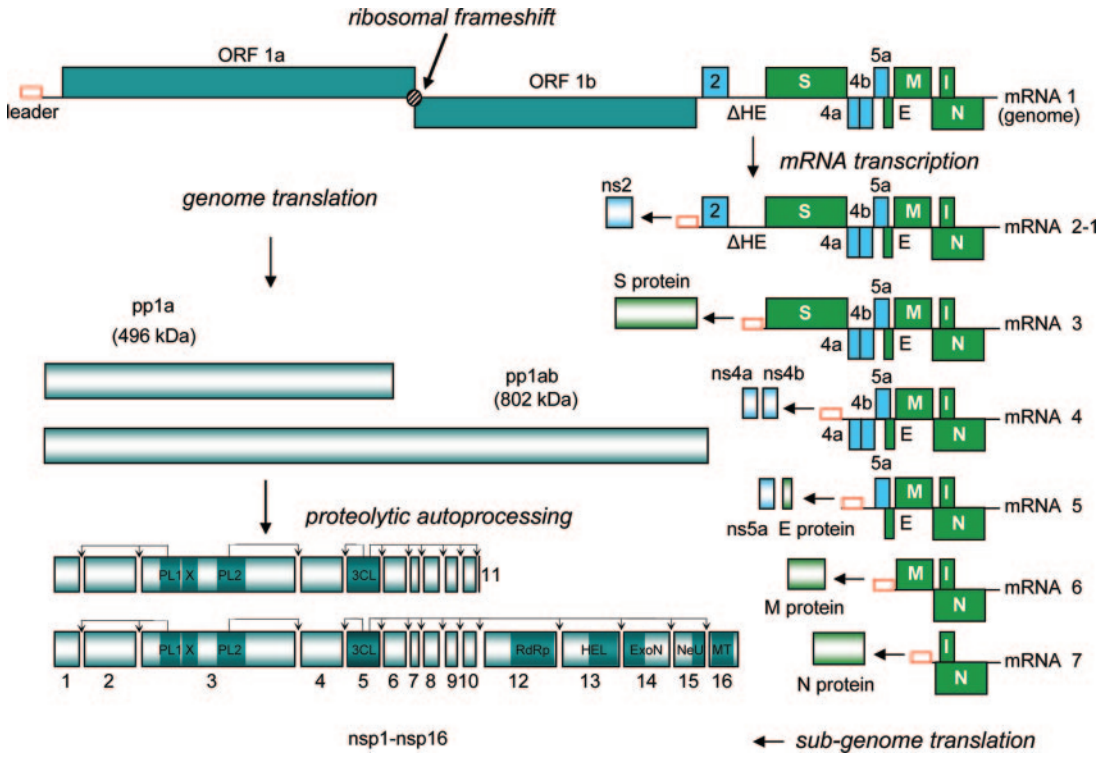

FIG. 1. Organization and expression of the MHV-A59 genome. The structural relationships of the MHV-A59 genome- and subgenome-length mRNAs are shown. The virus ORFs are depicted in teal (nsp1-nsp16 genes), blue (ns2, ns4a, ns4b, and ns5a genes), and green (S, M, E, N, and I structural protein genes). The ORFs are defined by the genomic sequence of MHV-A59 as published by Coley et al. (20). The open red box represents the common 59-leader sequence, and the barred circle represents the programmed (−1) frameshifting element. The translation products of the genome- and subgenome-length mRNAs are depicted, and the autoproteolytic processing of the ORF1a and ORF1a/ORF1b polyproteins into proteins nsp1 to nsp16 is shown. A number of confirmed and putative functional domains in the nsp proteins are also indicated. NeU, uridylate-specific endoribonuclease; PL1, papain-like protease 1; PL2, papain-like protease 2.

specific protein. The junction of the leader and body elements in each mRNA can be identified by a characteristic short, AU-rich motif of about 10 nucleotides that is known as the *t*ranscription-*r*egulating *s*equence (TRS). In the genome, functional TRS motifs are found at the 3′ end of the leader (leader TRS) and in front of each ORF that is destined to become 5′ proximal in one of the subgenome-length mRNAs (body TRSs).

Our explanation of how the subgenomic mRNAs are generated has two central tenets: (i) that the process of discontinuous transcription occurs during the synthesis of minus-strand subgenome-length templates, and (ii) that the process of discontinuous transcription resembles the mechanism of similarity-assisted or high-frequency copy-choice RNA recombination.

Mechanistically, the process of discontinuous transcription during minus-strand synthesis can be viewed as a number of consecutive events. (i) The components of a functional RTC are recruited and minus-strand synthesis is initiated at the 3′ end of a genomic RNA. (ii) Elongation of nascent minus-strand RNA continues until the first functional body TRS motif is encountered. A fixed proportion of RTCs will either (iii) disregard the TRS motif and continue to elongate the nascent strand or (iv) stop synthesis of the nascent minus strand and relocate and complete its synthesis. (v) This relocation will be guided by complementarity between the 3′ end of the nascent minus strand and the leader TRS motif. The translocated minus strand will be extended by copying the 5′ end of a genome. The completed minus-strand RNA would then serve as a template for mRNA synthesis. The evidence to support this model is now extensive and has been discussed in detail in recent reviews (49, 63).

There are many unanswered questions that are central to the model.

First, we have only a vague idea of what constitutes the signal that stops, or attenuates, minus-strand synthesis at each of the body TRS motifs. Three parameters that are considered important are the stability of TRS base pairing between the template and nascent minus strand, the nature of sequences flanking the TRS motifs, and the location of the TRS motif relative to the promoter for minus-strand synthesis (21, 25, 77, 92a, 97). Also, it is often proposed that protein-to-protein interactions may be important for attenuation but, in our opinion, the lack of rigid sequence specificity in the TRS base-pairing interaction implies that protein cofactors will not be major players in TRS recognition. Rather, specific RNA motifs (*cis*-acting and higher-order RNA structures) and their accessibility may be of importance, along with TRS base-pairing potential. Further evidence for the extremely tight regulation of body TRS attenuation is provided by a series of experiments done with the *Equine arteritis virus* (EAV) system; EAV belongs to a family that is distantly related to coronaviruses, with

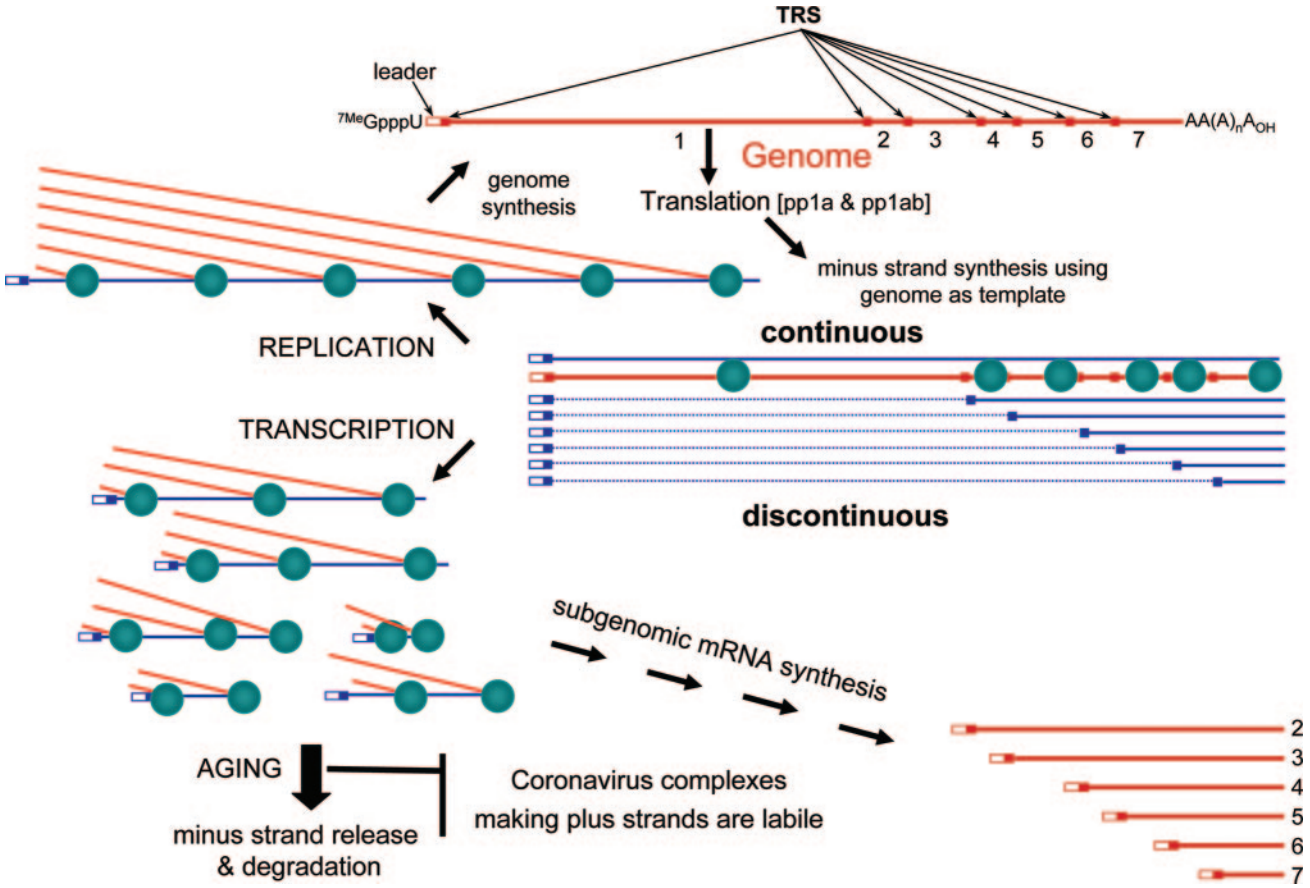

FIG. 2. Model for coronavirus replication-transcription. The ORF1 of genomic RNA (red) is translated to produce pp1a and pp1ab, which assemble into an RTC (teal oval) that recognizes *cis*-acting elements at the 5′ and 3′ ends of the genome. This RTC copies the genome either continuously into genome-length template or discontinuously into the various subgenome-length minus-strand templates. The minus strands (blue) are used as templates for genomic and subgenomic mRNA synthesis. Only genomes are used as templates for minus-strand synthesis, i.e., replication. The RTCs engaging in plus-strand synthesis age and release their minus-strand templates, which are then degraded specifically.

similar, if not identical, mechanisms of subgenome-length mRNA synthesis (49). In this system, it has been shown that the functional inactivation of a 3′-proximal body TRS element by minimal mutagenesis does not, as might have been predicted, increase the frequency with which more 3′-distal body TRS elements are used (50). In other words, the TRS element still enforces attenuation, i.e., the "launching" of the RTC, even when the "landing" is prevented.

Second, there is no information on how the relocation of the 3′ end of the nascent minus strand to the 5′ end of the genomic template is mediated or how the realignment of complementary bases on the template is facilitated. One idea is that this relocation might be mediated by protein-to-protein interactions between the polymerase attached to the growing minus strand and a protein associated with the 5′ end of the genome. It also could occur by the polymerase binding directly to the sequences downstream of the leader RNA. A variation on this theme is that the 5′ end of the genomic RNA is actually bound to the polymerase attached to the growing minus strand and essentially scans the newly synthesized RNA for the synthesis of the complementary TRS sequence (25). This is an attractive idea, but it implies that, at any one time, each genomic RNA template engages in the synthesis of only one subgenome-length minus strand, and it is contradictory to the idea that each template generates concurrently a complete set of genome- and subgenome-length minus strands. The difference should be experimentally testable once sensitive methods are developed. With regard to the realignment of complementary bases on the template, it has been suggested that coronavirus polymerase might copy the template RNA in a fashion analogous to DNA-dependent RNA polymerases, where regulatory elements in the template and nascent RNA disrupt elongation (59, 89) and cause retraction at pause sites (40, 41) and where the polymerase remains associated with the growing nascent strand rather than with the template. This would endow the 3′ end of the nascent minus strand with primer capability. Of interest, a similar mechanism was recently proposed for proofreading by RNA polymerases (71), and several gene products of ORF1b of coronaviruses could plausibly have a role in this sort of process (see section below on proteins involved in coronavirus transcription).

Kinetics of plus-strand and minus-strand synthesis. Coronaviruses resemble other plus-stranded RNA viruses and produce plus strands at 50- to 100-fold excess of their minus-strand templates. Based on studies with the A59 strain of *Mouse hepatitis virus* (MHV-A59), coronavirus RNA synthesis

is detectable as early as 2 to 3 h postinfection when metabolic labeling ([^{32}P]orthophosphate or [^{3}H]uridine) is employed and by 90 min postinfection when reverse transcription-PCR is used (63; data not shown). Throughout infection, syntheses of the various species of plus strands remain constant, i.e., in a fixed molar ratio, relative to each other. In addition, the same ratio is retained under conditions where overall viral RNA synthesis declines late in infection, after recovery from the inhibition of protein synthesis by cycloheximide, or after transfer from restrictive to permissive temperatures in the case of temperature-sensitive (*ts*) mutants. The pattern of plus-strand synthesis reflects a basic principle of coronavirus transcription; namely, the amount of genome- and subgenome-length mRNA synthesis is determined at the level of minus-strand synthesis.

Minus-strand synthesis begins 75 to 90 min after infection. At this time, genome-length and subgenome-length minus strands and their plus-strand complements can be detected simultaneously by reverse transcription-PCR (S. Sawicki, unpublished data). Therefore, once RTCs form, they immediately start to produce minus-strand RNA. Minus strands accumulate exponentially, and their rate of synthesis peaks at 5 to 6 h postinfection; their synthesis then declines but does not cease (62). Why the assembly of RTCs is hindered beyond 6 h postinfection is not known.

Coronavirus RIs/TIs and RFs/TFs. The templates in viral RTCs are recoverable after deproteinization of infected-cell lysates as a multistranded *r*eplication *i*ntermediate (RI) with genome-length templates or multistranded *t*ranscription *i*ntermediates (TIs) with templates corresponding in length to subgenome-length mRNAs and the similarly sized double-stranded *r*eplication *f*orm (RF) with genome-length templates and double-stranded *t*ranscription *f*orms (TFs) with templates corresponding in length to subgenome-length mRNAs. The native RF/TF structures are essentially single-stranded, but they become largely double-stranded following deproteinization. The TIs and TFs are authentic transcription structures that are active in subgenomic mRNA synthesis (60, 64).

In MHV-infected cells, all of the RTCs making minus and plus strands are labile. The short-lived nature of MHV minus-strand-synthesizing complexes is a constant feature throughout infection. It is demonstrated by inhibiting the synthesis of nsp's with cycloheximide (62), which means that only newly made viral proteins function in minus-strand synthesis and suggests that polyprotein intermediates probably function in minus-strand synthesis. The instability of plus-strand-synthesizing RTCs is more difficult to observe but can be seen under certain conditions. For instance, at 7 to 8 h postinfection, plus-strand synthesis starts to decline, but it is difficult to study because with MHV-A59 cell fusion also occurs. However, by use of a mutant of MHV-A59 that does not cause cell fusion (61), RNA synthesis can be seen to decline to undetectable levels by 12 to 15 h postinfection. This occurs even though viral proteins, at least the structural proteins, are being produced at high levels. Another way to demonstrate the lability of plus-strand-synthesizing RTCs is to inhibit protein synthesis early in infection when plus-strand synthesis is increasing. Plus-strand synthesis stops increasing almost immediately, starts to decline an hour later, and disappears after 4 to 5 h. Under these circumstances, the synthesis of all species of viral plus strands is equally affected.

What is the basis of the instability shown by plus-strand-synthesizing RTCs? One clue is that RI/RF and TI/TF structures disappear coincidently in time with the loss of plus-strand synthesis (63). This could reflect activation of specific RNase activities targeting minus strands. Our hypothesis is that coronavirus RTCs age and lose their activity and then their minus-strand templates; the virus is then required to produce new RTCs throughout infection.

Biogenesis of minus strands. The current model of coronavirus RNA synthesis proposes that minus strands arise from copying the genome continuously to form genome-length templates and discontinuously to form subgenome-length templates. While early experiments ruled out a role for splicing in the generation of viral subgenome-length mRNAs, it remained possible that the genome-length minus strands are subject to splicing, which would result in the formation of subgenome-length minus-strand templates with the same 5′ and 3′ ends. To test this possibility, we determined the sensitivity of MHV minus-strand synthesis to UV irradiation (data not shown). The kinetics of overall minus-strand sensitivity showed a dose dependency that was not linear and did not follow the inactivation of synthesis of any of the individual plus strands. Rather, it reflected the sum of their individual sensitivities. This result supports the argument that the subgenome-length minus-strand templates do not arise via splicing of genome-length minus strands. This conclusion is also supported by studies using replicons derived from the infectious clone of *Human coronavirus*, strain 229E (HCoV-229E) where, in the absence of N protein expression, the synthesis of a subgenome-length mRNA for green fluorescent protein (GFP) occurred even when there was little or no replication of the replicon-length RNA (68). Since the subgenome-length mRNA encoding GFP had a leader fused to its 5′ end and was produced in the absence of demonstrable replicon-length minus-strand synthesis, discontinuous synthesis rather than splicing was likely responsible for the generation of the minus-strand template.

Although they are central to any understanding of coronavirus transcription, fundamental issues regarding the mechanisms of the RTCs responsible for minus-strand synthesis remain to be addressed. The most obvious remaining question is the mechanism of discontinuous synthesis. However, it is also important to ask other questions. Can a single genome serve as a template for the synthesis of only one minus strand, or can it serve simultaneously for all species of minus strands? Is the ratio of each minus-strand product per genome 1 or greater than 1? We believe that the fixed ratio of different minus strands produced early and late during infection suggests that each genome serves as a template for the complete nested set of minus strands, with *cis*-active signals and the 3′ position relative to the genome influencing or determining the final abundance or molar ratios of each product. This prediction can be tested, however, only when a sensitive method is devised to analyze the templates and products of RTCs engaged in minus-strand synthesis.

Promoters for minus- and plus-strand synthesis. It is difficult to reconcile experiments investigating the sequences and structures found at the 5′ and 3′ ends of coronavirus RNA with simple models for the initiation of plus- and minus-strand

TABLE 1. Enzymatic activities and characteristics of coronavirus nsp protein domains

Activity	Location and designation	Protein family	Architecture	Comments	References
Papain-like proteinase	One or two PL^{pro} domains within nsp3	Deubiquitinating enzyme family	Finger-palm-thumb	Fingertips contain zinc-binding domain	5, 33, 56
ADP-ribose 1″-phosphatase	ADRP (or X) domain within nsp3	Macro-HS2A-fold family	Three-layered α/β/α	Also found in other plus-strand RNA viruses	53, 58
3C-like cysteine proteinase	nsp5 ($3CL^{pro}$ or main protease, M^{pro})	Two-β-barrel proteinase family	Twelve antiparallel β-strands	Extra, α-helical, carboxyl-terminal domain III	3, 4, 95
RNA-dependent RNA polymerase	RdRp domain within nsp12	Viral RdRp family	Finger-palm-thumb (predicted)	RdRp motif VI signature GDD replaced with SDD	19, 87
5′-to-3′ helicase (associated, NTPase, and RNA 5′-triphosphatase activities)	HEL domain within nsp13	Superfamily 1 helicase	Modeled to *Escherichia coli* Rep helicase	Adjacent to amino-proximal, binuclear, zinc-binding domain	9, 35, 36, 70
3′-to-5′ exonuclease	ExoN domain within nsp14	DEDD superfamily	Hexahelical bundle (predicted)	Unable to cleave ribose 2′-O-methylated RNA substrates	45, 75
Uridylate-specific endoribonuclease	NendoU domain within nsp15	XendoU family	Wing-body-wing (butterfly fold)	Active in hexameric form; RNA bound to internal cavity	10, 11, 31, 34, 56a, 88
S-adenosylmethionine-dependent 2′-*O*-methyltransferase	nsp16 (MT)	Rrmj methyltransferase family	Methyltransferase fold (predicted)	Activity yet to be determined	75, 86

synthesis. Two regions of the 3′ untranslated region (UTR) have been suggested as containing *cis*-acting regulatory elements that play a role in coronavirus RNA synthesis. The first region of ~150 nucleotides adjoins the poly(A) stretch and is predicted to form a number of different stem-loop structures (37, 57). One of these is known as the stem-loop II motif and has been identified in the *Severe acute respiratory syndrome coronavirus* (SARSCoV) genome (57). This region also contains the sequence 5′-GGAAGAGC-3′ that is present in all coronavirus genomes. The second region is upstream of the terminal 150 nucleotides and contains two structures, known as the bulged stem-loop and the hairpin-type pseudoknot. It is interesting that the pseudoknot structure involves nucleotides at the base of the bulged stem-loop structure, which means the structures are mutually exclusive. This may represent a form of "molecular switch" related in an as-yet-unknown way to different modes of RNA synthesis (27). Structures implicated in RNA synthesis have also been predicted in the 5′ UTR of the coronavirus genome (14, 54, 55). These include four higher-order structures, stem-loops I to IV, that may function as *cis*-acting elements in RNA replication and transcription. Stem-loops I and III demonstrate a *cis*-acting function in *Bovine coronavirus* (BCoV) defective interfering (DI) RNA replication. Stem-loop II harbors the leader TRS motif, and stem-loop IV may be involved in the initiation of minus-strand synthesis (55).

In a model analogous to the models for picornavirus replication-transcription (8), we propose that the 3′ and 5′ ends of the coronavirus genome 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 would explain why they are not able to replicate (43, 54, 55). Similarly, the 3′ end of minus strands presumably must function as part of the promoter for plus-strand synthesis. Again, we would propose that the 3′ and 5′ ends of the minus strand might interact to circularize the template and create functional plus-strand promoters. DI minus strands are capable of replicating when introduced into helper virus-infected cells (38), and this indicates that minus-strand templates are capable of being recruited in *trans* into a functional RTC to produce plus strands. Thus, either the minus-strand templates of RTCs are released after completion of synthesis of the plus strand, allowing the RTC to initiate RNA synthesis on another minus-strand template, or newly assembled RTCs might recognize minus strands in *trans* and not be obliged to first create a minus-strand template by copying a genome.

PROTEINS INVOLVED IN CORONAVIRUS TRANSCRIPTION

As mentioned above, sequence analyses of the nsp proteins of MHV, SARSCoV, and other coronaviruses predict that they include domains associated with at least eight enzymatic activities (75). The enzymatic activities and characteristics of these protein domains are listed in Table 1. Crystallographic or nuclear magnetic resonance structures have been determined for the PL^{pro} and ADRP (or X) domains of nsp3, nsp5, nsp7, nsp8, nsp9, and nsp10 (3, 39, 51, 56, 58, 82, 93). In addition, a number of other features, including domains with conserved cysteine and histidine residues (C/H domains in nsp3, nsp13, and nsp14), putative transmembrane domains (TM domains in nsp3, nsp4, and nsp6), and domains with conserved features (the A [acidic] and Y domains within nsp3) have also been identified in coronavirus nsp proteins (94).

In addition to the proteins for which enzymatic functions have been predicted or demonstrated, the structures of four other coronavirus nsp proteins have been reported. The SARSCoV proteins nsp7 and nsp8 cocrystallize to produce a supercomplex that can be viewed as a hollow, cylinder-like structure assembled from eight copies of nsp8 and eight copies of nsp7. This complex has a central channel with positive electrostatic properties favorable for nucleic acid binding. It has been suggested that the role of this structure may be to confer

processivity to the viral RdRp (51, 93). Second, the crystal structure of SARSCoV nsp9 has been reported as comprising a single β-barrel with a fold that resembles a carboxyl-extended oligonucleotide-oligosaccharide binding (OB) fold. The crystal structure suggests that the protein is dimeric, and gel shift assays show that it is able to bind single-stranded RNA (24, 82). And finally, recent studies have elucidated the crystal structure of the SARSCoV nsp10 protein (39). The structure predicts a single domain protein consisting of a pair of antiparallel amino-terminal helices stacked against an irregular β-sheet, together with a coil-rich carboxyl terminus and two zinc fingers. Twelve subunits assemble to form a unique dodecameric superstructure. The nsp10 protein is the first representative of a new family of zinc-finger proteins which, so far, are found exclusively in coronaviruses (39, 81).

Many review articles on coronavirus RNA synthesis include references to a nonstructural protein, ns2, which is encoded in the genome of a subset of group II coronaviruses, including MHV, BCoV, and HCoV-OC43. The ns2 gene lies immediately downstream of ORF1b, and the protein is translated from a subgenome-sized mRNA. This protein has been predicted to have cyclic phosphodiesterase (CPD) activity, although this has not yet been experimentally verified. It is tempting to envisage this activity, together with the ADRP activity of nsp3, as acting in the regulation of a pathway involving the processing of (viral) RNA by enzymes (for example, the NendoU activity of nsp15) that generate intermediates with terminal cyclic phosphate residues (78). It is worth remembering, however, that only a few coronaviruses encode this protein, that it is not essential for virus replication in cell culture (69), and that there is no evidence to suggest that it is recruited into the coronavirus RTC.

GENETICS OF CORONAVIRUS TRANSCRIPTION

Forward genetics. The classical approach to the genetic analysis of coronavirus replication and transcription, i.e., the characterization of conditionally lethal, usually *ts*, virus mutants that are unable to synthesize RNA when the infection is initiated and maintained at the nonpermissive temperature, has been adopted in only a few laboratories. This is disappointing, because the essential feature of such mutants is that they are likely to be defective in different aspects of viral RNA synthesis, and a detailed characterization of their genotype and phenotype should provide insights into the mechanisms of RNA synthesis, the functions of individual viral replicase proteins, and the protein-RNA and protein-protein interactions that regulate the activity of the RTC. These conditional-lethal mutants may also be used in a *cis-trans* test to define the number of complementation groups, or cistrons, that contribute to a specific phenotype. This sort of analysis can provide valuable insight into the possible pathways that polyproteins must travel to assume functional configurations.

MHV-A59 mutants have been produced in a number of laboratories over a period of 20 years (67, 79, 80). In a recent analysis of 19 *ts* mutants that are unable to produce viral RNA at 39°C, the nonpermissive temperature, four complementation groups were identified using classical and molecular complementation assays (66). Most of the 19 mutants, including Alb *ts*6, Alb *ts*16, and LA *ts*6, were members of complementation group 1. The lesions in these three mutants were located in nsp4, nsp5, and nsp10. Thus, by definition, these individual nsp's are *cis*-acting, i.e., their *ts* defects cannot be complemented in *trans* by nsp's made by a second virus. This result suggests three possibilities: (i) nsp4-nsp10 function as a polyprotein before cleavage into individual polypeptides, (ii) nsp4-nsp10 first assemble into an RTC and are then cleaved, with a gain of function(s) expressed in individual polypeptides, or (iii) *ts*-induced alteration to the folding or interaction of protein domains within the nsp4-nsp10 region of pp1a/pp1ab interferes with polyprotein processing or function. In the same study, five further mutants could be assigned to three additional complementation groups. Alb *ts*22 (mutation in nsp12) comprises group 2, Alb *ts*17 and Wü *ts*38 (mutations in nsp14) comprise group 4, and Wü *ts*18 and Wü *ts*36 (mutation in nsp16) comprise group 6. Thus, each of these nsp's is *trans*-active and diffusible between pp1ab complexes. Taken together, the results of this analysis are consistent with the idea that nsp4 to nsp10 of pp1a act together as a complex, multidomain structure or scaffold onto which the *trans*-acting nsp's (e.g., nsp12, nsp14, and nsp16) and viral RNA associate. It will be interesting to discover whether other complementation groups can be assigned to nsp1, nsp2, nsp3, nsp13, or nsp15 and if any of the individual nsp's in nsp4 to -10 have later and separate functions, i.e., are *trans*-acting. This information will be especially interesting for nsp's in pp1a that are thought to function as multimers.

Another major advantage of using *ts* mutants is that they can be analyzed by temperature-shift protocols; i.e., by allowing them to gain function at the permissive temperature, shifting to the nonpermissive temperature, and then determining if the function is lost. Generally, their failure to lose function after the temperature shift indicates that the amino acid change in the mutant has affected the formation of the complex or activity. If they lose function, the mutation probably directly affects function. When this analysis was done for the mutants described above, we were surprised to find that MHV-A59 mutants from cistrons 2, 4, and 6 were of the latter type. In one case, Alb *ts*22, where the mutation lies in the virus RdRp, it is easy to rationalize this phenotype. However, we also found that mutations in nsp14 and nsp16 have an immediate effect on plus-strand RNA synthesis by preformed RTCs after the shift to 39°C, showing that the activity of the MHV-A59 RTC is dependent on a complex structure of interacting domains contributed by nsp's encoded in ORF1b. In contrast, mutations in nsp4, nsp5, and nsp10 (cistron 1, i.e., Alb *ts*6, Alb *ts*16, and LA *ts*6) did not inhibit ongoing plus-strand synthesis following the temperature shift. This result is consistent with the notion that the nsp4-to-nsp10 portion of pp1a is providing structural and not catalytic components of the complex (see Addendum in Proof).

An RNA-negative phenotype, i.e., the failure to synthesize viral RNA at the nonpermissive temperature, might be due to the inability to form a minus-strand RTC or the inability to convert the minus strand into a RTC-making plus strand (the so-called conversion phenotype) (22). A more detailed analysis of the cistron 1 mutants showed that mutant LA *ts*6 was defective in continuing minus-strand synthesis after the shift to the nonpermissive temperature. This result implicates nsp10 in minus-strand synthesis. In contrast, Alb *ts*16 appeared to have

a conversion phenotype: Alb *ts*16-infected cells made minus strands but did not increase the rate of plus-strand synthesis after shift to 39°C. Alb *ts*16 has a mutation in the carboxyl-terminal domain of nsp5 (3C-like cysteine proteinase [$3CL^{pro}$]), and it will be important to determine if this mutation affects the activity of the proteinase (72, 84). It might affect the folding of pp1a or pp1ab, or the nsp5 C-terminal domain itself could have a function in plus-strand RNA synthesis. Nevertheless, because minus-strand RNA synthesis ceased in Alb *ts*16-infected cells that were treated with cycloheximide to block translation of new nsps at the time of the shift to nonpermissive temperature (66), we propose that the Alb *ts*16 RTC does not retain artifactually its activity for minus-strand synthesis. Rather, it fails to gain plus-strand synthesis activity at the nonpermissive temperature. We favor a model where the activity that makes plus strands is gained at the expense or loss of the activity to make minus strands.

Reverse genetics. The development of infectious cDNA constructs for coronaviruses is a recent event, and the use of reverse genetics in the analysis of coronavirus transcription is now gathering momentum. Already, a number of important findings have been made. First, reverse genetics, in particular the method of targeted RNA recombination (44), has been used to analyze *cis*-acting elements that regulate coronavirus transcription, as discussed in the above section on coronavirus RNA replication and transcription. Second, reverse genetics has been used to confirm the involvement of coronavirus nsp proteins in RNA synthesis and to discriminate between essential and nonessential functions (6, 26, 83). For example, mutation at the active site of the HCoV-229E nsp15 NendoU domain or the active site of the HCoV-229E nsp14 3′-to-5′ exonuclease (ExoN) domain suggests that these functions are essential for virus RNA synthesis in cell culture (34) although, in some cases, residual viral RNA synthesis was detected in cells transfected with mutant RNA. In contrast, mutation of the active site of the HCoV-229E nsp3 ADRP domain had no significant effect on virus RNA synthesis or virus titer, and no reversion to wild-type sequence was observed when the mutant virus was passed in cell culture (53).

Reverse genetics has been used to show that the MHV and SARSCoV nsp2 proteins are not essential for virus replication but that their deletion attenuates virus growth and virus RNA synthesis (30). In a similar fashion, MHV mutants that are incapable of liberating nsp1 from the nascent polyprotein (i.e., nsp1/nsp2 cleavage mutants) exhibit delayed replication, low titers, small plaque morphology, and reduced RNA synthesis compared to wild-type virus (23). A more extensive deletion and site-specific mutagenesis study of the MHV nsp1 has identified domains and residues that are important for polyprotein processing and virus RNA synthesis (16). Although informative, this sort of reverse genetic analysis must be approached with caution. The frequency of lethal mutation is likely to be high, and there is, as always, the possibility of indirect effects, such as phenotypes caused by the disruption of *cis*-acting RNA structures.

ROLE OF THE N PROTEIN

The coronavirus N protein has been implicated in virus RNA synthesis by three lines of evidence. First, the N protein was shown to bind specific RNA sequences, including the leader sequence, the TRS sequence, and sequences located at the 3′ end of the virus genome (73). Second, in addition to a cytoplasmic distribution in the host cell, at least a fraction of the N protein colocalizes with RTCs early in infection (13, 85). And third, there is clearly a requirement for sustained translation of the N protein in *trans* or in *cis* for optimal replication of BCoV DI RNA and *Transmissible gastroenteritis virus*-derived replicons (1, 18). The importance of the N protein was also emphasized when it was shown that the rescue of recombinant coronaviruses from cells transfected with infectious RNA was greatly enhanced by, if not dependent upon, the expression of N protein in the same cell (2, 17, 20, 90–92).

These observations were followed up by experiments showing that, in the absence of N protein, HCoV-229E replicons expressing GFP from a subgenomic mRNA produced low levels of GFP and little or no amplification of the replicon. In contrast, the expression of N in the same cells, in *cis* or in *trans*, significantly increased the number of transduced cells, the levels of GFP expression and, concomitantly, the amplification of the replicon (68). Our interpretation of these data is that the N protein has a role in determining the ratio of genome- to subgenome-length minus strands and, in the absence of N, this ratio is perturbed, with the underproduction of genome-length templates. In the virus-infected cell, there should always be sufficient N protein, either introduced with the nucleocapsid structure or synthesized within 90 min of infection (see the section on coronavirus RNA replication and transcription), to fulfill this role. Therefore, according to this interpretation, N does not function as a replication-transcription switch.

What could the role of the N protein be? One possibility is that it is acting as an RNA chaperone as proposed for the N protein of hantaviruses (46), where chaperone activity results in the transient dissociation of RNA structures, including RNA duplexes with adjoining single-stranded regions, which may be required for facilitating correct higher-order RNA structure. For coronaviruses, such chaperone activity could be important for the initiation of minus-strand synthesis or, perhaps, for suppressing attenuation and/or template switching at the TRS element during discontinuous synthesis (25). Second, coronaviruses possess helical nucleocapsids. This is not common among plus-stranded RNA viruses, *Tobacco mosaic virus* being another exception, but is typical of minus-stranded RNA viruses. It is possible that the role of the coronavirus N protein is to associate with the genomic RNA to produce a template that is "configured" to balance the ratio of RTCs engaged in either transcription or replication, as has been proposed for measles virus (12). In this respect, it is also worth noting that replication and transcription from the genome of EAV, a virus that has an icosahedral nucleocapsid structure, does not appear to involve N protein function (47).

WHY IS CORONAVIRUS TRANSCRIPTION SO COMPLEX?

Two reasons are most often given for the complexity of coronavirus transcription. (i) The generation of multiple subgenomic mRNAs and the process of discontinuous transcription during minus-strand synthesis demand complex replication-tran-

scription machinery. (ii) The large size of the coronavirus genome demands unusual activities to maintain genetic stability.

With regard to the first reason, it is true that the generation of multiple minus-strand RNAs from a single template must be a complex process but, essentially, it is only the process of discontinuous transcription during minus-strand synthesis that is unique. The initiation of plus-strand and minus-strand synthesis, elongation, and termination are carried out by all RNA viruses, usually with a smaller number of replicase components than are found in the coronavirus RTC. Also, it is important to realize that arteriviruses, which, as already mentioned, are a family of viruses related to coronaviruses and have similar, if not identical, mechanisms of subgenome-length mRNA synthesis, do not encode proteins with functions analogous to the ExoN, *S*-adenosylmethionine-dependent 2′-*O*-methyl transferase (MT), ADRP, or CPD functions of coronaviruses (75). This implies that these functions, although they may be an integral part of the coronavirus RTC, are not needed to allow for discontinuous transcription. Biological advantages to discontinuous transcription include economies of coding and the ability to regulate individual mRNA abundance, but these do not seem to justify the extraordinary genetic investment that coronaviruses have made in replicase-transcriptase and niche-specific proteins.

An alternative view is proposed by Gorbalenya and colleagues (28). Basically, these authors argue that the acquisition of the coronavirus enzymatic activities may have improved the fidelity of RNA replication and transcription to allow for genome expansion. This, in turn, would provide coronaviruses with the opportunity, for example, to expand their host range and adapt rapidly to changing environmental conditions while maintaining genomic stability. Specifically, it is proposed that the HEL, ExoN, NendoU, and MT functions may provide RNA specificity and that the ADRP and CPD functions (when present) modulate the pace of a reaction in a common pathway, which could be part of an oligonucleotide-directed repair mechanism (75). The existence of such a repair mechanism in coronaviruses, with similarities to the "proofreading" or repair activities associated with DNA replication, would require a paradigm shift in our view of RNA virus replication.

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ADDENDUM IN PROOF

It was recently shown that SARSCoV encodes a second, noncanonical RdRp residing in nsp8, and it was proposed that the nsp8 RdRp produces primers utilized by the primer-dependent nsp12 RdRp (I. Imbert, J. C. Guillemot, J. M. Bourhis, C. Bussetta, B. Coutard, M. P. Egloff, F. Ferron, A. E. Gorbalenya, and B. Canard, EMBO J. **25:**4933–4942, 2006).

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