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Viral and Cellular Proteins Involved in Coronavirus Replication

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Abstract As the largest RNA virus, coronavirus replication employs complex mechanisms and involves various viral and cellular proteins. The first open reading frame of the coronavirus genome encodes a large polyprotein, which is processed into a number of viral proteins required for viral replication directly or indirectly. These proteins include the RNA-dependent RNA polymerase (RdRp), RNA helicase, proteases, metal-binding proteins, and a number of other proteins of unknown function. Genetic studies suggest that most of these proteins are involved in viral RNA replication. In addition to viral proteins, several cellular proteins, such as heterogeneous nuclear ribonucleoprotein (hnRNP) A1, polypyrimidine-tract-binding (PTB) protein, poly(A)-binding protein (PABP), and mitochondrial aconitase (m-aconitase), have been identified to interact with the critical *cis*-acting elements of coronavirus replication. Like many other RNA viruses, coronavirus may subvert these cellu-

lar proteins from cellular RNA processing or translation machineries to play a role in viral replication.

1 Introduction

Studies of diverse groups of positive-stranded RNA viruses reveal that they employ common strategies for replication, although the precise nature of these proteins varies for each virus (Pogue et al. 1994). In general, the formation of viral translation and RNA replication complexes require multiple viral and cellular proteins. By analogy with the phage Qβ, which recruits four host (bacterial) proteins to be an integral part of the replicase complex together with the viral polymerase (Blumenthal and Carmichael 1979), it is likely that replication complexes of positive-stranded RNA viruses consist of both virus- and host-encoded proteins. In addition, viral and cellular proteins interact with various *cis*-acting elements on viral RNAs and play essential roles in the regulation of viral replication. They may mediate the cross talk between the 5′ and 3′ ends of the viral RNA and bring other distant *cis*-acting elements close together to carry out complex processes, such as subgenomic RNA transcription, coupling between translation and RNA replication, and asymmetric production of excess genomic positive- over negative-strand RNAs. The switch between translation and replication in poliovirus has been shown to involve the cellular protein poly(rC)-binding protein (PCBP), which upregulates viral translation, and the viral protein 3CD, which represses viral translation and promotes negative-strand synthesis (Gamarnik and Andino 1998). Identification of the roles of viral and cellular proteins should provide valuable insights into the mechanisms of viral replication.

The replication of the genome is considered as the most fundamental aspect of the biology of positive-stranded RNA viruses. Like all other positive-stranded RNA viruses, coronavirus replicates its genome through the synthesis of a complementary negative-strand RNA using the genomic RNA as a template. The negative-strand RNA, in turn, serves as the template for synthesizing more progeny positive-strand RNAs. Analysis of the structure of mouse hepatitis virus (MHV) defective-interfering (DI) RNAs indicates that approximately 470 nucleotides (nt) at the 5′ terminus, 436 nt at the 3′ terminus, and about 135 internal nt are required for coronavirus DI RNA replication and suggests that these sequences contain signals necessary for viral RNA replication

(Kim et al. 1993; Kim and Makino 1995b; Lin and Lai 1993; Lin et al. 1996). Both of the 5′ and 3′ ends of the genome are necessary for positive-strand synthesis (Kim et al. 1993; Lin and Lai 1993), whereas the *cis*-acting signals for the synthesis of negative-strand RNA exist within the last 55 nt and the poly(A) tail at the 3′ end of the MHV genome (Lin et al. 1994). One unique feature of coronaviruses is the expression of their genetic information by transcription of a 3′ coterminal nested set of subgenomic mRNAs that contain a common 5′ leader sequence derived from the 5′ end of the RNA genome. The interaction between the leader sequence and an intergenic (IG) sequence upstream of each open reading frame (ORF), also named transcription-regulating sequence (TRS), is required for the transcription of subgenomic mRNAs (Chang et al. 1994; Liao and Lai 1994; Zhang and Lai 1995b). Logically, these *cis*-acting sequences for viral genomic RNA replication and subgenomic RNA transcription serve as ideal signals to recruit viral factors and possibly cellular proteins for the formation of the RNA replication and transcription complex.

Apart from the findings that continuous synthesis of viral proteins is a prerequisite for the synthesis of both positive- and negative-strand RNA and subgenomic mRNAs (Perlman et al. 1986; Sawicki and Sawicki 1986), little information is currently available concerning the identities and functions of the viral proteins that participate in coronavirus replication. Because of the unparalleled size of the coronavirus RNA genome, genetic approaches to the analysis of replicase gene function have been limited to date. Nevertheless, studies of the temperature-sensitive mutants of coronavirus demonstrate the importance of ORF 1 polyprotein (also known as the polymerase or replicase protein) in coronavirus RNA synthesis and suggest that different domains of this polyprotein are involved in different steps of viral RNA synthesis (Baric et al. 1990a; Fu and Baric 1994; Leibowitz et al. 1982; Schaad et al. 1990). Evolutionarily, the virus genome is composed of relatively constant replicative genes that are indispensable for viral replication and more flexible genes coding for virion structural proteins and various accessory proteins (Koonin and Dolja 1993). Despite the high mutation frequency that is typical of RNA viruses, viral proteins mediating the replication and expression of virus genomes contain arrays of conserved sequence motifs. Proteins with such motifs include RdRp, putative RNA helicase, chymotrypsin-like and papain-like proteases, and metal-binding proteins, all of which are present in the coronavirus ORF 1 polyprotein as shown by sequence comparisons (Bonilla et al. 1994; Bredenbeek et al. 1990; Gorbalenya et al. 1989b; Lee et al. 1991). Strategically located as the

5′-most gene in the viral genome, the coronavirus ORF 1 is translated into a large polyprotein immediately upon virus entry and processed by viral proteases into functional proteins, which are responsible for RNA replication and transcription. The processing scheme of the coronavirus ORF 1 polyprotein has been largely delineated by a number of recent studies. As a result, the functions of the domains that have not been identified before are beginning to emerge. In addition to the proteins with apparent enzymatic activities required for viral RNA synthesis, a number of other coronavirus proteins have also been implicated in viral replication.

Many studies have shown that viruses use cellular proteins for multiple purposes in their replication cycles, including the attachment and entry into the cells, the initiation and regulation of RNA replication/transcription, the translation of their mRNAs, and the assembly of progeny virions. Because many aspects of the replication cycles of different types of viruses are unique, the cellular proteins used by different types of viruses also differ. Nevertheless, viruses typically subvert the normal components of cellular RNA processing or translation machineries to play an integral or regulatory role in the replication/transcription and translation of viral RNA (Lai 1998). These cellular proteins include, but are not limited to:

1. Heterogeneous nuclear ribonucleoproteins and other RNA processing factors: hnRNP A1 (Black et al. 1995, 1996; Li et al. 1997; Shi et al. 2000; Wang et al. 1997) and other hnRNP type A/B proteins (Bilodeau et al. 2001; Caputi et al. 1999; Shi et al. 2003), hnRNP C (Gontarek et al. 1999; Sokolowski and Schwartz 2001; Spangberg et al. 2000), hnRNP E (PCBP) (Gamarnik and Andino 1997; Parsley et al. 1997), hnRNP H (Caputi and Zahler 2002), hnRNP I (PTB) (Black et al. 1995, 1996; Chung and Kaplan 1999; Gontarek et al. 1999; Hellen et al. 1994; Ito and Lai 1997; Li et al. 1999; Wu-Baer et al. 1996), hnRNP L (Gutierrez-Escolano et al. 2000; Hahm et al. 1998), HuR (Spangberg et al. 2000), and Lsm1p-related protein (Diez et al. 2000).
2. Translation factors: elongation factors EF-1α (Blackwell and Brinton 1997; Harris et al. 1994; Joshi et al. 1986), -β and -γ (Das et al. 1998), EF-Tu (Blumenthal and Carmichael 1979), and eukaryotic initiation factor eIF-3 (Osman and Buck 1997; Quadt et al. 1993).
3. Noncanonical translation factors: hnRNP A1, PTB, and La antigen (Meerovitch et al. 1993; Pardigon and Strauss 1996; Svitkin et al. 1996).
4. Cytoskeletal or chaperone proteins: tubulin (Huang et al. 1993; Moyer et al. 1990; Moyer et al. 1986), actin (De et al. 1991), and heat shock protein (Oglesbee et al. 1996).

These cellular proteins typically bind to viral RNAs or polymerase to form replication or translation complexes (Lai 1998). Remarkably, most of them can interact with RNAs of several different viruses or bind to viral RNA in one virus but associate with viral polymerase in another.

Coronavirus RNA synthesis, including replication of viral genome and transcription of subgenomic mRNAs, has been shown to be regulated by several viral RNA elements, including 5′-untranslated region (UTR), *cis*- and *trans*-acting leader RNAs (Liao and Lai 1994; Zhang et al. 1994; Zhang and Lai 1995b), IG sequence (Makino et al. 1991), and 3′-UTR (Lin et al. 1996). Biochemical evidence suggests that these regulatory sequences likely interact with each other either directly or indirectly, probably through protein-RNA and protein-protein interactions involving both viral and cellular proteins (Zhang and Lai 1995b). Indeed, hnRNP A1 (Huang and Lai 2001; Li et al. 1997; Shi et al. 2000), PTB (Huang and Lai 1999; Li et al. 1999), PABP (Spagnolo and Hogue 2000), and mitochondrial aconitase (Nanda and Leibowitz 2001), have been identified as binding specifically to the known *cis*-acting regulatory sequences. The functional importance of hnRNP A1 (Shi et al. 2000) and PTB (Huang and Lai 1999) in viral RNA synthesis has also been established, further supporting the notion that cellular proteins play an integral or regulatory role in viral replication.

Viruses invariably rely on cellular architecture as an important structural element of their replication machineries. The replication complexes of numerous positive-stranded RNA viruses have been found to be membrane associated (Bienz et al. 1994; Chambers et al. 1990; Froshauer et al. 1988; Miller et al. 2001; Schwartz et al. 2002; van Dinten et al. 1996). Thus, many cellular membrane proteins are expected to serve as scaffolds to provide support for the formation of viral replication complexes, for localized protein translation, and for viral assembly. Very little is currently known about these cellular factors. In this chapter, we focus on the proteins that are the integral parts of the replication complexes. Left out are the cellular factors involved in other aspects of viral replication, such as virus entry and virus assembly.

2 Viral Proteins in Coronavirus Replication

Although the mechanism of coronavirus RNA replication is still controversial, the consensus is that coronavirus RNA replication is directed by *cis*-acting sequences present on the viral RNAs with the help of *trans*-

acting factors encoded by the virus. Indeed, continuous protein synthesis is required for RNA synthesis, due to the fact that the application of inhibitors of protein synthesis at any time during the viral life cycle inhibits viral RNA synthesis (Perlman et al. 1986; Sawicki and Sawicki 1986). A similar observation has been made with an inhibitor of cysteine protease, which inhibits the processing of the MHV ORF 1 (termed the polymerase or the replicase gene) polyprotein (Kim et al. 1995), suggesting that continuous production of the polymerase gene products is required for viral RNA synthesis. The precise nature of many of these products, however, is largely unknown.

2.1 The Polymerase Gene Products

The coronavirus polymerase gene accounts for approximately two-thirds of the genome. It contains two overlapping ORFs, ORF 1a and ORF 1b, which overlap by 76 nt (Fig. 1). The expression of the downstream

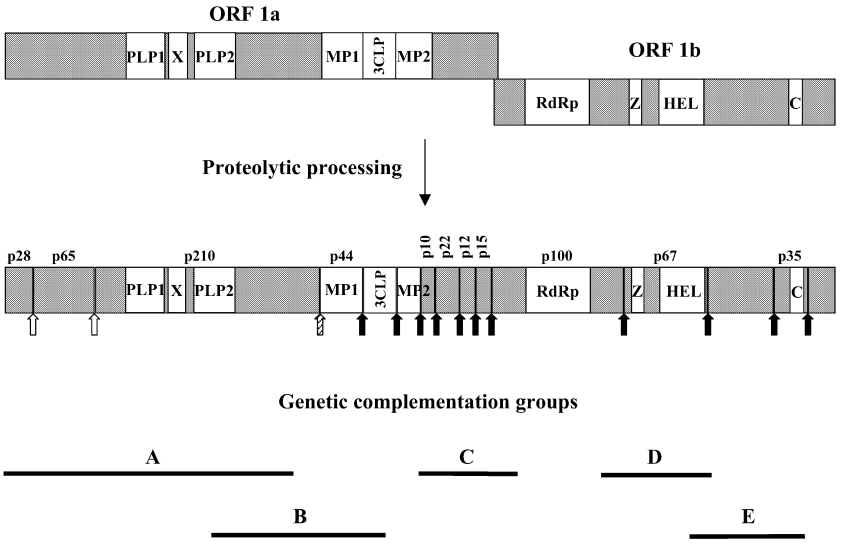

Fig. 1. The domain structure and processing scheme of the MHV polymerase gene products and the approximate location of genetic complementation groups (Baric et al. 1990a). *PLP*, papain-like protease; *3CLP*, 3C-like protease; *MP*, membrane protein; *RdRp*, RNA-dependent RNA polymerase; *Z*, zinc-binding domain; *HEL*, helicase; *C*, conserved domain. The *open*, *hatched*, and *closed arrows* indicate the PLP1, PLP2, and 3CLP cleavage sites, respectively

ORF 1b is mediated by a ribosomal frameshift event that is aided by the formation of a pseudoknot structure within the overlapping region (Bredenbeek et al. 1990; Brierley et al. 1987; Herold and Siddell 1993). To date, the full-length product of ORF 1 has not been detected in coronavirus-infected cells, most probably because it is cotranslationally and auto-proteolytically processed into numerous intermediates and mature nonstructural proteins. Based on the primary sequences of several different coronaviruses, the degree of amino acid identity for this gene product is greater than that is observed for any other coronavirus gene products. A combination of computer-based motif prediction and experimental analysis has identified a number of functional domains in the ORF 1 polyprotein (Fig. 1) (Gorbalenya et al. 1991; Lee et al. 1991). ORF 1a contains the papain-like cysteine proteases (PLPs), a chymotrypsin/picornaviral 3C-like protease (3CLP), and membrane-associated proteins (MP). The more conserved ORF 1b includes domains for an RdRp, a zinc-finger nucleic acid-binding domain (metal-binding domain), and a nucleoside triphosphate (NTP)-binding/helicase domain. Both the synthesis and the processing of the ORF 1 polyprotein have been shown to be essential throughout infection to sustain RNA synthesis and virus replication (Denison et al. 1995b; Kim et al. 1995; Shi et al. 1999).

The importance of the polymerase gene products in viral replication has been established by the study of temperature-sensitive (ts) mutants, which are a practical tool for investigating the roles of viral proteins in replication. The ts mutants are grouped into two categories, RNA^- and RNA^+, based on the ability of these mutants to support viral RNA synthesis at the restrictive temperature (Leibowitz et al. 1982; Robb and Bond 1979). Complementation analysis of ts mutants suggests that at least five RNA^- complementation groups are encoded in the MHV genome (Baric et al. 1990a; Koolen et al. 1983; Leibowitz et al. 1982; Martin et al. 1988; Schaad et al. 1990). All of the RNA^- complementation groups are mapped within the ORF 1 region, suggesting that the coronavirus ORF 1 encodes all of the proteins required for viral RNA replication. Different complementation groups within MHV ORF 1 have been demonstrated to affect distinct steps of RNA synthesis, including the synthesis of leader RNA, negative-strand RNA, and positive-strand RNA, suggesting that different steps of RNA synthesis require different viral proteins (Baric et al. 1990b). Among the five RNA^- complementation groups, A, B, C, D, and E, identified by Baric et al. (Fig. 1) (Baric et al. 1990a), groups A and B are defective in the synthesis of all viral RNAs, whereas the rest of the groups are only defective in certain steps of viral RNA synthesis. The group C mutants encode a function required early

in viral transcription to synthesize negative-strand RNA, whereas the group E mutants are blocked at a later stage in the virus growth cycle. The group D mutants are incapable of subgenomic mRNA transcription. Taken together, at least four cistrons are required for positive-strand RNA synthesis whereas the group C cistron functions during negative-strand RNA synthesis. A comparison of three disparate panels of MHV ORF 1 mutants, one for JHM (Robb and Bond 1979) and two for A59 (Koolen et al. 1983; Schaad et al. 1990), concluded that there are at least eight genetically complementable, *trans*-acting functions encoded by ORF 1 (Stalcup et al. 1998).

Genetic recombination analysis revealed that the five RNA^- complementation groups of MHV are arranged in alphabetical order in the 5′ to 3′ direction, with some overlaps between the group A/B and D/E mutants (Fig. 1) (Baric et al. 1990a, b). Group A most likely includes the PLP1 and PLP2 domains, whereas group B encompasses the 3CLP domain. Group C spans the ORF 1a/ORF 1b junction, including the site of ribosomal frameshifting and the N-terminal part of the putative RdRp. Group D is mapped approximately in the middle part of the ORF 1b, possibly encoding the C-terminal part of the putative RdRp and the helicase domain. Group E is located at the C terminus of ORF 1b, about 20–22 kb from the 5′ end of the genome (Fu and Baric 1994). Further characterization of the ts mutants showed that one group C mutant carries a mutation in the 5′ end of ORF 1b encoding the putative RdRp, which is the only mutation found in a domain with an assigned function. Because most of the mutations in other ts mutants have not been identified, it is still not possible to correlate all the genetic defects with the processed products of the ORF 1 polyprotein.

Studies of the localization and interactions of MHV replicase proteins in infected cells have also provided critical insights into the possible roles of these proteins during viral replication. The localization of polymerase gene products, including PLP1 and PLP2, 3CLP, RdRp, and helicase, to cytoplasmic foci active in viral RNA synthesis has been well documented, suggesting that they may participate in the formation and function of the viral replication complexes (Denison et al. 1999; Shi et al. 1999; van der Meer et al. 1999).

2.1.1 RNA-Dependent RNA Polymerase

The RdRp is the most conserved domain of all RNA viruses and is certainly the most fundamental component of the viral replication machin-

ery. It functions as the catalytic subunit of the viral replicase required for the replication of all positive-stranded RNA viruses (Buck 1996). The vast majority of RdRps, including the coronavirus RdRp, have been identified solely on the basis of sequence similarity. Most viral RNA polymerases contain a signature GDD motif, which is considered to be the most characteristic sequence of the RdRps of positive-stranded RNA viruses. In coronavirus, an SDD motif is detected instead of GDD; the effect of this substitution on the activity of coronavirus RdRp is not clear (Gorbalenya et al. 1989b). Based on sequence analysis, the coronavirus RdRp is encoded by the 5′ end of the ORF 1b gene, synthesized as part of the gene 1 polyprotein, and processed by cysteine proteases into an approximately 100-kDa protein (Fig. 1) (Gorbalenya et al. 1989b; Lee et al. 1991). The viral proteins that contain the putative RNA polymerase domain have been detected by immunofluorescence or immunoprecipitation in cells infected with MHV (Shi et al. 1999; van der Meer et al. 1999), IBV (Liu et al. 1994), and HCoV-229E(Grotzinger et al. 1996) but it is not known whether they represent the functional RdRp.

Earlier studies on transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCV), and MHV demonstrated viral polymerase activities in membrane fractions of virus-infected cells (Brayton et al. 1982, 1984; Dennis and Brian 1982; Mahy et al. 1983). Two temporally and enzymatically distinct RdRp activities have been detected in MHV-infected cells (Brayton et al. 1982), suggesting that the enzyme represents two different species of RNA polymerase that perform different roles in virus-specific RNA synthesis. The early polymerase is most likely responsible for negative-strand RNA synthesis, whereas the late polymerase is responsible for the positive-stranded RNA synthesis (Brayton et al. 1984). It is unknown whether the protein components of these two complexes are different or whether the same polymerase is modified by other viral or cellular proteins to perform distinct functions. Because coronaviruses are known to have a unique mechanism of subgenomic RNA synthesis quite distinct from that of genome replication, it is possible that the viruses could have more than one RNA polymerase. After the initial detection of polymerase activities in the fractions of coronavirus-infected cells, several in vitro RNA synthesis systems were also reported (Baker and Lai 1990; Compton et al. 1987; Leibowitz and DeVries 1988). The nature of the polymerases in these systems, however, has not been characterized.

The catalytic activity of the coronavirus RdRp has so far not been demonstrated biochemically. In fact, only a handful of viral RdRps, such as Qβ replicase subunit II (Landers et al. 1974), poliovirus 3D pol pro-

tein (Neufeld et al. 1991; Rothstein et al. 1988; Van Dyke and Flanegan 1980), hepatitis C virus NS5B protein (Behrens et al. 1996; Lohmann et al. 1997; Yuan et al. 1997), dengue virus NS5 protein (Tan et al. 1996), and tobacco vein mottling virus (TVMV) nuclear inclusion protein NIb (Hong and Hunt 1996), have been shown to possess RNA replicating activities in vitro. It is likely that the extremely hydrophobic nature of the coronavirus RdRps prevents the purification and biochemical characterization of this protein. Thus, the precise role of coronavirus RdRps in viral RNA synthesis has not been established.

2.1.2 Helicase

The RNA helicase is the second most conserved component of the RNA virus replication machinery (Gorbalenya et al. 1988, 1989a; Gorbalenya and Koonin 1989; Koonin and Dolja 1993). Nearly all double-stranded and positive-stranded RNA viruses are predicted to encode putative helicases (Gorbalenya and Koonin 1989). RNA helicases are a diverse class of enzymes that use the energy of NTP hydrolysis to unwind duplex RNA. There is extensive genetic evidence suggesting a key function for helicases in the life cycle of positive-stranded RNA viruses (Buck 1996; Kadare and Haenni 1997). They are involved in virtually every aspect of RNA metabolism, including transcription, splicing, translation, export, ribosome biogenesis, mitochondrial gene expression, and the regulation of mRNA stability (de la Cruz et al. 1999; Linder and Daugeron 2000; Lohman and Bjornson 1996; Schmid and Linder 1992). The idea of involvement of RNA helicase in RNA replication came from the observation that helicase mutants of BMV are defective in template recruitment for RNA replication and the synthesis of negative-strand or subgenomic RNA (Ahola et al. 2000).

The RNA helicase domains of coronaviruses are encoded by ORF 1b and processed by 3CLP (Denison et al. 1999). They have been proposed to represent a separate phylogenetic lineage of the RNA virus superfamily 1 (SF1) helicases, which include the majority of putative RNA virus helicases (Gorbalenya and Koonin 1989; Kadare and Haenni 1997; Koonin and Dolja 1993). The putative MHV RNA helicase, which is processed from the ORF 1b polyprotein by 3CLP, has been detected in MHV-infected cells throughout the viral life cycle (Denison et al. 1999). Numerous attempts to detect the predicted RNA duplex-unwinding activity of these proteins have failed until recently when duplex-unwinding activity was observed for the human coronavirus (HCoV) helicase, pro-

viding valuable insights into the functions of this protein in viral replication (Seybert et al. 2000). Biochemical characterization revealed that this helicase has both RNA and DNA duplex-unwinding activities with a 5′ to 3′ polarity, in contrast to the previously characterized RNA virus SF2 helicases. A zinc finger/nucleic acid-binding domain, which has been found in numerous cellular helicases (Fig. 1) (Gorbalenya and Koonin 1993), is also present in the coronavirus ORF 1b, upstream of the helicase domain, but it is not known whether it contributes to the activity of the coronavirus helicase.

Although there is no direct evidence indicating the involvement of the helicase in coronavirus RNA replication and transcription, the helicase was localized to the perinuclear sites where active viral RNA synthesis was observed (Denison et al. 1999). It was further detected by biochemical analysis in membrane fractions that contain viral RNAs, suggesting that helicase is a component of the viral replication complex (Bost et al. 2000, 2001; Denison et al. 1999; Sims et al. 2000). Furthermore, because double-stranded replicative intermediates are believed to be the predominant RNA structures in coronavirus RNA synthesis, it is tempting to speculate that, in analogy to models described for the DNA replisome (Baker and Bell 1998), the coronavirus helicase cooperates with the RdRp by providing the single-stranded RNA template for processive RNA synthesis. It is noteworthy that the vaccinia virus NPH-II RNA helicase was recently shown to be a highly processive enzyme that unwinds long duplex RNA structures, supporting the hypothesis that at least some viral RNA helicases might be directly involved in RNA replication (Jankowsky et al. 2000).

2.1.3 Proteases

The coronavirus replicase is translated from the genomic RNA as a large precursor polyprotein, which is then processed by viral proteases to generate functional replicase proteins. Whereas the RdRp and RNA helicase play direct roles in viral RNA synthesis, the proteases are involved in viral replication through the processing of viral polyproteins into mature products critical for the appropriate localization, assembly, and function of the replicase complex. They also play an important regulatory role in the generation of specific protein functions at certain stages of the viral life cycle. This controlled proteolysis is thought to be determined mainly by the substrate specificity of the proteases and the accessibility of cleavage sites in the context of specific intermediate products

(van Dinten et al. 1997, 1999; Ziebuhr and Siddell 1999). Sequence analysis of coronavirus genomic RNA led to the prediction of two or three protease domains in ORF 1a: one or two PLPs and a 3CLP (Gorbalenya et al. 1991; Lee et al. 1991). All of these proteases have been shown to function during viral replication and drive the processing of the MHV ORF 1 replicase polyprotein into at least 15 products (Fig. 1) (Baker et al. 1989, 1993; Bonilla et al. 1994, 1995; Bost et al. 2000; Denison et al. 1992, 1995a, 1999; Gao et al. 1996; Lu et al. 1995, 1996, 1998; Lu and Denison 1997; Pinon et al. 1999; Schiller et al. 1998; Shi et al. 1999). Comparable, but distinct, proteolytic processing pathways have also been reported for some other coronaviruses, most notably IBV (Liu et al. 1994, 1998; Liu and Brown 1995) and HCoV-229E (Ziebuhr et al. 2000).

The coronavirus ORF 1 polyprotein can be divided into an N-terminal region that is processed by one or two PLPs and a C-terminal region that is processed by the 3CLP (Ziebuhr et al. 2000). The N-terminal region of the polyprotein spans from the initiator Met to the N terminus of the hydrophobic domain MP1 (Fig. 1). All coronaviruses, except IBV, encode two paralogous and sequentially positioned PLP1 and PLP2 that flank a conserved X domain from both sides (Fig. 1) (Gorbalenya et al. 1991; Lee et al. 1991). At least three proteins, p28, p65, and p210 (also known as p240), are produced from this region of the ORF 1a polyprotein in MHV (Denison and Perlman 1987; Denison et al. 1995a; Schiller et al. 1998). The MHV p210 protein is autocatalytically released through cleavages mediated by PLP1 at the N-terminal site (Bonilla et al. 1995, 1997) and PLP2 at the C-terminal site (Kanjanahaluethai and Baker 2000). PLP1 also cleaves the p28-p65 junction (Baker et al. 1989, 1993; Dong and Baker 1994; Hughes et al. 1995), which, except for IBV, is conserved in all coronaviruses (Herold et al. 1998). Accordingly, a PLP1-mediated cleavage at this site, resulting in the production of a small N-terminal protein (p9, p28 equivalent), was also detected in HCoV-infected cells (Herold et al. 1998). The single IBV PLP corresponds to the PLP2 domain of other coronaviruses. It is part of a p195 protein, which is cleaved to produce an N-terminal product, p87 (Lim and Liu 1998; Lim et al. 2000).

Coronavirus PLPs contain a transcription factor-like zinc finger (Herold et al. 1999), suggesting that they might also be directly involved in coronavirus RNA synthesis. This hypothesis is strongly supported by a recent report showing the equine arteritis virus (EAV) nonstructural protein 1, which is likely a distant homolog of the coronavirus PLPs, to

be a transcriptional factor indispensable for subgenomic mRNA synthesis (Tijms et al. 2001).

The C-terminal part of the ORF 1 polyprotein encompasses all of the major conserved domains starting from the hydrophobic domain MP1 and extending to the C terminus of the replicase polyprotein. The 3CLP, flanked on either side by membrane-spanning regions MP1 and MP2 (Bonilla et al. 1994; Lee et al. 1991; Lu et al. 1995), is believed to be the principal viral protease responsible for the processing events leading to the formation of the viral replicase complex. At least 12 processing products, including the 3CLP itself, RdRp, and helicase, are generated by 3CLP-mediated cleavage (Fig. 1) (Gorbalenya et al. 1991; Lee et al. 1991). Treatment of infected cells with E64d, a known inhibitor of the 3CLP, results in the inhibition of viral RNA replication in these cells (Kim et al. 1995), demonstrating the importance of the action of the 3CLP in the events leading to viral replication. The importance of 3CLP cleavages was demonstrated with an infectious clone of the related arterivirus EAV (van Dinten et al. 1999). Introduction of mutations into the candidate ORF 1b 3CLP cleavage sites had drastic effects on RNA synthesis and virus replication. 3CLP has also been localized to the site of viral RNA synthesis by immunofluorescence staining and biochemical fractionation studies (Bost et al. 2000, 2001; Denison et al. 1999; Shi et al. 1999; Sims et al. 2000).

2.1.4
Other Polymerase Gene Proteins

Apart from the RdRp, helicase, and proteases, the identities of many of the ORF 1 products have not been established. Thus, their roles in viral replication remain unknown. By immunofluorescence staining and confocal microscopy, several studies have shown that a number of ORF 1a products, p65, p10, p22, p12, and p15, and an ORF 1b product, p35, are associated with the site of viral RNA synthesis (Fig. 1) (Bost et al. 2000; Shi et al. 1999). However, biochemical studies revealed two distinct but tightly associated membrane populations, only one of which appears to be a site for viral RNA synthesis (Sims et al. 2000). p28, helicase, 3CLP, and nucleocapsid (N) protein cosegregated with the viral RNA and, therefore, are likely to be the components of the viral replication complexes, whereas p65 and p22 are present in different membrane fractions and may serve roles during infection that are distinct from viral RNA transcription or replication (Sims et al. 2000).

The hydrophobic domains, MP1 and MP2, within the ORF 1a polyprotein were postulated to mediate the association of the coronavirus replicase with cellular membrane structures. MP1 has indeed been detected in microsomal membranes (Pinon et al. 1997), but its role in membrane association and coronavirus replication is largely speculative. A recent study on the related arterivirus demonstrated that the EAV nonstructural proteins (nsp) 2 and 3, which contain one or two hydrophobic regions, induce the formation of double-membrane structures where EAV RNA synthesis takes place (Snijder et al. 2001). Similarly, the membrane proteins of coronavirus may serve to alter the cell architecture so that it is more favorable for viral replication.

2.2 The N Protein

The coronavirus N protein associates with the genomic RNA to form a helical nucleocapsid. In addition to its role as a major structural component of virions, N may also be involved in viral RNA replication and translation control. In an in vitro replication system for MHV, it was demonstrated that antibodies against the N protein could inhibit RNA synthesis (Compton et al. 1987). Optimal replication of the bovine coronavirus (BCV) DI RNA also requires the translation of most, if not all, of the N protein *in cis* (Chang and Brian 1996). Structural analysis of DI RNAs shows that the presence of gene 1 and N gene is sufficient for viral RNA replication (Kim and Makino 1995a). In addition, the MHV N protein was detected in membrane fractions containing viral RNA (Sims et al. 2000) and colocalized with putative replicase proteins in virus-infected cells, providing further support that N may be involved in RNA replication (Denison et al. 1999; van der Meer et al. 1999). However, a mutational study of an infectious cDNA clone of EAV, a close relative of coronavirus, reported that all structural proteins, including N, are dispensable for genome replication and subgenomic mRNA transcription (Molenkamp et al. 2000). The coronavirus replicase gene products were also shown to be sufficient for discontinuous subgenomic mRNA transcription with a partial cDNA clone representing the 5′ and 3′ ends of the HCoV-229E genome, the HCoV-229E replicase gene, and a reporter gene located downstream of a regulatory element for coronavirus mRNA transcription (Thiel et al. 2001). The RNA replication levels observed in these systems are much lower than those containing the wild-type full-length viral genome, indicating that factors other than the replicase polyprotein are required for efficient RNA replication.

Because the N protein has the ability to interact with viral RNA, it most likely functions in viral RNA synthesis by binding to RNA and forming a ribonucleoprotein (RNP) complex. The N protein binds to the leader RNA sequences present at the 5′ end of genomic RNA and all six subgenomic mRNAs in MHV-infected cells (Baric et al. 1988; Nelson et al. 2000). Biochemical analysis measured a dissociation constant of 14 nM for bacterially expressed MHV N-binding to the leader RNA (Nelson et al. 2000). The MHV negative-stranded RNA was also immunoprecipitated by the anti-N monoclonal antibody. These data indicate that the MHV N protein is associated with MHV-specific RNAs and RNA intermediates and may play an important functional role during MHV transcription and replication. Furthermore, the N-leader-RNA-containing RNP complexes were also immunoprecipitated from BCV-infected cells (Cologna et al. 2000). The interactions between the N protein and the RNA encompassing the N ORF may also contribute to the formation of the N-RNA complexes that are present in coronavirus-infected cells (Cologna et al. 2000).

The N protein of MHV is also involved in positive translational control (Tahara et al. 1993, 1998). It stimulated translation of a chimeric reporter mRNA containing an intact MHV 5′-untranslated region and the chloramphenicol acetyltransferase (CAT)-coding sequence. Preferential translation of viral mRNA in MHV-infected cells is stimulated in part by the interaction between the N protein and a 12-nt tract at the 3′ end of the leader.

Other coronavirus proteins, including structural protein hemagglutinin-esterase (HE) (Luytjes et al. 1988; Yokomori et al. 1991) and nonstructural proteins NS2 (Schwarz et al. 1990), NS4 and NS5 (Yokomori and Lai 1991), are not essential for coronavirus replication. However, it is not clear whether any of these proteins can modulate viral replication.

3
Cellular Proteins in Coronavirus Replication

Coronavirus replication involves not only the viral proteins, but also cellular proteins, which are subverted from the normal functions of the host to play roles in the viral replication cycle. No coronavirus proteins in the infected cell extract could be cross-linked to the viral RNA in vitro, suggesting that viral proteins may interact with viral RNA only indirectly through cellular proteins. Several cellular proteins have been shown to bind to the regulatory elements of MHV RNA, including the 5′

and 3′ ends of the genomic RNA and the 3′ end of the negative-strand RNA and IG sites. So far, only a handful of them have been identified, among which hnRNP A1 and PTB are the only two proteins found to interact with regions other than the 3′ end of the coronavirus genome. These proteins are likely to serve as mediators to bring the *cis*-regulatory regions together to form viral replication complexes. They may also help recruit and stabilize the RdRp to the initiation sites of viral RNA synthesis.

3.1 HNRNP A1

UV cross-linking experiments using cytoplasmic extracts of uninfected cells and the IG sequence showed that three different cellular proteins bind to IG of the template RNA (Zhang and Lai 1995a). Deletion analyses and site-directed mutagenesis of IG further demonstrated a correlation between protein binding and transcription efficiency, suggesting that these RNA-binding proteins are involved in the regulation of coronavirus mRNA transcription. One of these proteins was identified by partial peptide sequencing to be hnRNP A1 (Li et al. 1997). hnRNP A1 is an RNA-binding protein that contains two RNA-binding domains (RBDs) and a glycine-rich domain responsible for protein-protein interaction. It is predominantly a nuclear protein but also shuttles between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss 1992). A 38-amino acid sequence, termed M9, located near the C terminus of hnRNP A1 between amino acids 268 and 305 has been determined to be the signal that mediates shuttling (Michael et al. 1995; Siomi and Dreyfuss 1995; Weighardt et al. 1995). The nuclear hnRNP A1 is known to be involved in pre-mRNA splicing and transport of cellular RNAs (Dreyfuss et al. 1993), whereas the cytoplasmic hnRNP A1 is capable of high-affinity binding to AU-rich elements and thus modulating mRNA turnover and translation (Hamilton et al. 1993, 1997; Henics et al. 1994). Another function of hnRNP A1 in the cytoplasm is to promote ribosome binding by a cap-mediated mechanism and to prevent spurious initiations at aberrant translation start sites (Svitkin et al. 1996).

hnRNP A1 binds MHV negative-strand leader and IG sequences (Furuya and Lai 1993; Li et al. 1997), which are critical elements for the discontinuous viral RNA transcription (Fig. 2). Site-directed mutagenesis of the IG sequences demonstrated that the extent of binding of hnRNP A1 to the IG sequences correlated with the efficiency of transcription from the IG site (Furuya and Lai 1993; Li et al. 1997; Zhang and Lai

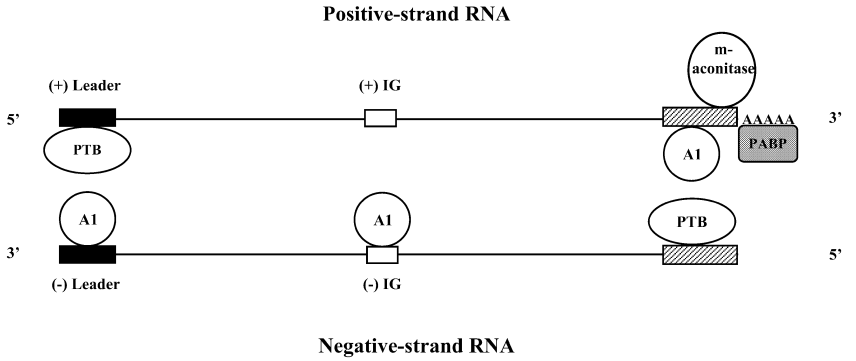

Fig. 2. Schematic drawings of the cellular proteins that interact with coronavirus RNA. hnRNP A1 interacts with the negative-strand leader and IG sequences as well as the positive-strand 3′-UTR, whereas PTB interacts with the positive-strand leader and the complementary sequence of 3′-UTR. These two proteins bind to sequences that are complementary to each other at both the 5′ and 3′ ends of coronavirus RNAs. The poly(A) tail and the 3′-most 42 nt of the genomic RNA serve as binding domains for PABP and m-aconitase, respectively

1995b). Immunostaining of hnRNP A1 showed that hnRNP A1 relocated to the cytoplasm of MHV-infected cells, where viral RNA synthesis occurs (Li et al. 1997). hnRNP A1 also interacts with the MHV N protein (Wang and Zhang 1999), which also binds to the MHV RNA directly (Baric et al. 1988; Stohlman et al. 1988). Furthermore, hnRNP A1 mediates the formation of a ribonucleoprotein complex containing the MHV negative-strand leader and IG sequences (Zhang et al. 1999), suggesting that it may serve as a protein mediator for distant RNA regions to interact with each other to form a transcription initiation complex. Remarkably, hnRNP A1 has also been shown to bind the positive-stranded 3′-UTR and may play a role in negative-strand RNA synthesis (Fig. 2) (Huang and Lai 2001).

The functional importance of hnRNP A1 in coronavirus RNA replication was shown in cells stably expressing the wild-type hnRNP A1 or a dominant-negative mutant of hnRNP A1, which lacks the C-terminal nuclear localization domain (Shi et al. 2000). Viral RNA synthesis was accelerated by the overexpression of hnRNP A1 but delayed by the expression of the mutant hnRNP A1 in the cytoplasm. Thus, the truncation mutant of hnRNP A1 interferes with viral RNA replication in a dominant-negative fashion. In addition to the general inhibition of viral RNA synthesis, the hnRNP A1 mutant also caused a preferential inhibition of

the replication of DI RNAs, suggesting that the inhibition of MHV replication by the hnRNP A1 mutant was most likely a direct effect on viral RNA synthesis rather than an indirect effect on other aspects of cellular or viral functions. Because hnRNP A1 binds directly to the *cis*-acting MHV RNA sequences critical for MHV RNA transcription (Li et al. 1997) and replication (Huang and Lai 2001), it is most likely that hnRNP A1 may participate in the formation of the transcription/replication complex.

However, a mouse erythroleukemia cell line, CB3, that lacks detectable hnRNP A1 expression (Ben-David et al. 1992) can still support efficient MHV replication (Shen and Masters 2001). Because hnRNP A1 protein is involved in a variety of important cellular functions, it is conceivable that other cellular gene products may substitute for the function of hnRNP A1 in both uninfected and virus-infected CB3 cells. Indeed, a number of CB3 cellular proteins comparable to hnRNP A1 in size were found to interact with the MHV negative-strand leader RNA. All of these proteins were identified to be hnRNP A1-related proteins, including hnRNP A/B, hnRNP A2/B1, and hnRNP A3 (Shi et al. 2003). These hnRNPs have primary sequence structure, biochemical properties, and function similar to those of hnRNP A1 (Dreyfuss et al. 1993; Ma et al. 2002; Mayeda et al. 1994). They also have binding specificity and affinity similar to MHV RNA compared with hnRNP A1 (Shi et al. 2003). One of these proteins, hnRNP A2/B1, can substitute for hnRNP A1 in regulating the splicing of cellular (Mayeda et al. 1994) and viral (Bilodeau et al. 2001; Caputi et al. 1999) pre-mRNAs. Together, these multiple hnRNP A1-related proteins may perform similar functions in MHV replication.

3.2 PTB

PTB, which is also known as hnRNP I, binds to the UC-rich RNA sequences typically found near the 3′ end of introns. Similar to hnRNP A1, PTB shuttles between the nucleus and cytoplasm and plays a role in the regulation of alternative splicing of pre-mRNAs and translation of cellular and viral RNAs (Kaminski et al. 1995; Svitkin et al. 1996; Valcarcel and Gebauer 1997). Studies of picornaviruses revealed that PTB plays a role in internal ribosome entry site (IRES)-mediated translation by mechanisms distinct from those governing the cap-dependent translation of most eukaryotic mRNAs (Jackson and Kaminski 1995). PTB was found to be associated with the IRES elements of encephalomyocarditis virus and foot-and-mouth-disease virus and to stimulate translation ini-

tiated from these IRES elements (Kaminski et al. 1995; Niepmann 1996; Niepmann et al. 1997).

UV cross-linking and immunoprecipitation studies using cellular extracts and a recombinant PTB established that PTB binds specifically to the MHV positive-strand leader RNA (Fig. 2) (Li et al. 1999), which is required for MHV RNA synthesis (Kim et al. 1993; Liao and Lai 1994) and regulates translation (Tahara et al. 1994). The PTB-binding sites were mapped to the UCUAA pentanucleotide repeats within the leader RNA; deletion of these binding sites significantly inhibits RNA transcription (Li et al. 1999). Interestingly, PTB also interacts with the complementary strand of the 3′-UTR (c3′-UTR) (Fig. 2) (Huang and Lai 1999). A strong PTB-binding site was mapped to nt 53–149, and another weak binding site was mapped to nt 270–307 on c3′-UTR. Partial substitutions of the PTB-binding nucleotides reduced PTB binding in vitro. Furthermore, DI RNAs harboring these mutations showed substantially reduced ability to synthesize subgenomic mRNA. Remarkably, the binding of PTB to nt 53–149 caused a conformational change in the neighboring RNA region. Partial deletions within the PTB-binding sequence completely abolished the PTB-induced conformational change in the mutant RNA even when the RNA retained partial PTB-binding activity. Correspondingly, the MHV DI RNAs containing these deletions lost their ability to transcribe mRNAs. Thus, the conformational change in the c3′-UTR caused by PTB binding may play a role in mRNA transcription.

It is interesting to note that hnRNP A1 and PTB bind to the precisely complementary sites on the negative- and positive-stranded RNA, respectively, of the leader region of MHV RNA, and also the 5′- and 3′-ends of both the positive- and negative-strand RNAs (Fig. 2) (Huang and Lai 2001; Huang and Lai 1999; Li et al. 1997, 1999;). Furthermore, hnRNP A1 and PTB together mediate the formation of an RNP complex involving the 5′- and 3′ end fragments of MHV RNA in vitro (Huang and Lai 2001). The interaction between hnRNP A1 and PTB have also been detected in a splicing complex in uninfected cells (Bothwell et al. 1991). All of these findings support the notion that hnRNP A1 and PTB may be involved in the formation of a ribonucleoprotein complex, which functions in MHV RNA synthesis.

Most coronavirus mRNAs are capped at the 5′ end and translated by a cap-dependent mechanism. The binding of PTB to the coronavirus leader RNA, which regulates MHV RNA translation (Tahara et al. 1994), suggests a possible role of PTB in coronavirus mRNA translation as well. Surprisingly, PTB was found to have no direct effect on the cap-dependent MHV RNA translation (Choi and Lai, unpublished data). It is, how-

ever, still possible that PTB may affect the IRES-mediated translation of coronavirus ORF 5b, which encodes the envelope (E) protein (Lai and Cavanagh 1997; Thiel and Siddell 1994). The ORF 5b IRES has been shown to serve as a binding site for cellular proteins (Jendrach et al. 1999), although it is not known whether PTB is among these proteins.

3.3 PABP

The 3′-UTRs of coronavirus RNA are necessary for the synthesis of negative-strand viral RNA (Lin et al. 1994) and both genomic and subgenomic positive-strand RNA synthesis (Kim et al. 1993; Lin and Lai 1993; Lin et al. 1996). They contain structures that are conserved among divergent coronaviruses (Hsue et al. 2000; Hsue and Masters 1997; Liu et al. 2001). It is possible that these secondary structural elements serve as binding sites for cellular proteins and function in viral replication. Indeed, the mutations at the 3′ end of the viral genomic RNA that abolished the binding of cellular proteins also inhibited both negative-strand and positive-strand RNA synthesis, although the correlation between protein binding and RNA synthesis was not absolute (Liu et al. 1997; Yu and Leibowitz 1995a).

A number of cellular proteins have been found to interact with multiple sites within the 3′ end of positive-strand MHV RNA (Huang and Lai 2001; Liu et al. 1997; Spagnolo and Hogue 2000; Yu and Leibowitz 1995a, b). Several cellular proteins have also been shown to interact with the BCV 3′-UTR [287 nt plus poly(A) tail] (Huang and Lai 2001; Liu et al. 1997; Spagnolo and Hogue 2000; Yu and Leibowitz 1995a, b). Competition with the MHV 3′-UTR [301 nt plus poly(A) tail] suggests that the interactions are conserved for the two viruses (Huang and Lai 2001; Liu et al. 1997; Spagnolo and Hogue 2000; Yu and Leibowitz 1995a, b). Proteins with molecular masses of 99, 95, 73, 40–50, and 30 kDa were detected, among which the 73-kDa protein was identified to be poly(A)-binding protein (PABP) by immunoprecipitation experiments. PABP is known to interact specifically with poly(A), which is an important *cis*-acting signal for coronavirus RNA replication (Fig. 2) (Lin et al. 1994). RNAs with shortened poly(A) tails exhibited less in vitro PABP binding. Furthermore, binding of PABP to the 3′-UTR of the DI RNA replicons correlated with the ability of the DI RNA to replicate, suggesting that the interaction between PABP and the poly(A) tail may affect coronavirus RNA replication (Huang and Lai 2001; Liu et al. 1997; Spagnolo and Hogue 2000; Yu and Leibowitz 1995a, b).

PABP is a highly abundant cytoplasmic protein (Gorlach et al. 1994) that binds the 3′ poly(A) tail on eukaryotic mRNAs and helps promote both efficient translation initiation and mRNA stability. It interacts with the translation factor eukaryotic initiation factor (eIF) 4G (Imataka et al. 1998; Le et al. 1997; Tarun and Sachs 1996; Tarun et al. 1997), which is part of the eIF4F triple complex that binds mRNA cap structures during translation, and PABP-interacting protein (PAIP-1), a protein with homology to eIF-4G (Craig et al. 1998). This interaction, known as the closed-loop model of translation initiation, mediates the cross talk between the 5′ and 3′ ends of mRNAs (Gallie 1998; Sachs et al. 1997). Because coronavirus RNA is capped and polyadenylated like the host mRNAs, PABP is likely involved in the translation of the coronavirus genome upon virus entry into the cell. Because translation is required for efficient coronavirus RNA replication, it is conceivable that PABP can indirectly modulate RNA synthesis through its effect on translation. It is also possible that the PABP-poly(A) interaction may play a more direct role in coronavirus RNA replication in view of the apparent requirement for both the 5′ and 3′ ends, including the poly(A) tail, of the coronavirus genome for DI RNA replication and mRNA transcription (Kim et al. 1993; Lai 1998; Liao and Lai 1994; Lin et al. 1994, 1996). Indeed, hnRNP A1 and PTB together have been shown to mediate the interaction between the 5′ and 3′ ends of MHV RNA (Huang and Lai 2001). PABP may be another cellular factor that facilitates a similar interaction of the ends.

3.4
Mitochondrial Aconitase

The 3′-most 42 nt of the MHV genomic RNA has been shown to interact with host factors and form at least three RNA-protein complexes (Nanda and Leibowitz 2001). Four proteins of approximately 90, 70, 58, and 40 kDa were resolved from these complexes, and the 90-kDa protein was identified as mitochondrial aconitase (m-aconitase), which catalyzes stereospecific interconversion of citrate into isocitrate through a *cis*-aconitase intermediate in the Krebs cycle (Beinert and Kennedy 1993). UV cross-linking studies indicate that the highly purified m-aconitase binds specifically to the MHV 3′ protein-binding element despite the absence of a consensus RNA-binding domain (Fig. 2) (Burd and Dreyfuss 1994). Colocalization of m-aconitase with the MHV N protein was observed in virus-infected cells, suggesting a possible interaction of m-aconitase with the MHV replication complexes (Nanda and Leibowitz 2001).

A cytoplasmic homolog of m-aconitase, cytoplasmic aconitase (c-aconitase), also known as iron regulatory protein 1, is a well-recognized RNA-binding protein (Kennedy et al. 1992). The binding properties of m-aconitase and the functional relevance of RNA binding appear to parallel those of c-aconitase. c-Aconitase is a bifunctional protein, which has been shown to interact with iron-responsive elements located in the 5′-UTR of ferritin mRNA and the 3′-UTR of transferrin receptor (TfR) mRNA and to function to coordinate posttranscriptional regulation of cellular iron metabolism (Hentze and Kuhn 1996; Kuhn and Hentze 1992). Similarly, m-aconitase can function as a posttranscriptional regulator as well (Beinert and Kennedy 1993; Klausner et al. 1993). A link between cellular iron status and m-aconitase expression has also been established (Kim et al. 1996; Schalinske et al. 1998). Increasing the intracellular level of m-aconitase of MHV-infected cells by iron supplementation resulted in increased RNA-binding activity of cell extracts and increased virus production as well as viral protein synthesis at early hours of infection (Nanda and Leibowitz 2001). It is possible that the binding of m-aconitase to the 3′-UTR increases the stability of the viral mRNAs and hence augments the translation of viral proteins, similar to the role of IRP in regulating TfR (Kuhn and Hentze 1992).

3.5 Other Cellular Proteins

Accumulating evidence indicates the presence of additional cellular proteins that interact with coronavirus RNA. The 3′-UTRs of murine and bovine coronaviruses were reported to contain bulged stem-loop (Hsue et al. 2000; Hsue and Masters 1997) and pseudoknot (Williams et al. 1995) structures, which are essential for viral replication. These motifs are potential binding sites for the proteins shown to interact with the 3′-UTR. Indeed, a number of cellular proteins have been shown to interact with different regions within the 3′-UTR of MHV (Liu et al. 1997; Nanda and Leibowitz 2001; Yu and Leibowitz 1995a, b). The 3′-most 42-nt sequence interacts with at least four proteins 90, 70, 58, and 40 kDa in size, among which the 90-kDa protein was identified as m-aconitase (Nanda and Leibowitz 2001). A distinct host cellular protein-binding element was also mapped within a 26-nt sequence at positions 154–129 from the 3′ end of the MHV-JHM genome (Liu et al. 1997). The resulting RNA-protein complex contains six host cellular proteins with one protein of 120-kDa molecular mass, two poorly resolved species approximately 55 kDa in size, a second pair of poorly resolved 40-kDa proteins, and a

minor component of 25 kDa. This region contains multiple stem-loop and hairpin-loop structures, which are shown by mutational analysis to be important for efficient MHV replication (Liu et al. 2001). In the study that identified PABP, several other proteins with molecular masses of 99, 95, 40–50, and 30 kDa were also shown to interact with the 3′-UTRs of both BCV and MHV (Spagnolo and Hogue 2000). These cellular proteins have the potential to regulate viral RNA synthesis through their binding to the 3′ ends of the coronavirus genomes; however, their identities and functions remain to be determined.

3.6 Proposed Functions of Cellular Proteins

The *cis*-acting signals for viral RNA replication or transcription often consist of multiple distant sequences on the viral RNA. In many cases, there appears to be a cross talk between the 5′ and 3′ ends of viral RNAs so that the 3′ end sequence often can regulate RNA synthesis or translation initiated from the 5′ end of the RNA. The 5′- and 3′-UTRs of both positive- and negative-sense RNA and the IG sequences are thought to contain important sequence and structural elements that function in the initiation and regulation of RNA replication, transcription, and translation. The 3′ end of the MHV RNA has been shown to regulate mRNA synthesis starting from an upstream internal promoter (Lin et al. 1996). The poly(A) tail is also involved in coronavirus RNA synthesis (Huang and Lai 2001; Liu et al. 1997; Spagnolo and Hogue 2000; Yu and Leibowitz 1995a, b). Furthermore, there is an apparent interaction between the leader and IG sequences, which regulates the synthesis of coronavirus subgenomic mRNAs (Lai and Cavanagh 1997; Zhang et al. 1994). When no sequence complementarity exists between the 5′ and 3′ ends, RNA-protein and protein-protein interactions must be involved. hnRNP A1 and PTB have the ability to interact with each other, thus allowing different RNA regions to interact (Fig. 3A). By analogy to translation regulation, the binding of PABP to the 3′ end of the coronavirus genome may also facilitate the cross talk between the 3′ end and the other upstream *cis*-acting sequences. Furthermore, because most of the viral RdRps do not appear to bind directly to the *cis*-acting regulatory or promoter sequences on the RNA, their ability to initiate RNA synthesis at specific sites probably depends on their interactions with the cellular proteins that bind directly to the viral RNA template. These cellular proteins may serve as a platform on which other proteins, both viral and

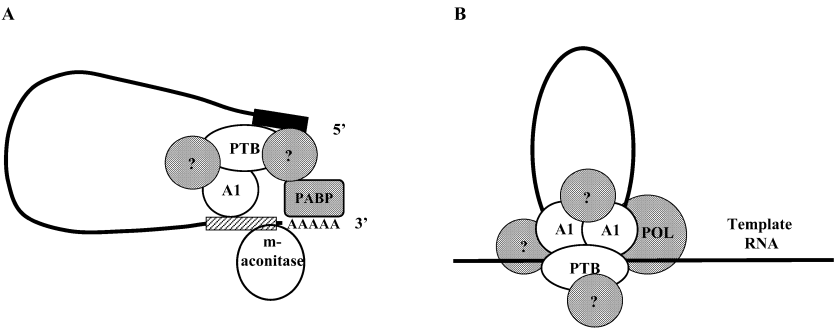

Fig. 3A, B. Proposed functions of cellular proteins in coronavirus replication. **A** Interactions between distant RNA elements are mediated by hnRNP A1, PTB, and PABP. **B** Formation of coronavirus replication/transcription complexes through the recruitment of additional viral and cellular proteins by hnRNP A1 and PTB

cellular, subsequently bind to form functional replication and transcription complexes (Fig. 3B).

Together, cellular proteins play important roles in coronavirus replication. Identification of these proteins and analysis of their functions in virus replication are critical to furthering our understanding of virus-host interactions and will provide clues to unveil the replication strategies of other positive-stranded RNA viruses.

4 Perspectives

Although an increasing body of literature supports the importance of various viral and cellular proteins in coronavirus replication, our current understanding of the roles of these proteins is still limited. The availability of the reverse genetics approach for coronaviruses is expected to greatly accelerate the understanding of coronavirus replication as well as the functional importance of viral and cellular factors in coronavirus replication. In addition, the growing knowledge of the properties of the individual protein products of the coronavirus ORF 1 should help in understanding the makeup of the replication machinery. The recent advances in gene knockout by RNA interference (RNAi) in mammalian cells will likely be a valuable tool in establishing the functional relevance of these cellular proteins. Nevertheless, the ultimate unraveling of the viral and cellular proteins involved in coronavirus replication is expected

to come after the purification of coronavirus RdRp and the reconstitution of virus replication in vitro.

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