CHAPTER 28

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# Coronaviridae



## HISTORY

Coronaviruses are enveloped RNA viruses that are broadly distributed among humans, other mammals, and birds, causing acute and persistent infections. Members of this family were isolated as early as the 1930s as the causative agents of infectious bronchitis in chickens,[25] transmissible gastroenteritis in pigs,[142] and severe hepatitis and neurologic diseases in mice.[75,186] It was not until the 1960s, however, that these viruses,[27,32] as well as certain human respiratory viruses,[8,391] were recognized to share characteristics that merited their being grouped together. Their most notable common feature, revealed by electron microscopy, was a fringe of widely spaced, club-shaped spikes that projected from the virion surface; these spikes were morphologically distinct from the surface projections of ortho- and paramyxoviruses. The halo of spikes was described as giving the viral particle the appearance of the solar corona, which prompted the name that was adopted for this new virus group.[7]

Over the next 40 years, coronaviruses were studied mainly because they cause economically significant respiratory and gastrointestinal diseases in domestic animals and because they provide unique models for viral pathogenesis. In humans, two coronaviruses were known to be responsible for a substantial fraction of common colds, particularly those that circulate in winter months. This situation changed dramatically with the emergence in 2002 of a devastating new human disease, severe acute respiratory syndrome (SARS), which was caused by a previously unknown coronavirus.[143,288,440] Research stimulated by the SARS outbreak has led to great strides in our understanding of coronaviruses; by 2005, two additional, widespread human respiratory coronaviruses had been discovered.[573,615] Moreover, the search for animal virus reservoirs has nearly tripled the total number of identified coronaviruses,[255,394,616] although most of the recently discovered species are known only as genomic sequences and have yet to be isolated or propagated experimentally.

## CLASSIFICATION

The coronaviruses are the largest group within the *Nidovirales* (Fig. 28.1), an order that comprises the families *Coronaviridae, Arteriviridae,*[524] and *Roniviridae.*[102] The arteriviruses, a small group of mammalian pathogens, are discussed in Chapter 29. The roniviruses, which infect shrimp, and a very recently isolated mosquito-borne virus,[416,663] which is not yet classified, are currently the only members of the order having invertebrate hosts. Nidoviruses are membrane-enveloped, nonsegmented positive-strand RNA viruses that are set apart from other RNA viruses by certain distinctive characteristics.[194] Their most significant common features are (a) an invariant general genomic organization, with a very large replicase gene upstream of the structural protein genes; (b) the expression of the replicase-transcriptase polyprotein by means of ribosomal frame shifting; (c) a collection of unique enzymatic activities contained within the replicase-transcriptase protein products; and (d) the expression of downstream genes via transcription of multiple

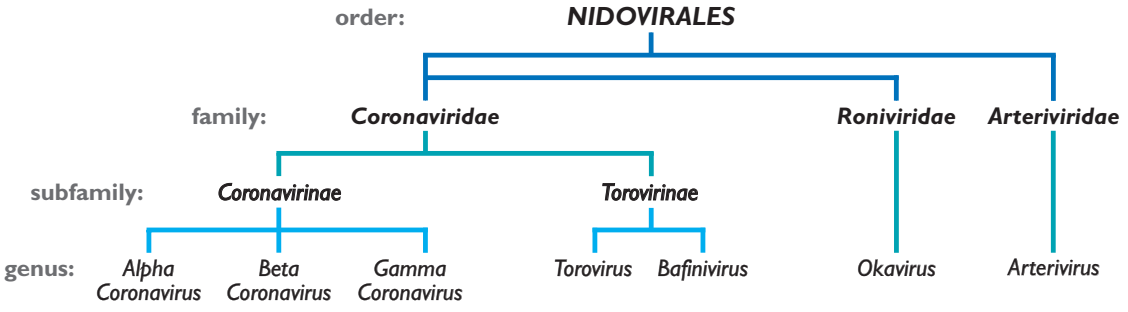



**FIGURE 28.1. Taxonomy of the order *Nidovirales*.**

3′-nested subgenomic messenger RNAs (mRNAs). This last property has provided the name for the order, which comes from the Latin *nido,* for "nest".[157] It should be noted that the replicative similarities among the three nidovirus families are offset by marked differences in the numbers, types, and sizes of their structural proteins and great variation among the morphologies of their virions and nucleocapsids.

Coronaviruses are now classified as one of two subfamilies (*Coronavirinae*) in the family *Coronaviridae* (see Fig. 28.1). The other subfamily, *Torovirinae,* includes the toroviruses, which are pathogens of cattle, horses, and swine,[523] and the bafiniviruses, whose sole member is the only nidovirus currently known to infect fish.[505] This chapter will concentrate almost exclusively on the *Coronavirinae.*

Coronaviruses have long been sorted into three groups, originally on the basis of serologic relationships and, subsequently, on the basis of phylogenetic clustering.[193,195] Following proposals that were recently ratified by the International Committee on Taxonomy of Viruses (ICTV),[57] these groups—the alpha-, beta-, and gammacoronaviruses—have now been accorded the taxonomic status of genera (see Fig. 28.1). The ICTV classifications have also established rigorous criteria for coronavirus species definitions, in a manner consistent with those used for other viral families. As a consequence, some viruses previously considered to be separate species are currently recognized as a single species—for example, the viruses now grouped within alphacoronavirus 1 or betacoronavirus 1 (Table 28.1). Additionally, the new classification criteria resolve any previous uncertainty about the taxonomic assignment of the virus that caused SARS (severe acute respiratory syndrome coronavirus [SARS-CoV]) as a betacoronavirus.[153,197,374,473,483,521,534,535]

Almost all alpha- and betacoronaviruses have mammalian hosts. In contrast, the gammacoronaviruses, with a single exception, have been isolated from avian hosts. Several of the viruses listed in Table 28.1 have been studied for decades, specifically those included in the species alphacoronavirus 1, betacoronavirus 1, murine coronavirus, and avian coronavirus. The focus on these viruses came about largely because they were amenable to isolation and growth in tissue culture. However, since 2004, molecular surveillance and genomics efforts initiated in the wake of the SARS epidemic have led to the discovery of a multitude of previously unknown coronaviruses that now constitute most members of this subfamily.[616] Notably, most of the newly recognized species were identified in bats, which constitute one of the largest orders within the mammals. Diverse coronaviruses have been described from bats, principally in Asia but also in Africa, Europe, and North and South America. These viruses include likely predecessors of SARS-CoV[308,332] but also four unique species of alphacoronaviruses and three species of betacoronaviruses. Birds have also proven to be a rich source of new viruses. Novel avian coronaviruses have been found to infect geese, pigeons, and ducks,[255] and highly divergent coronaviruses recently identified in bulbuls, thrushes, and munias[617] have the potential to define a fourth genus in the *Coronavirinae.* It has been proposed that bats and birds are ideally suited as reservoirs for the incubation and evolution of coronaviruses, owing to their common ability to fly and their propensity to roost and flock.[616]

Five of the viruses in Table 28.1 are associated with human disease. The most categorically harmful of these, SARS-CoV, which is discussed at length later in this chapter, does not currently infect the human population. The remaining four human coronaviruses (HCoVs), the alphacoronaviruses HCoV-229E and HCoV-NL63, and the betacoronaviruses HCoV-OC43 and HCoV-HKU1, typically cause common colds. Remarkably, HCoV-NL63 and HCoV-HKU1 were only discovered recently, in the post-SARS era,[573,615] despite the fact that each has a worldwide prevalence and has been in circulation for a long time.[461,618] Although generally associated with upper respiratory tract infections, the extant HCoVs can also cause lower respiratory tract infections and have more serious consequences in the young, the elderly, and immunocompromised individuals. In particular, HCoV-NL63 is strongly associated with childhood croup,[574] and the most severe HCoV-HKU1, -OC43, and -229E infections are manifest in patients with other underlying illnesses.[460]

## VIRION STRUCTURE

### Virus and Nucleocapsid

Virions of coronaviruses are roughly spherical and exhibit a moderate degree of pleomorphism. In the earlier literature, viral particles were reported to have average diameters of 80 to 120 nm but were far from uniform, with extreme sizes from 50 to 200 nm.[389] The spikes of coronaviruses, typically described as club-like or petal-shaped, emerge from the virion surface as stalks with bulb-like distal termini. Some of the variation in particle size and shape was likely attributable to stresses exerted by virion purification or distortions introduced by negative staining of samples for electron microscopy. More recent studies, employing cryo-electron microscopy and cryo-electron tomography,[21,30,413,415] have produced images (e.g., Fig. 28.2A) in which virion size and shape are far more regular, although still

**TABLE 28.1 Classification of Coronaviruses**

| Species[a] | GenBank accession[b] | Previous names for viruses included in newly defined species |
|---|---|---|
| **Genus *Alphacoronavirus*** | | |
| Alphacoronavirus 1 | EU186072 | Feline coronavirus type I (FeCoV I) |
| | AY994055 | Feline coronavirus type II (FeCoV II), Feline infectious peritonitis virus (FIPV) |
| | GQ477367 | Canine coronavirus (CCoV) |
| | AJ271965 | Transmissible gastroenteritis virus (TGEV) |
| Human coronavirus 229E (HCoV-229E) | AF304460 | |
| Human coronavirus NL63 (HCoV-NL63) | AY567487 | |
| Porcine epidemic diarrhea virus (PEDV) | AF353511 | |
| *Rhinolophus* bat coronavirus HKU2 (*Rh*-BatCoV HKU2) | EF203067 | |
| *Scotophilus* bat coronavirus 512 (*Sc*-BatCoV 512) | DQ648858 | |
| *Miniopterus* bat coronavirus 1 (*Mi*-BatCoV 1) | EU420138 | |
| *Miniopterus* bat coronavirus HKU8 (*Mi*-BatCoV HKU8) | EU420139 | |
| **Genus *Betacoronavirus*** | | |
| Betacoronavirus 1[c] | U00735 | Bovine coronavirus (BCoV) |
| | EF446615 | Equine coronavirus (EqCoV) |
| | AY903460 | Human coronavirus OC43 (HCoV-OC43) |
| | DQ011855 | Porcine hemagglutinating encephalomyelitis virus (PHEV) |
| Murine coronavirus[d] | AY700211 | Mouse hepatitis virus (MHV) |
| | FJ938068 | Rat coronavirus (RCoV) |
| Human coronavirus HKU1 (HCoV-HKU1) | AY597011 | |
| Severe acute respiratory syndrome–related coronavirus (SARSr-CoV) | AY278741 | Human severe acute respiratory syndrome coronavirus (SARS-CoV) |
| | DQ022305 | Severe acute respiratory syndrome–related *Rhinolophus* bat coronavirus HKU3 (SARSr-*Rh*-BatCoV HKU3) |
| | DQ071615 | Severe acute respiratory syndrome–related *Rhinolophus* bat coronavirus Rp3 (SARSr-*Rh*-BatCoV Rp3) |
| *Tylonycteris* bat coronavirus HKU4 (*Ty*-BatCoV HKU4) | EF065505 | |
| *Pipistrellus* bat coronavirus HKU5 (*Pi*-BatCoV HKU5) | EF065509 | |
| *Rousettus* bat coronavirus HKU9 (*Ro*-BatCoV HKU9) | EF065513 | |
| **Genus *Gammacoronavirus*** | | |
| Avian coronavirus[e] | AJ311317 | Infectious bronchitis virus (IBV) |
| | EU022526 | Turkey coronavirus (TuCoV) |
| Beluga whale coronavirus SW1 | EU111742 | |

[a]Listed viruses are those for which complete genome sequences are available. Novel viruses that have not yet been formally classified include Bulbul coronavirus HKU11,[617] Thrush coronavirus HKU12,[617] Munia coronavirus HKU13,[617] Asian leopard cat coronavirus,[139] and Mink coronavirus.[592]

[b]Representative GenBank accession numbers are given for viruses in each species; in many cases, multiple genomic sequences for a given virus are available.

[c]Other viruses included in the species Betacoronavirus 1 are Human enteric coronavirus (HECoV) and Canine respiratory coronavirus (CRCoV), for which only partial genomic sequences are available.

[d]Other viruses included in the species Murine coronavirus are Puffinosis virus (PCoV) and Sialodacryoadenitis virus (SDAV), for which only partial genomic sequences are available.

[e]Other viruses included in the species Avian coronavirus are Pheasant coronavirus (PhCoV), Goose coronavirus (GCoV), Pigeon coronavirus (PCoV), and Duck coronavirus (DCoV), for which only partial genomic sequences are available.[255]

pleomorphic. These studies, which examined a number of alpha- and betacoronaviruses, converge on mean particle diameters of 118 to 136 nm, including the contributions of the spikes, which project some 16 to 21 nm from the virion envelope.

Enclosed within the virion envelope is the nucleocapsid—a ribonucleoprotein that contains the viral genome. The structure of this component is relatively obscure in images of whole virions; however, its makeup has been partially displayed by electron micrographs of spontaneously disrupted virions or of virions solubilized with nonionic detergents.[59,109,183,269,366] Such studies revealed another distinguishing characteristic of coronaviruses: They have helically symmetric nucleocapsids.

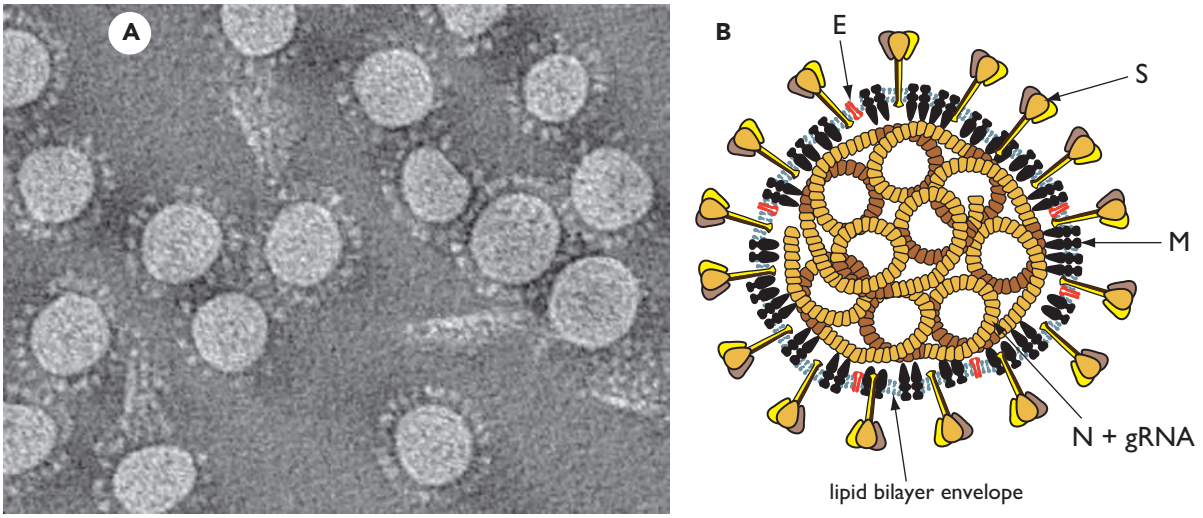



**FIGURE 28.2. Coronavirus structure. A:** Cryo-electron tomographic image of purified virions of mouse hepatitis virus (MHV), reconstructed as described in reference 415. (Courtesy of Benjamin Neuman, David Bhella, and Stanley Sawicki.) **B:** Schematic showing the major structural proteins of the coronavirus virion: S, spike protein; M, membrane protein; E, envelope protein; and N, nucleocapsid protein.

Helical symmetry is common for negative-strand RNA virus nucleocapsids, although it is highly unusual for positive-strand RNA animal viruses, almost all of which have icosahedral capsids. The best-resolved images of the coronavirus nucleocapsid, which were obtained with HCoV-229E, showed filamentous structures 9 to 13 nm in diameter, with 3- to 4-nm-wide central canals[59]; these filaments were thinner and less sharply segmented than paramyxovirus nucleocapsids. However, widely ranging and sometimes discrepant parameters have been reported for the nucleocapsids of other coronaviruses,[378] varying with both the viral species and the method of preparation.[109,183,269,366,476] Thus, further work is needed to clearly define the diameter, symmetry, length, and protein: RNA stoichiometry of this virion component in isolation. More recent coronavirus ultrastructural studies suggest that when packaged within the virion envelope, the helical nucleocapsid is quite flexible, forming coils and other structures that fold back on themselves.[21,413]

## Virion Structural Proteins

Coronaviruses contain a canonical set of four major structural proteins: the spike (S), membrane (M), and envelope (E) proteins, all of which are located in the membrane envelope, and the nucleocapsid (N) protein, which is found in the ribonucleoprotein core (see Fig. 28.2B).

The distinctive surface spikes of coronaviruses are composed of trimers of S molecules.[30,129,529] S is a class I viral fusion protein[41] that binds to host cell receptors and mediates the earliest steps of infection.[95] In some cases, S protein can also induce cell–cell fusion late in infection. The S monomer is a transmembrane protein of 128 to 160 kDa, composed of a very large N-terminal ectodomain and a tiny C-terminal endodomain (Fig. 28.3). This protein is inserted, via a cleaved signal peptide,[62] into the endoplasmic reticulum (ER), where it obtains N-linked glycosylation increasing its mass by some 40 kDa.[224,487] Comprehensive mapping of glycosylation sites has not been carried out for any S protein; however, an analysis of the SARS-CoV S protein showed that at least half of its 23 candidate sites are glycosylated.[287] The early steps of glycosylation occur co-translationally, and this modification assists monomer folding and proper oligomerization; terminal glycosylation is then completed subsequent to trimerization.[129] S protein monomer folding is also accompanied by the formation of intramolecular disulfide bonds among a subset of the numerous cysteine residues of the ectodomain.[425] The positions of S protein cysteines are well conserved in each coronavirus genus[2,153]; disulfide linkages have yet to be mapped.

In many beta- and gammacoronaviruses (e.g., mouse hepatitis virus [MHV], bovine coronavirus [BCoV], and infectious bronchitis virus [IBV]), the S protein is partially or completely cleaved by a furin-like host cell protease into two polypeptides, denoted S1 and S2, which are roughly equal in size. Correspondingly, in coronaviruses that do not have detectably cleaved mature S proteins, the N-terminal and C-terminal halves of the molecule are also designated S1 and S2, respectively. S protein cleavage occurs immediately downstream of a highly basic pentapeptide motif,[2,62,361] and the extent of proteolysis correlates with the number of positively charged residues in the motif.[36] The S1 domain is extremely variable, exhibiting very low homology across the three genera and often diverging extensively among different isolates of a single coronavirus.[181,430,597] By contrast, the S2 domain is highly conserved.[111] For those coronaviruses in which it occurs, S1-S2 cleavage is a late event in virion assembly and release from infected cells. For many other coronaviruses, an alternative type of S protein cleavage (S2′) takes place during the initiation of infection, activating the molecule for fusion.[28] The differing functions of S1 and S2 and the role of proteolysis are discussed later (see the Viral Entry and Uncoating section).

A complete high-resolution structure has not yet been determined for any coronavirus S protein, although a cryo-electron microscopic reconstruction of the SARS-CoV S protein is available,[30] and partial crystal structures have been solved for particular S protein domains.[144,208,323,325,624,630,655]

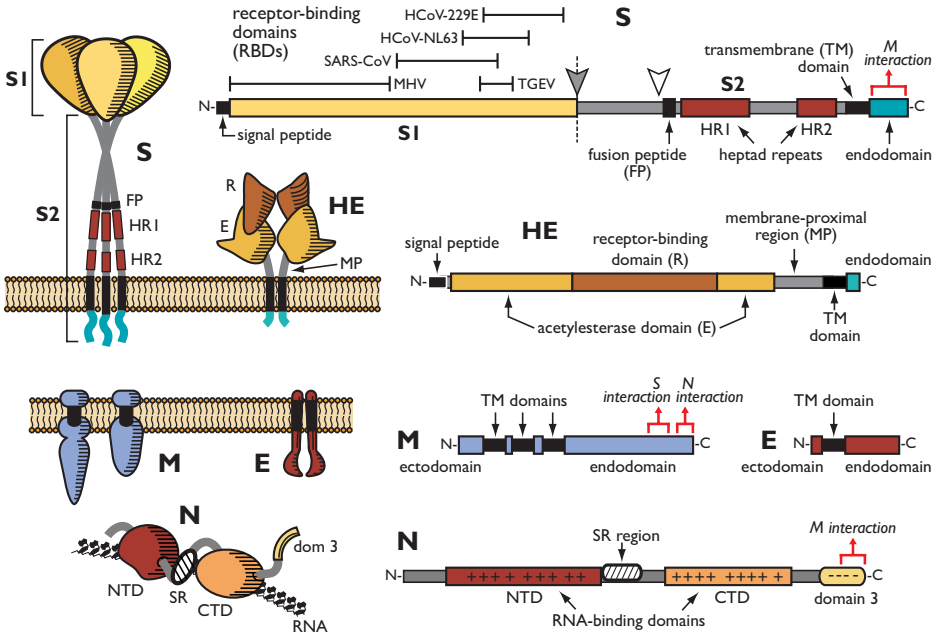



**FIGURE 28.3. Virion structural proteins.** Folded and linear representations of the spike (S), hemagglutinin-esterase (HE), membrane (M), envelope (E), and nucleocapsid (N) proteins. The size scale for the linear diagram of S is half of that for the other proteins. In the linear diagram of S, *solid* and *open arrowheads* indicate the S1-S2 and alternative (S2′) cleavage sites, respectively. In the linear diagrams of S, M, and N, *red brackets* indicate mapped regions involved in assembly interactions (see the Assembly and Release of Virions section).

Nevertheless, all currently available structural and biochemical evidence accords well with an early proposal that S is functionally analogous to the influenza HA protein.[111] In this model, the S1 domains of the S protein oligomer make up the bulbous, receptor-binding portion of the spike. The narrow stalk of the spike, distancing the bulb from the membrane, is a coiled-coil structure formed by association of heptad repeat regions (HR1 and HR2) of the S2 domains of monomers (see Fig. 28.3).

The most abundant structural protein in coronaviruses—the M protein[544,546]—gives the virion envelope its shape. The M monomer, which ranges from 25 to 30 kDa, is a polytopic membrane protein that is embedded in the envelope by three transmembrane domains.[14,486] At its amino terminus is a very small ectodomain; the C-terminal endodomain of M accounts for the major part of the molecule and is situated in the interior of the virion or on the cytoplasmic face of intracellular membranes (see Fig. 28.3). Although it is inserted co-translationally into the ER membrane, the M protein generally does not bear an amino-terminal signal peptide.[62,486] For IBV and MHV, either the first or the third transmembrane domain of M alone suffices as a signal for insertion and anchoring of the protein in its native membrane orientation.[350,363,384] Anomalously, M proteins of the alphacoronavirus 1 species do contain cleavable N-terminal signal peptides, although it is not clear whether these are necessary for membrane insertion.[263,584] The ectodomain of M is modified by glycosylation, which is usually N linked.[60,251,402,536,632] However, a subset of betacoronavirus M proteins exhibit O-linked glycosylation, and the MHV M protein has served as a model for study of this type of post-translational modification.[116,349,419] Glycosylation of M influences both organ tropism and the interferon (IFN)-inducing capacity of some coronaviruses.[72,113,311]

M proteins are moderately well conserved within each coronavirus genus but diverge considerably across genera. The most variable part of the molecule is the ectodomain. By contrast, a short segment, overlapping the third transmembrane domain and the start of the endodomain, exhibits a high degree of sequence conservation that is seen even in torovirus M proteins.[132] Like most multispanning membrane proteins, the M protein has been refractory to crystallization; however, recent cryo-electron microscopic and tomographic reconstructions have provided a glimpse of the structure of this protein within the virion envelope.[21,413,415] These studies reveal that the large carboxy terminus of M extends some 6 to 8 nm into the viral particle and is compressed into a globular domain, consistent with early work showing that the endodomain is very resistant to proteases.[61,384,486,490] The observed M structures are likely to be dimers, the monomers of which are associated through multiple interacting regions. M dimers appear to adopt two different conformations: a compact form that promotes greater membrane curvature and a more elongated form that contacts the nucleocapsid.[415]

The E protein is a small polypeptide of 8 to 12 kDa that is found in limited amounts in the virion envelope.[189,344,647] Despite its minor presence, no wild-type coronavirus has been discovered to lack this protein. Engineered knockout or deletion of the *E* gene has effects ranging from moderate[124] to severe[293,296] to lethal.[105,428] Thus, although E is not always essential, it is critical for coronavirus infectivity (see the Assembly and Release of Virions section). E protein sequences are widely divergent, even among closely related coronaviruses.[293] However, all E proteins share a common architecture: a short hydrophilic amino terminus, followed by a large hydrophobic region, and, lastly, a large hydrophilic C-terminal tail (see Fig. 28.3). E is an integral membrane protein,[100,335,582] but it does not have a cleavable signal peptide[465] and is not glycosylated. Beta- and gammacoronavirus E proteins are palmitoylated on cysteine residues downstream and adjacent to the hydrophobic region[38,101,335,354,647]; this modification remains to be found in an alphacoronavirus E protein.[189] The membrane topology of E is not completely resolved. Most evidence indicates that this polypeptide transits the membrane once, with an N-terminal exodomain and a C-terminal endodomain.[101,420,465,564,582] Contrary to this are reports that E has a

hairpin conformation, placing both of its termini on the cytoplasmic face of membranes,[12,368] or that E can have multiple membrane topologies.[648] Also unresolved is the oligomeric state of E protein. The hydrophobic region of the SARS-CoV E protein forms multimers, from dimers through pentamers.[564,610] A pentameric alpha-helical bundle structure has been solved for this domain,[449] although it is not yet clear whether this reflects the organization of the native protein.

Residing in the interior of the virion, the N protein is the sole protein constituent of the helical nucleocapsid.[222] Monomers of this 43- to 50-kDa protein bind along the RNA genome in a beads-on-a-string configuration common to other helical viral nucleocapsids (see Fig. 28.2B). However, unlike the nucleoproteins of rhabdo- and paramyxoviruses, the coronavirus N protein provides little or no protection for its genome against the action of ribonucleases.[366,408] The bulk of the N protein monomer is made up of two independently folding domains—designated the N-terminal domain (NTD) and the C-terminal domain (CTD)—although neither includes its respective terminus of the N molecule (see Fig. 28.3). Crystal or solution structures have been determined for NTDs and CTDs of SARS-CoV, IBV, and MHV.[76,164,200,234,253,493,555,646] Flanking the NTD and CTD are three spacer segments, the central one of which contains a serine- and arginine-rich tract (the SR region), which was noted to resemble the SR domains of RNA-splicing factors.[442] Another functionally distinct region of N, the carboxy-terminal domain 3, has been defined genetically.[236,279,441,442] The spacer segments and domain 3 are each likely to be intrinsically disordered polypeptides.[66,67] Most of the N molecule, including the NTD and CTD, is highly basic; by contrast, domain 3 is acidic. There is only a moderate degree of sequence homology among N proteins across the three genera, with the exception of a stretch of 30 amino acids within the NTD that is highly conserved among all coronaviruses.[380]

The N protein is a phosphoprotein,[272,352,515,542] modified at a limited number of serine and threonine residues. Phosphorylation sites have been mapped for a representative coronavirus from each genus, and targeted sites, collectively, fall in every domain and spacer region of the N molecule.[55,77,604,619] Thus, a general pattern for N protein phosphorylation cannot yet be discerned, nor have all responsible kinases been identified, although there is evidence linking glycogen synthase kinase-3 to phosphorylation of the SR region.[619] The role of phosphorylation is also not known but is thought to have regulatory impact. Phosphorylation has been suggested to trigger a conformational change in N protein,[541] and it may enhance the affinity of N for viral versus nonviral RNA.[77]

The most conspicuous function of the N protein is to bind to viral RNA. Nucleocapsid formation must involve both sequence-specific and nonspecific modes of RNA binding. Specific RNA substrates that have been identified for N protein include the transcription-regulating sequence (TRS)[200,412,539] (see the Viral RNA Synthesis section) and the genomic RNA packaging signal[96,396] (see the Assembly and Release of Virions section). The NTD and the CTD are each separately capable of binding to RNA ligands *in vitro,* and the structures of these domains offer some clues as to how this is accomplished. The NTD consists of a U-shaped $\beta$-platform with an extruding $\beta$-hairpin, which presents a putative RNA-binding groove rich in basic and aromatic amino acid residues.[164,200,493] The CTD forms a tightly interconnected dimer, which exhibits a potential RNA-binding groove lined by basic $\alpha$-helixes.[253,555] Some work suggests that in the intact N protein, optimal RNA binding requires concerted contributions from both the NTD and the CTD.[67,235] A significant fraction of nucleocapsid stability also results from interactions among N monomers.[408] This level of association is generally attributed to the CTD[67,164,253,646]; however, additional regions of N–N interaction have been mapped to the NTD and to domain 3.[164,235,253] Another crucial function of N protein is to bind to M protein.[162,546] This capability is provided by domain 3 of N.[236,295,585]

A fifth prominent structural protein—the hemagglutinin-esterase (HE) protein—is found in only a subset of the betacoronaviruses, including murine coronavirus, betacoronavirus 1, and HCoV-HKU1. In virions of these species, HE forms a secondary set of short projections of 5 to 10 nm arrayed beneath the canopy of S protein spikes.[204,435,550] The 48-kDa HE monomer is composed almost entirely of an N-terminal ectodomain; this is followed by a transmembrane anchor and a very short C-terminal endodomain (see Fig. 28.3). HE is inserted into the ER by means of a cleaved signal peptide and acquires an additional 17 kDa of N-linked glycosylation at multiple sites.[221,271,640] The assembled protein is a homodimer, the subunits of which are connected by disulfide bonds.[221] As its name indicates, the HE protein contains a pair of associated activities. First, it is a hemagglutinin—that is, it has the capability to bind to sialic acid moieties found on cell surface glycoproteins and glycolipids.[54,272] Second, HE exhibits acetylesterase activity with specificity for either 9-*O*- or 4-*O*-acetylated sialic acids.[274,472,520,590,591] These characteristics are thought to allow HE to act as a cofactor for S protein, assisting attachment of virus to host cells, as well as expediting the travel of virus through the extracellular mucosa.[99] Consistent with this notion, the presence of HE in MHV dramatically enhances neurovirulence in the mouse host.[265] Conversely, the HE protein is a burden to the virus in tissue culture, where its expression is rapidly counterselected.[343] The two activities of the HE protein are strikingly similar to the receptor-binding and receptor-destroying activities found in influenza C virus,[590,591] and, remarkably, the coronavirus *HE* gene is clearly related to the influenza C virus *HEF* gene.[359] Moreover, toroviruses also possess a homolog of the *HE* gene,[99,305] raising the possibility that all three of these virus groups evolved from a common ancestor.[359,522] This kinship is further corroborated by the crystal structure of the BCoV HE protein, which reveals separate receptor-binding and acetylesterase domains perched atop a truncated membrane-proximal region.[650] The HE protein thus resembles a squat version of its influenza virus counterpart, shortened because it lacks the fusion domain stalk of the HEF protein.

## GENOME STRUCTURE AND ORGANIZATION

### Basic and Accessory Genes

The coronavirus genome, which ranges from 26 to 32 kb, is the largest among all RNA viruses, including RNA viruses that have segmented genomes. This exceptional RNA molecule acts in at least three capacities[50,194]: as the initial mRNA of the infectious cycle (see the Expression of the Replicase-Transcriptase Complex section), as the template for RNA replication and transcription (see the Viral RNA Synthesis section), and as the substrate for packaging into progeny viruses (see

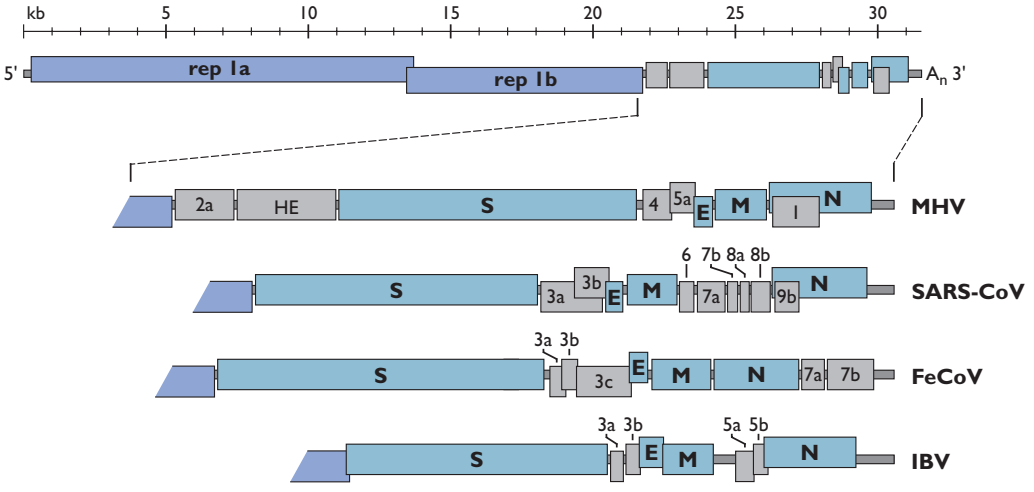



**FIGURE 28.4. Coronavirus genome organization.** A schematic of the complete genome of MHV is shown at the top. The replicase gene constitutes two ORFs, rep 1a and rep 1b, which are expressed by a ribosomal frameshifting mechanism (see the Expression of the Replicase-Transcriptase Complex section). The expanded region shows the downstream portion of the genomes of two betacoronaviruses (MHV and SARS-CoV), an alphacoronavirus (FeCoV), and a gammacoronavirus (IBV). The sizes and positions of accessory genes are indicated, relative to the basic genes *S*, *E*, *M*, and *N*. MHV, mouse hepatitis virus; ORFs, open reading frames; SARS-CoV, severe acute respiratory syndrome coronavirus; FeCoV, feline coronavirus; IBV, infectious bronchitis virus.

the Assembly and Release of Virions section). Consistent with its role as an mRNA, the coronavirus genome has a standard eukaryotic 5′-terminal cap structure[301] and a 3′ polyadenylate tail.[302,351,503,599] The genome comprises a basic set of genes in the invariant order 5′-*replicase-S-E-M-N-3′*, with the huge *replicase* gene occupying two-thirds of the available coding capacity (Fig. 28.4). The replicase-transcriptase is the only protein translated from the genome; the products of all downstream open reading frames (ORFs) are derived from subgenomic mRNAs. The 5′-most position of the *replicase* gene is dictated by the requirement for expression of the replicase to set in motion all subsequent events of infection. The organization of the other basic genes, however, does not seem to reflect any underlying principle, because engineered rearrangement of the downstream gene order is completely tolerated.[121]

Dispersed among the basic genes in the 3′-most third of the genome, there are from one to as many as eight additional ORFs, which are designated accessory genes[378,407] (see Fig. 28.4). These can fall in any of the intergenic intervals downstream of the *replicase* gene,[616] except, curiously, never between the *E* and *M* genes. In some cases, an accessory gene can be partially or entirely embedded as an alternate reading frame within another gene—for example, the internal (*I*) gene of MHV or the *3b* gene of SARS-CoV. Accessory genes are generally numbered according to the smallest transcript in which they fall. Consequently, there is usually no relatedness among identically named accessory genes in coronaviruses of different genera, such as the *3a* genes of SARS-CoV, feline coronavirus (FeCoV), and IBV (see Fig. 28.4). Some of these extra ORFs are thought to have been acquired through ancestral recombination with RNA from cellular or heterologous viral sources. The *HE* gene is the best-supported example of this type of horizontal genetic transfer.[359] Two other such candidates are the *2a* gene found in murine coronavirus and betacoronavirus 1, which encodes a putative 2′,3′-cyclic phosphodiesterase,[385,485] and gene *10* of beluga whale coronavirus, which encodes a putative uridine-cytidine kinase.[394] Notably, the *2a* gene has a homolog embedded as a module within the *replicase* gene of the toroviruses,[522] which is a situation also consistent with horizontal transfer. The origin of most accessory genes, however, remains an open question. It is plausible that some of them evolved through intragenomic recombination, resulting in gene duplication and subsequent divergence, as suggested for several of the accessory genes of SARS-CoV.[241]

Almost all accessory genes that have been examined are expressed during infection, although their functions are incompletely understood. The protein products of most accessory genes are nonstructural; however, this rule is not without exception. The HE protein, the MHV I protein,[165] and the products of SARS-CoV ORFs 3a, 6, 7a, 7b, and 9b[231,407,502,627] are all components of virions. Mutational knockout or deletion of accessory genes has revealed that none are essential for viral replication in tissue culture. Conversely, accessory gene ablation,[103,115,206] or transfer to another virus,[452,559] can have profound effects on viral pathogenesis. In some cases, the basis for this is understood to result from interactions with host innate immunity (see the Immune Response and Viral Evasion of the Immune Response section). For other accessory genes, though, potential *in vivo* functions have not yet been elucidated.[125,165,645]

## Coronavirus Genetics

Classical coronavirus genetics focused principally on two types of mutants.[299] The first were naturally arising viral variants, particularly deletion mutants, which offered clues to genetic changes responsible for different pathogenic traits.[430,583,603] The second were temperature-sensitive (*ts*) mutants isolated from MHV following chemical mutagenesis.[282,477,501,545] Some of these proved to be valuable in analyses of the functions of structural proteins.[279,360,380,474] However, owing to the large target size of the *replicase* gene, most of such randomly generated mutants

had conditional-lethal, RNA-negative phenotypes. Complementation analyses of these latter mutants yielded early insights into the multiplicity of functions entailed by coronavirus RNA synthesis.[22,176,177,501] There has been a recent resurgence of interest in classical replicase *ts* mutants, which are currently sorted into five complementation groups, because they can now be fully examined by the tools of reverse genetics.[138,499,543]

The development of coronavirus reverse genetics proceeded in two phases.[130] Initially, a method called *targeted RNA recombination* was devised at a time when it was uncertain whether the construction of full-length infectious complementary DNA (cDNA) clones of coronavirus genomes would ever become technically feasible. With this method, a synthetic donor RNA bearing mutations of interest is transfected into cells that have been infected with a recipient parent virus possessing some characteristic that can be selected against.[279,377,380] In its current form, for manipulation of MHV, the technique uses a chimeric recipient parent virus designated fMHV (Fig. 28.5A). The fMHV chimera is a mutant of MHV that contains the S protein ectodomain from the FeCoV feline infectious peritonitis virus (FIPV) and can therefore only grow in feline cells (see the Virion Attachment to Host Cells section). The restoration of its ability to grow in murine cells, via recombination with donor RNA containing the MHV *S* gene, enables a strong selection for viruses bearing site-specific mutations[292,381]; unwanted secondary crossover events distal to the *S* gene are eliminated owing to the rearrangement of downstream genes in fMHV.[190] Targeted RNA recombination remains a powerful method to recover structural or accessory protein or 3′ untranslated region (UTR) mutants.

To obtain access to the major part of the coronavirus genome, however, it was necessary to create full-length cDNAs, despite the barriers presented by the huge size of the *replicase* gene and the high instability of various regions when propagated in bacterial clones. Three innovative strategies were developed to overcome these inherent difficulties.[130] In the first (see Fig. 28.5B), a full-length cDNA copy of a coronavirus genome is assembled downstream of a cytomegalovirus (CMV) promoter in a bacterial artificial chromosome (BAC) vector, which is stable by virtue of its low copy number.[5,6] The infection is then launched from transfected BAC DNA through transcription of infectious coronavirus RNA by host RNA polymerase II. This method of initiating infection obviates potential limitations of *in vitro* capping and synthesis of genomic RNA. In the second strategy (see Fig. 28.5C), a full-length genomic cDNA is assembled by *in vitro* ligation of smaller cloned cDNA fragments, some of the boundaries of which have been chosen so as to interrupt regions of instability.[642,643] The ligation occurs in a directed order that is dictated by the use of asymmetric restriction sites. Infectious genomic RNA is then transcribed *in vitro* and used to transfect susceptible host cells. An extension of this method has demonstrated the construction of a coronavirus genome entirely from synthetic cDNAs.[26] In the third strategy (see Fig. 28.5D), the genome of vaccinia virus is used as the cloning vector for a full-length coronavirus cDNA that is generated by long-range reverse transcription polymerase chain reaction (RT-PCR).[94,561] The cDNA is then amenable to manipulation by the repertoire of techniques available for poxvirus reverse genetics.[51,94] Infections are launched from *in vitro*–synthesized RNA or else from transfected cDNA

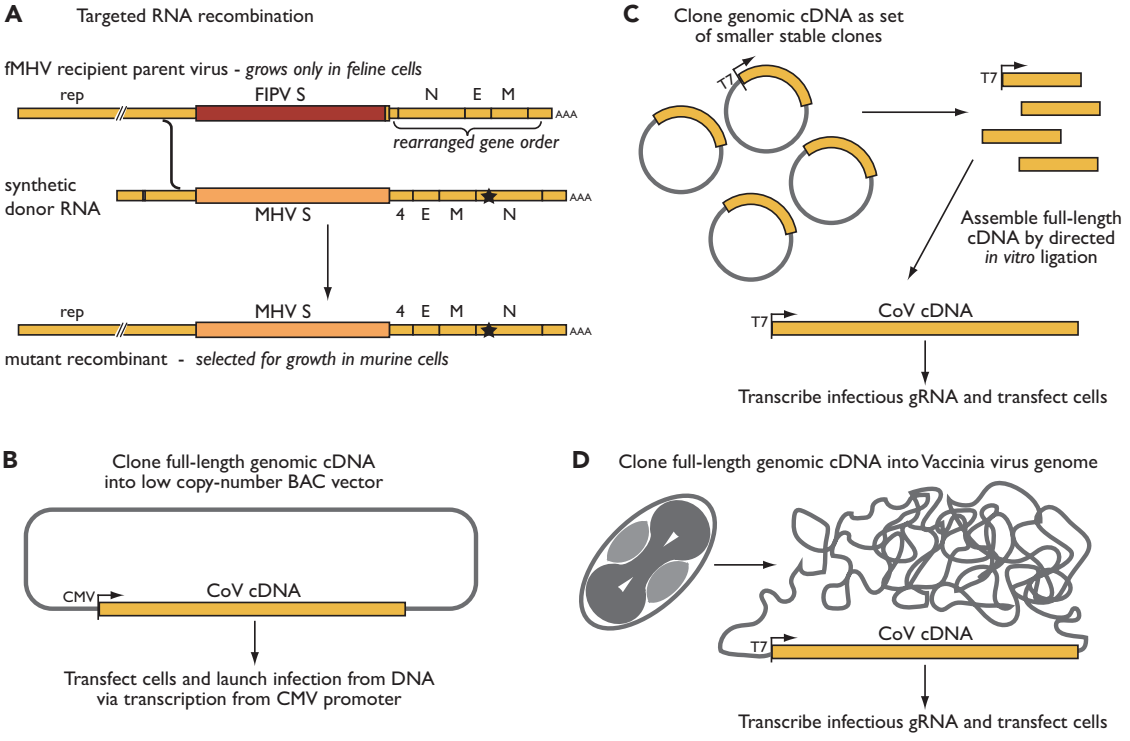



**FIGURE 28.5. Methods for coronavirus reverse genetics. A:** Targeted RNA recombination, which is applicable to the downstream third of the genome, shown here for transduction of a mutation (*star*) into the mouse hepatitis virus *N* gene. **B–D:** Three schemes developed for complete reverse genetics, based on stable production of full-length genomic complementary DNAs.

transcribed *in vivo* by fowlpox-encoded T7 RNA polymerase.[58] Collectively, these systems developed for complete reverse genetics provide an important pathway toward unraveling the complexities of the coronavirus replicase.

## CORONAVIRUS REPLICATION

### Virion Attachment to Host Cells

Coronavirus infections are initiated by the binding of virions to cellular receptors (Fig. 28.6). There then follows a series of events culminating in the delivery of the nucleocapsid to the cytoplasm, where the viral genome becomes available for translation. Individual coronaviruses usually infect only one or a few closely related hosts. The interaction between the viral S protein and its cognate receptor constitutes the principal determinant governing coronavirus host species range and tissue tropism. This has been most convincingly shown in two ways. First, the expression of a particular receptor in nonpermissive cells of a heterologous species renders those cells permissive for the corresponding coronavirus.[127,146,330,331,399,567,639] Second, the engineered replacement of the S protein ectodomain changes the host cell species specificity or tissue tropism of a coronavirus in a predictable fashion.[207,292,410,453,495] The amino-terminal, more variable half of the spike protein, S1, is the part that binds to receptor. Binding leads to conformational changes that result in fusion between virion and cell membranes, mediated by the more conserved half of the spike protein, S2. The region of S1 that contacts the receptor—the receptor-binding domain (RBD)—varies among different coronaviruses (see Fig. 28.3). For MHV, the RBD maps to the N-terminal section of S1.[290,554] By contrast, RBDs for SARS-CoV,[614,625] HCoV-NL63,[337] transmissible gastroenteritis virus (TGEV),[188] and HCoV-229E[34] fall in the middle or C-terminal sections of S1.

The known cellular receptors for alpha- and betacoronaviruses are listed in Table 28.2; to date, no receptors have been identified for gammacoronaviruses. The MHV receptor mCEACAM1 was the first discovered coronavirus receptor (as well as one of the first receptors defined for *any* virus).[606,607] That this molecule is the only biologically relevant receptor for MHV was made clear by the demonstration that homozygous *Ceacam1*$^{-/-}$ knockout mice are totally resistant to infection by high doses of MHV.[215] CEACAM1 is a member of the carcinoembryonic antigen (CEA) family within the immunoglobulin (Ig) superfamily and, in its full-length form, contains four Ig-like domains.[146] A diversity of two- and four-Ig domain isoforms is generated by multiple alleles and alternative splicing variants of *Ceacam1*.[97,145,147,422,423,641] The wide range of pathogenicity of MHV in mice is thought to be strongly affected by the interactions of S proteins of different virus strains with the array of receptor isoforms that are expressed in mice of different genetic backgrounds. Although their S proteins are phylogenetically very close to that of MHV, the betacoronaviruses BCoV and HCoV-OC43 do not use CEACAMs to infect their

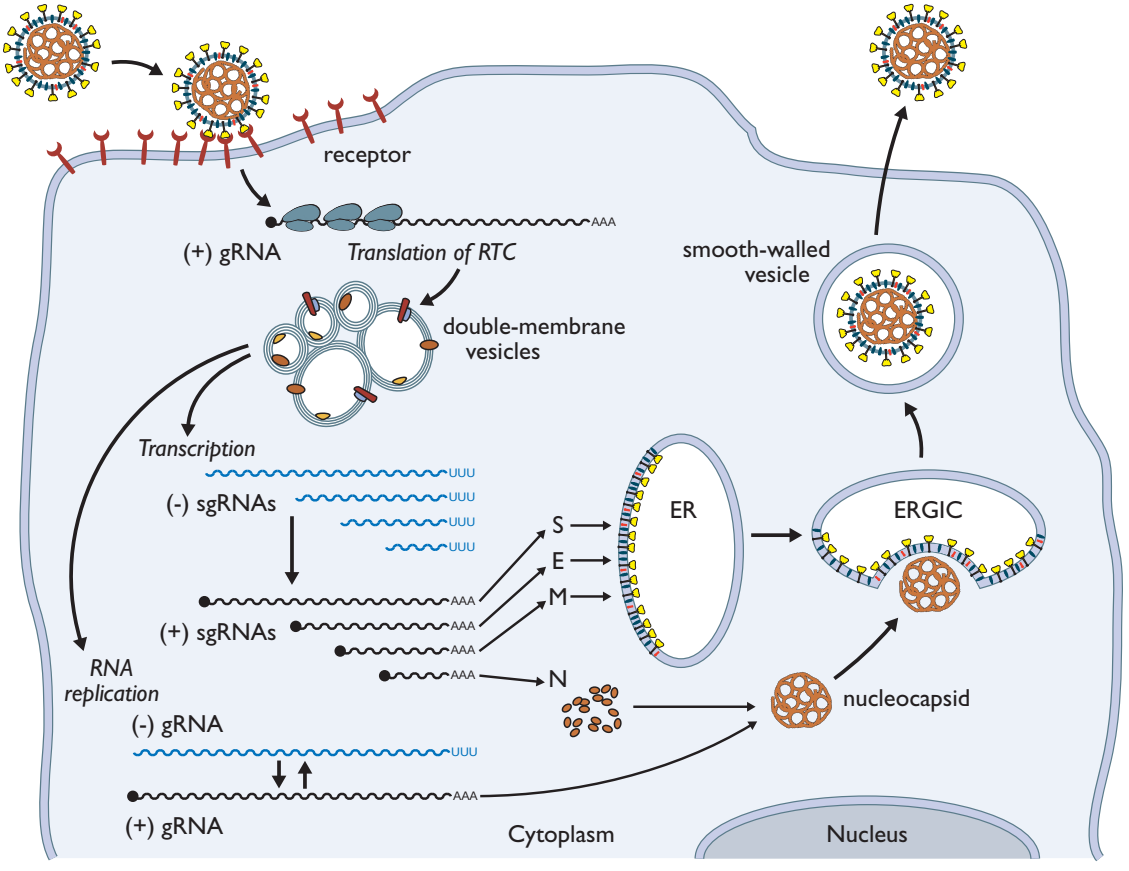



**FIGURE 28.6. Overview of coronavirus replication (see text for details).**

**TABLE 28.2 Coronavirus Receptors**

| Virus | Receptor | References |
|---|---|---|
| **Alphacoronaviruses** | | |
| TGEV | pAPN[a] | 127 |
| PRCoV | pAPN | 128 |
| PEDV | pAPN | 322 |
| FeCoV II, FIPV | fAPN[b] | 567 |
| FeCoV I | Unknown, but *not* fAPN[b] | 148,223 |
| CCoV | cAPN | 29 |
| HCoV-229E | hAPN | 639 |
| HCoV-NL63 | ACE2 | 219 |
| **Betacoronaviruses** | | |
| MHV | mCEACAM1[c] | 411,606 |
| BCoV | N-acetyl-9-*O*-acetylneuraminic acid | 504 |
| HCoV-OC43 | N-acetyl-9-*O*-acetylneuraminic acid | 291 |
| SARS-CoV | ACE2[d] | 331 |

TGEV, transmissible gastroenteritis virus; pAPN, porcine aminopeptidase N; PRCoV, porcine respiratory coronavirus; PEDV, porcine epidemic diarrhea virus; FeCoV, feline coronavirus; fAPN, feline aminopeptidase N; FIPV, feline infectious peritonitis virus; CCoV, canine coronavirus; cAPN, canine aminopeptidase N; HCoV, human coronavirus; hAPN, human aminopeptidase N; ACE2, angiotensin-converting enzyme 2; MHV, mouse hepatitis virus; mCEACAM1, murine carcinoembryonic antigen–related adhesion molecule 1; BCoV, bovine coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus.

[a]Mammalian aminopeptidase N is also known as CD13.

[b]Although the receptor for FeCoV I remains to be identified, the lectin fDC-SIGN serves as a coreceptor for both FeCoV I and FeCoV II.[471]

[c]The related molecule mCEACAM2 functions weakly as an MHV receptor in tissue culture; however, it is not an alternate receptor in the mouse host *in vivo*.[215]

[d]Human CD209L (L-SIGN), a lectin family member, can also act as a receptor for SARS-CoV but with much lower efficiency than ACE2[254]; a related lectin, DC-SIGN, can serve as a coreceptor.[376,635]

hosts; rather, the only currently known attachment factor for these viruses is N-acetyl-9-*O*-acetylneuraminic acid.[291,504] The recently solved structure of the MHV RBD complexed with mCEACAM1 has allowed the identification of key residues at the S protein–receptor interface.[443] Coupled with mutational analysis, this structure reveals why the S proteins of BCoV and HCoV-OC43 cannot bind the MHV receptor and, conversely, why MHV does not bind to bovine or human CEACAMs.

Many alphacoronaviruses use aminopeptidase N (APN) of their respective host species as a receptor (see Table 28.2).[127,567,639] APN (also called CD13) is a cell-surface, zinc-binding protease that is resident in respiratory and enteric epithelia and in neural tissue. The APN molecule is a heavily glycosylated homodimer. Mutational and inhibitor studies have shown that its enzymatic activity is not required for viral attachment and entry.[126] In general, the receptor activities of APN homologs are not interchangeable among species[126,281]; however, feline aminopeptidase N (fAPN) can serve as a receptor not only for FIPV but also for canine coronavirus (CCoV), TGEV, and HCoV-229E.[567] This circumstance has been exploited for the construction of chimeric APN molecules to map the basis for receptor recognition. Such studies have found three small, linearly discontinuous determinants in APN that govern the species specificity of this subgroup of alphacoronaviruses.[29,214,280,569]

The receptor for SARS-CoV—angiotensin-converting enzyme 2 (ACE2)—was discovered with notable rapidity following the isolation of the virus.[331] ACE2 is a cell-surface, zinc-binding carboxypeptidase involved in regulation of cardiac function and blood pressure. It is expressed in epithelial cells of the lung and the small intestine, which are the primary targets of SARS-CoV, as well as in heart, kidney, and other tissues.[209] As with APN, the receptor role of ACE2 appears to be independent of its enzymatic activity. Although the SARS-CoV S protein binds to the catalytic domain of ACE2, active-site mutation or chemical inhibition does not detectably affect the ability of ACE2 to associate with S protein or to promote syncytia formation.[331,333,398] The crystal structure of the SARS-CoV S protein RBD in complex with ACE2 shows the RBD cradling one lobe of the claw-like catalytic domain of its receptor.[325] Remarkably, ACE2 also serves as the receptor for the alphacoronavirus HCoV-NL63,[219] and the corresponding structural complex for that virus reveals that the HCoV-NL63 RBD and the SARS-CoV RBD bind to the same motifs.[624] Because the SARS-CoV and HCoV-NL63 RBDs have neither sequence nor structural homology, this finding strongly supports the notion that they have independently evolved to bind to the same hotspot on the ACE2 surface.[623,624] Analyses of the SARS-CoV RBD–ACE2 interface have additionally demonstrated the structural basis for the final jump of SARS-CoV from palm civets to human hosts (see the Epidemiology section). These studies found that merely four critical residues constitute the major species barrier between the civet and human ACE2 molecules, and that mutation of only two key RBD residues was sufficient for civet SARS-CoV S protein to gain the ability to productively bind human ACE2.[323,333]

## Viral Entry and Uncoating

The entry of virions into cells results from large-scale rearrangements of the S protein that lead to the fusion of viral and cellular membranes.[41] These rearrangements are triggered by some combination of receptor binding, proteolytic cleavage of S, and exposure to acidic pH. The S proteins of many coronaviruses are uncleaved in mature virions and require an encounter with a protease at the entry step of infection to separate the receptor-binding and fusion components of the spike. The details of proteolytic activation are still incompletely understood but have been best studied for SARS-CoV. In the cell types in which this virus is most commonly grown in tissue culture, viral entry depends on cathepsins, which are acid-activated endosomal proteases. The infectivity of SARS-CoV is thus suppressed by cathepsin inhibitors or by lysosomotropic agents.[517] However, cell-bound SARS-CoV can alternatively be activated by treatment with extracellular proteases, such as trypsin or elastase. This route of activation greatly enhances the infectivity of SARS-CoV and allows the virus to enter from the cell surface, thereby rendering the infection insensitive to lysosomotropic agents.[383] The same pattern of proteolytic activation—cathepsin-dependence and its circumvention by exogenously added protease—is observed with a particular strain of MHV (MHV-2) that is unique in having an uncleaved S protein.[464]

The site of cleavage of receptor-bound SARS-CoV S protein by cathepsin or by exogenous trypsin differs from that of the S1-S2 cleavage, which occurs in other coronaviruses upon exit from cells. Cleavage at entry takes place at a locus (S2′) within the S2 half of the molecule, immediately upstream of the putative fusion peptide[28] (see Fig. 28.3). It is not yet clear if cleavage at analogous S2′ sites is the pattern for all coronavirus S proteins; however, the emerging pattern is that proteolytic activation of S protein is required for infectivity and that coronaviruses have evolved in different ways to ensure that this occurs.[41] Recent studies provide evidence that for the SARS-CoV S protein, the most biologically relevant protease may be TMPRSS2.[187,382,514] This transmembrane serine protease, which is expressed in pneumocytes, co-localizes with and binds to ACE2. In cells expressing TMPRSS2, SARS-CoV enters at the cell surface and is insensitive to cathepsin inhibitors and lysosomotropic agents.

Just as the mechanism of S protein proteolytic activation is variable, so too is its location. Some coronaviruses, such as most strains of MHV, fuse with the plasma membrane,[547,601] whereas others, such as TGEV,[212] HCoV-229E,[421] and SARS-CoV,[517] can enter cells through receptor-mediated endocytosis and then fuse with the membranes of acidified endosomes. The boundary between these two modes of entry may easily shift. For one strain of MHV (MHV-4), as few as three amino acid changes in the heptad repeat region of S2 switches the virus from plasma membrane fusion to acid pH-dependent fusion.[180] It remains unresolved whether acidic pH, *per se,* is required for S protein conformational changes[90,154,324] or whether this reflects the requirements for activation of endosomal proteases during infection of some types of cells.[517]

The coronavirus S protein is a class I viral fusion protein with domains functionally similar to those of the fusion proteins of phylogenetically distant RNA viruses, such as influenza virus, human immunodeficiency virus (HIV), and Ebola virus, but on a much larger scale.[41,42] As in those other viral fusion proteins, the coronavirus S2 moiety contains two separated heptad repeats—HR1 and HR2—with a fusion peptide upstream of HR1 and the transmembrane domain immediately downstream of HR2 (see Fig. 28.3). The exact assignment of the fusion peptide is not agreed upon, however.[41,367,450] Receptor-mediated conformational changes in S1, and the dissociation of S1 from S2, are thought to initiate major rearrangements in the remaining S2 trimer that proceed through multiple intermediate states.[133,324] These rearrangements ultimately expose the fusion peptide, which interacts with the host cellular membrane, and the two heptad repeats in each monomer are brought together to form an antiparallel, six-helix bundle. The six-helix bundle is an extremely stable, rod-like complex, the biophysical properties of which have been extensively studied.[40,42,242,348,568] Highly similar crystallographic structures have been solved for the six-helix complexes from both the MHV S protein[629] and the SARS-CoV S protein.[144,552,630] These show the three HR1 helices forming a central, coiled-coil core some two to three times larger than its counterparts in other viruses. Arrayed around this, the three shorter HR2 helices, in an antiparallel orientation, pack into the grooves between the HR1 monomers via hydrophobic interactions. The outcome of the formation of the six-helix bundle is the juxtaposition of the viral and cellular membranes in sufficient proximity to allow mixing of their lipid bilayers and the deposition of the contents of the virion into the cytoplasm.

## Expression of the Replicase-Transcriptase Complex

Following delivery of the viral nucleocapsid to the cytoplasm, the next event is the translation of the *replicase* gene from the genomic RNA. This gene consists of two large ORFs—rep 1a and rep 1b—that share a small region of overlap (see Fig. 28.4). Translation of the entire replicase depends on a mechanism called *ribosomal frameshifting,* whereby, with a fixed probability, a translating ribosome shifts one nucleotide in the –1 direction, from the rep 1a reading frame into the rep 1b reading frame.[378] This repositioning is programmed by two RNA elements (Fig. 28.7A), embedded near the region of overlap, that were discovered in studies of IBV.[46,47] The first element is the 5′-UUUAAAC-3′ heptanucleotide slippery sequence, which is identical for all known coronaviruses and has apparently been selected as optimal for its role.[48,457] The second element, located a short distance downstream of the slippery sequence, is an extensively characterized RNA pseudoknot structure.[49,405] This latter component was initially thought to be a classic two-stem (H-type) pseudoknot; however, recent analyses of SARS-CoV frameshifting support a more elaborate structure that includes a third stem loop within pseudoknot loop 2.[20,141,456]

The two elements act together to produce the coterminal polyprotein products pp1a and pp1ab. During most rounds of translation, the elongating ribosome unwinds the pseudoknot and translation terminates at the rep 1a stop codon, yielding the smaller product, pp1a. Some fraction of the time, however, the pseudoknot blocks the mRNA entrance channel of the ribosome.[213,403,528] The consequent pause required for the ribosome to melt out the mRNA structure allows the simultaneous slippage of the P and A site transfer RNAs (tRNAs) into the rep 1b reading frame. This results in the synthesis of pp1ab when elongation resumes.[20,47] Studies of reporter gene expression suggest that the incidence of coronaviral ribosomal frameshifting is as high as 25% to 30%; however, the *in vivo* frequency in infected cells remains to be quantitated. It is thought that the role of programmed frameshifting is to provide a fixed ratio of translation products for assembly into a macromolecular complex.[457] It is also possible that frameshifting forestalls expression of the enzymatic products of rep 1b until the products of rep 1a have prepared a suitable environment for RNA synthesis.

Polyproteins pp1a (440–500 kDa) and pp1ab (740–810 kDa) are autoproteolytically processed into mature products that are designated nsp1 to nsp16 (except for the gammacoronaviruses, which do not have a counterpart of nsp1). From work begun with early studies of MHV,[134,135,525] complete processing schemes have now been solved for replicases of multiple coronaviruses representing all three genera[659,661] (see Fig. 28.7B). Processing also generates many long-lived partial proteolytic products, which may have functional importance. There are two types of polyprotein cleavage activity.[17,358] One or two papain-like proteases ($PL^{pro}$), which are situated in nsp3, carry out the relatively specialized separation of nsp1, nsp2, and nsp3. The main protease ($M^{pro}$)—nsp5—performs the remaining 11 cleavage events. $M^{pro}$ is often designated the 3C-like protease ($3CL^{pro}$) to point out its distant relationship to the 3C proteins of picornaviruses. Several crystal structures have been determined for $PL^{pro}$ and $M^{pro}$ of SARS-CoV and other

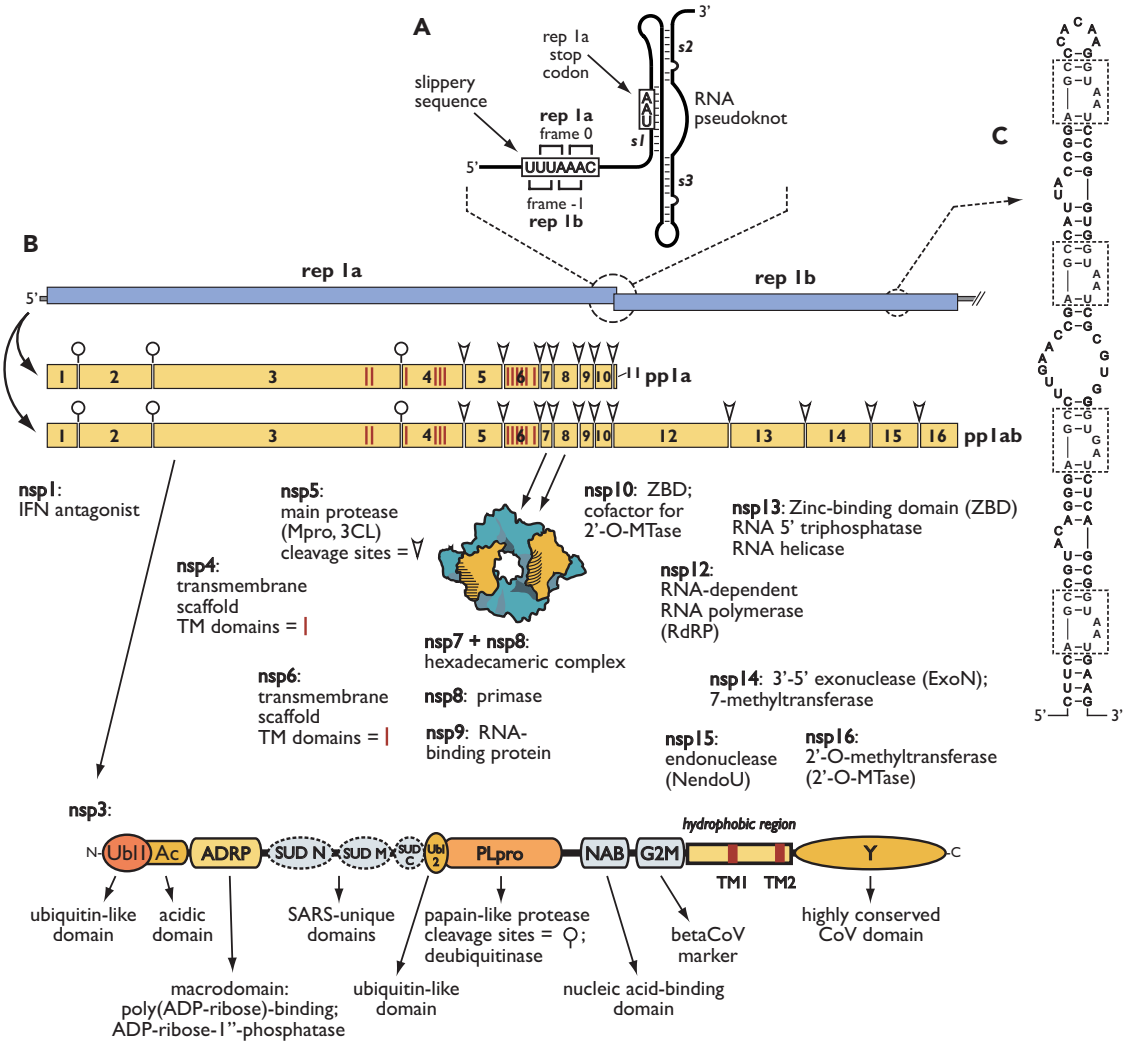



**FIGURE 28.7. Coronavirus replicase gene and protein products. A:** Ribosomal frameshifting elements of the SARS-CoV *replicase* gene. Pseudoknot stems are indicated as s1, s2, and s3. **B:** Polyprotein pp1a and pp1ab processing scheme for alpha- and betacoronaviruses. The gammacoronavirus processing scheme is identical, except for the absence of nsp1. Known functions and properties of nsp1 through nsp16 are listed; nsp11 is an oligopeptide generated when ribosomal frameshifting does not occur. Transmembrane domains in nsp3, nsp4, and nsp6 are indicated by *red vertical lines*. The nsp3 schematic shown is for SARS-CoV[414]; some modules differ in other coronaviruses. **C:** The RNA packaging signal located in the nsp15-encoding region of the MHV genome.[81] This element is found only in a subset of the betacoronaviruses (MHV, betacoronavirus 1, and HCoV-HKU1); repeat units are boxed. SARS-CoV, severe acute respiratory syndrome coronavirus; MHV, mouse hepatitis virus; HCoV, human coronavirus.

coronaviruses,[9,469,612,631] and these enzymes present attractive targets for antiviral drug design.[468,633,634]

The processed nsps assemble to form the coronavirus replicase, which is also referred to as the replicase-transcriptase complex (RTC).[660] The challenge of defining the roles of the many nsp components of the RTC was initially addressed by foundational studies in bioinformatics,[196,317] which is a discipline that continues to inform the analysis of this intricate molecular machinery.[414,521] Besides $PL^{pro}$ and $M^{pro}$, the products of rep 1a contain several activities that establish cellular conditions favorable for infection. Some of these are directly linked to RNA synthesis. Others are nonessential for viral replication in tissue culture; however, they can have major effects on virus–host interactions (see the Immune Response and Viral Evasion of the Immune Response section). The very first polyprotein product—nsp1—exhibits a broad repertoire of antagonistic activities that selectively inhibit host protein synthesis and IFN signaling.[230,258,259] By contrast, nsp2 is completely expendable and, as yet, has no demonstrated function.[199]

Nsp3 is by far the largest of the RTC proteins. It consists of a concatenation of individual structural modules that are arranged as globular domains separated by flexibly disordered linkers[414] (see Fig. 28.7B). At the amino terminus of nsp3 are ubiquitin-like (Ubl1) and acidic (Ac) domains[506] that interact with the SR region of the N protein.[237] It is proposed that this interaction tethers the genome to the assembling RTC to allow formation of the initiation complex for RNA synthesis. As mentioned earlier, located within nsp3 are one (in SARS-CoV and

gammacoronaviruses) or two $PL^{pro}$ modules (in most other coronaviruses). In addition to protease activity, $PL^{pro}$ domains possess deubiquitinase activity,[341,469,612] which forms another part of the viral arsenal that counters host innate immunity.[136,174] A highly conserved domain of nsp3 has adenosine diphosphate-ribose-1″-phosphatase (ADRP) and poly(adenosine diphosphate [ADP]-ribose)-binding activities,[152,494] which, although nonessential for replication, help confer resistance to host defenses.[158,297] At the C-terminus of nsp3 is a conserved region, designated the Y domain, containing three metal-binding clusters of cysteine and histidine residues.[414,662] The potential functions of other domains of nsp3 (NAB, G2M, SUD),[73,414,507,521] which appear only in various subsets of coronaviruses, remain to be elucidated.

Notably, the rep 1a products nsp3, nsp4, and nsp6 each contain multiple transmembrane helices that anchor the RTC to intracellular membranes.[262,424] These proteins also appear to be responsible for remodeling cellular membranes to form structures that are dedicated to viral RNA synthesis.[92,178] Recent cryo-electron tomographic imaging has revealed an extensive network of convoluted membranes, double-membrane vesicles (DMVs), and vesicle packets, all continuous with the ER, induced by coronavirus infection[277] (Fig. 28.8). Anchorage and compartmentalization of the RTC are thought to provide a scaffold for recruitment of soluble nsps, to offer protection from ribonucleases, and to sequester double-stranded viral RNA intermediates that might activate host innate immunity (see the Immune Response and Viral Evasion of the Immune Response section).

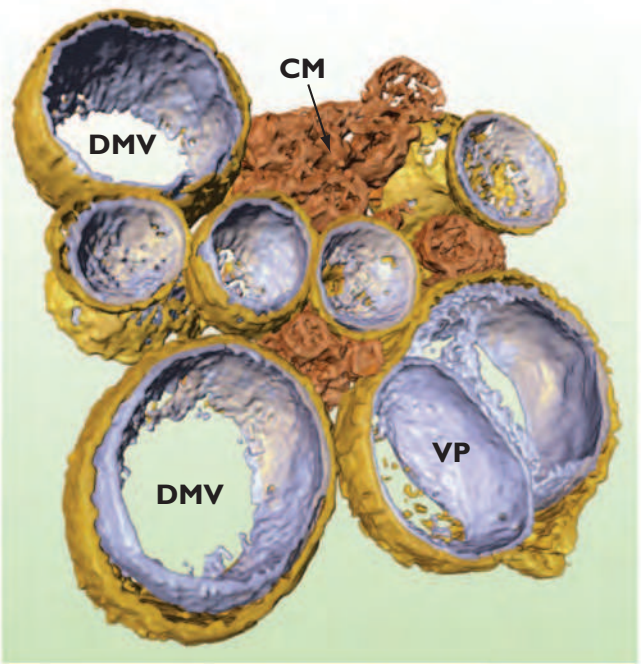



**FIGURE 28.8. Membranous compartments for RNA replication and transcription induced by coronavirus infection.** Shown is a cryo-electron tomographic reconstruction of the network of intracellular membrane rearrangements found in SARS-CoV-infected Vero cells. There are three types of structures: convoluted membranes (*CM*), which are the major sites of nsp accumulation; double-membrane vesicles (*DMV*), which appear to be the sites of active RNA synthesis; and vesicle packets (*VP*), which are formed by the merger of DMV. (From Knoops K, Kikkert M, Worm SH, et al. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol* 2008;6:e226.)

The most C-terminal rep 1a products are nsp7 through nsp10, a cluster of essential small proteins.[131] Structural studies have revealed that two of these—nsp7 and nsp8—form a hexadecameric supercomplex with a central channel large enough to accommodate double-stranded RNA.[651] This formidable assembly has thus been proposed to act as a processivity clamp for the RNA polymerase. Nsp9 is a single-stranded RNA-binding protein,[151,553] and nsp10 defines a novel structural class of zinc finger proteins.[257,548]

The processed products encoded by rep 1b contain several well-studied enzymatic activities, including many that are common to all positive-strand RNA viruses. Most prominent in this latter class is the coronavirus RNA-dependent RNA polymerase (RdRp), which is contained in nsp12. Sequence alignment and homology modeling indicate that nsp12 has the fingers, palm, and thumb domains characteristic of several viral RdRps and reverse transcriptases[628]; however, to date, this protein has proven refractory to structural determination. Additionally, nsp12 has an unusually large NTD, at least part of which mediates targeting to the RTC.[52] Coronavirus RdRp activity, *in vitro,* is primer dependent.[83,560] Remarkably, a second RdRp activity resides in nsp8 and is capable of synthesizing short RNA oligomers.[240] Nsp8 is thus the optimal candidate for the requisite primase. Another enzyme crucial to RNA synthesis is the helicase of nsp13. This activity unwinds RNA duplexes with a 5′ to 3′ polarity, suggesting that its role is to prepare the template ahead of the RdRp.[247,248] The nsp13 helicase has an amino-terminal zinc finger domain that is found only in nidoviruses.[510]

Like many RNA viruses, coronaviruses contain machinery capable of catalyzing multiple steps of the pathway for synthesis of the 5′-terminal cap structure of mRNA. An RNA 5′-triphosphatase, which would be required for the first step, is yet another property of nsp13.[247,248] Intriguingly, a guanylyltransferase has thus far not been identified among the nsps. The nsp14 C-terminus and nsp16, respectively, harbor N7-methyltransferase and 2′-*O*-methyltransferase activities.[82,123] These enzymes operate in an obligatory sequential manner, with guanosine-N7 methylation preceding ribose-2′-*O* methylation. Activation of the nsp16 methyltransferase requires nsp10 as a cofactor, and the crystal structure of a heterodimer of these two proteins suggests that nsp10 serves as a platform to stabilize nsp16.[43,122] Genetic evidence also implicates nsp10 as a regulator of polyprotein processing by the nsp5 $M^{pro}$.[138]

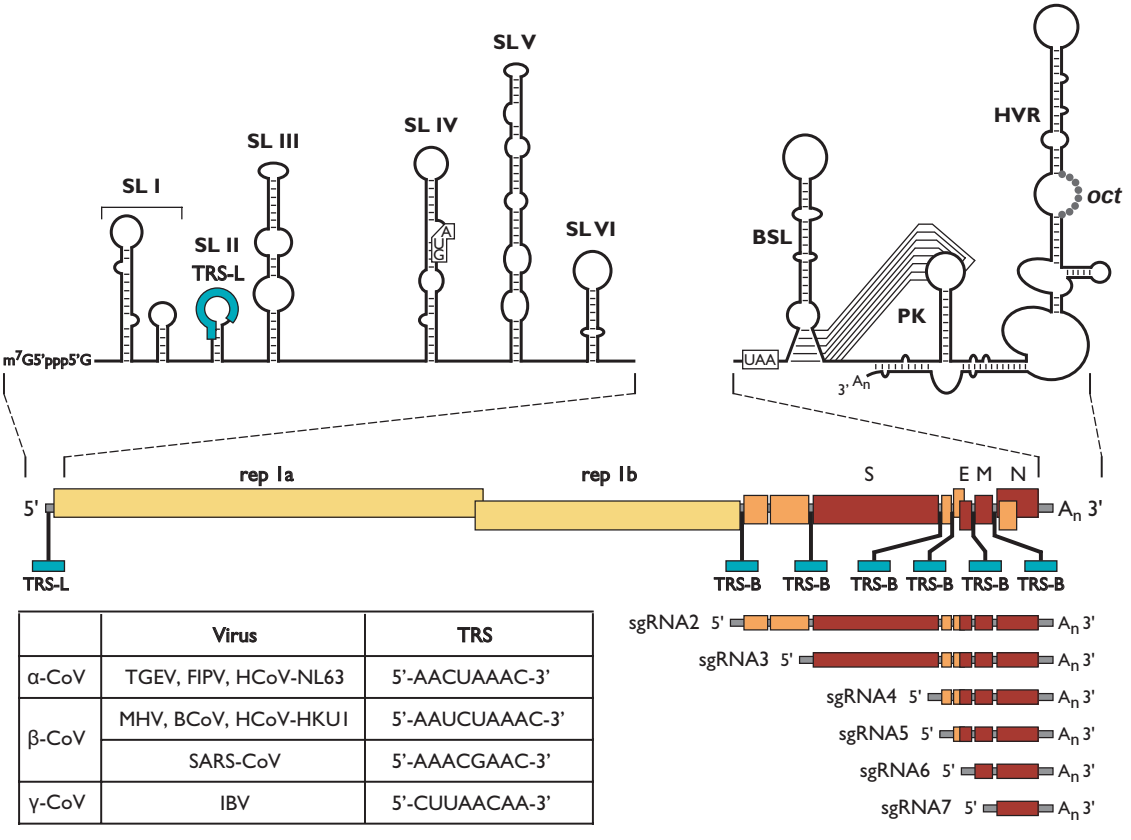
Finally, there are two rep 1b–encoded activities that are not found outside the order *Nidovirales*[194,521]; surprisingly, both are ribonucleases. The first is an endonuclease, designated NendoU, which resides in nsp15. NendoU hydrolyzes both single- and double-stranded RNA and specifically cleaves downstream of uridylate residues, producing 2′-3′ cyclic phosphates.[33,246] Although it bears homology to XendoU, an enzyme involved in small nucleolar RNA (snoRNA) processing, the potential role of NendoU in coronavirus RNA synthesis is not clear. It is also unresolved whether NendoU is essential or if lethal mutations constructed in nsp15 affect some other function of that protein.[246,260] The second activity is ExoN, a 3′-5′ exonuclease that is associated with the amino-terminal portion

of nsp14.[395] This enzyme is not essential for viral replication; however, nsp14 mutants have a greatly enhanced mutation rate, supporting the notion that ExoN provides a proofreading function for the coronavirus RdRp.[149,150] Such a corrective activity may be critical for maintenance of the stability of the exceptionally large coronavirus genome.

## Viral RNA Synthesis

Expression and assembly of the RTC sets the stage for viral RNA synthesis (see Fig. 28.6), a process resulting in the replication of genomic RNA and the transcription of multiple subgenomic RNAs (sgRNAs).[299,433,577] The latter species serve as mRNAs for the genes downstream of the *replicase* gene. Each sgRNA consists of a leader RNA of 70 to 100 nucleotides, which is identical to the 5′ end of the genome, joined to a body RNA, which is identical to a segment of the 3′ end of the genome. The fusion of the leader RNA to body RNAs occurs at short motifs—TRSs—examples of which are listed in Figure 28.9. Like the genome, the sgRNAs have 5′ caps and 3′ polyadenylate tails. Together, these transcripts form a 3′-nested set—the single most distinctive feature of the order *Nidovirales*.[157,194] Synthesis of both genomic RNA and sgRNAs proceeds through negative-strand intermediates.[24,509] The negative sense RNAs, which possess 5′ oligouridylate tracts[220] and 3′ antileaders,[508] are roughly a tenth to a hundredth as abundant as their positive sense counterparts.

At their 5′ and 3′ termini, coronavirus genomes contain *cis*-acting RNA elements that allow their selective recognition as templates for the RTC and play essential roles in RNA synthesis (see Fig. 28.9). The initial localization of these elements was carried out in studies of defective interfering (DI) RNAs, which are extensively deleted genomic variants that propagate by competing for the viral RNA synthesis machinery.[69,371,393,445,575] Manipulations of natural and artificially constructed DI RNAs, evaluated by transfection into helper-virus–infected cells, made possible the mapping of sequences




| | Virus | TRS |
|---|---|---|
| α-CoV | TGEV, FIPV, HCoV-NL63 | 5'-AACUAAAC-3' |
| β-CoV | MHV, BCoV, HCoV-HKU1 | 5'-AAUCUAAAC-3' |
| | SARS-CoV | 5'-AAACGAAC-3' |
| γ-CoV | IBV | 5'-CUUAACAA-3' |

**FIGURE 28.9. Coronavirus RNA synthesis.** Shown are a schematic of MHV genomic RNA and the nested set of transcribed subgenomic RNA species that are a defining feature of the order *Nidovirales*. The leader and body copies of the TRS (TRS-L and TRS-B, respectively) are denoted by *green boxes*. At the **left** are listed examples of consensus TRSs that have been experimentally confirmed[462,463,531,562]; the inferred TRSs of other coronaviruses are identical or highly similar to these. Expanded regions above the genome depict *cis*-acting RNA structures at the genome termini. The structures shown are those characterized for MHV.[190,202,346,667] Homologous structures exist in the BCoV[53,622] and SARS-CoV genomes,[192,261] and counterparts of some of these elements appear in other coronaviruses.[80,107,605] The 5′ expanded region represents the 210-nt 5′ UTR and the first 140 nt of the *rep 1a* gene; the elements shown are SLs I through VI, numbered as originally described for BCoV.[53,202] TRS-L is denoted in *green* in SL II; the start codon of rep 1a is *boxed* in SL IV. The 3′ expanded region represents the 301-nt 3′ UTR. The elements shown are the bulged stem loop (*BSL*), the pseudoknot (*PK*), the hypervariable region (*HVR*), and the conserved coronavirus octanucleotide motif (*oct*); the stop codon for the upstream *N* gene is *boxed*. MHV, mouse hepatitis virus; TRS, transcription-regulating sequence; BCoV, bovine coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; UTR, untranslated region; nt, nucleotide; SL, stem loop.

that are critical for the replication and transcription of DI RNA and, presumably, also for genomic RNA.[45,379] More recently, *cis*-acting RNA elements have been dissected through reverse genetics of the intact viral genome, complemented by *in vitro* biochemical and structural analyses. The most completely characterized structures and sequences are those of the betacoronaviruses MHV, BCoV, and SARS-CoV (see Fig. 28.9).

At the 5′ end of the genome, the elements that participate in viral RNA synthesis extend well beyond the 5′ UTR into the replicase coding region, making up a set of seven stem loops.[53,202,346,467] One of these displays the leader copy of the TRS (TRS-L) in its loop, and another sequesters the start codon of the *rep 1a* gene within its stem. Many, but not all, of these defined structures can be exchanged among the genomes of different betacoronaviruses.[202,261] Significantly, functional analyses have shown that either the stability[202,346] or the instability[329] of a given RNA stem can be critical for viral fitness, suggesting that these structures operate in a dynamic manner during RNA synthesis.

At the 3′ end of the genome, *cis*-acting RNA elements are confined entirely to the 3′ UTR[190] and are functionally interchangeable among the betacoronaviruses.[192,228,622] These elements consist of a bulged stem loop[228] and an adjacent pseudoknot[605] that have each been demonstrated to be essential for viral replication. Further downstream is a hypervariable region, which is completely dispensable for viral replication but yet harbors 5′-GGAAGAGC-3′, an octanucleotide motif that is universally conserved in the coronaviruses.[191,347] Notably, the bulged stem loop and the pseudoknot partially overlap, and they therefore can not fold up simultaneously. The two structures are thus thought to constitute a molecular switch between different steps of RNA synthesis.[190,227] In addition, the first loop of the pseudoknot forms a duplex with the extreme 3′ end of the genome and genetically interacts with the RTC subunits nsp8 and nsp9.[667] On this basis, a mechanism has been proposed in which alternate RNA conformations of the 3′ UTR facilitate the transition between initiation of negative-strand RNA synthesis by the nsp8 primase and elongation by the nsp12 RdRp. However, this scheme does not yet incorporate potential cross talk between the 5′ and 3′ ends of the genome,[329] and much remains to be learned about how *cis*-acting RNA elements are recognized by, and cooperate with, the RTC.

A central issue in coronavirus RNA synthesis is how the leader RNA becomes attached to the body segments of the sgRNAs. It became clear from early work that transcription involves a discontinuous process. Ultraviolet (UV) transcriptional mapping demonstrated that sgRNAs are not processed from a genome-length precursor,[250,537] and mixed infections with two different strains of MHV showed that leader RNAs could reassort between separate sgRNA body segments.[372] It was also clearly established by DI RNA studies, and later confirmed by genomic reverse genetics,[527,664] that the TRSs play key roles in sgRNA formation. The efficiency of fusion at an individual body TRS (TRS-B) is, in part, governed by how closely it conforms to the leader TRS (TRS-L).[217,369,576] Nonetheless, factors such as the local sequence context of the TRS and the position of the TRS relative to the 3′ end of the genome also profoundly influence transcription levels.[286,429,580]

Originally, the leader-to-body fusion event was envisioned to occur by a leader-primed mechanism during positive-strand RNA synthesis.[298,300,652] However, there is now broad, although not universal, agreement that fusion takes place through discontinuous extension of negative-strand RNA synthesis.[433,496,498,664] In this model, both genomic and subgenomic negative-strand RNAs are initiated by the RTC at the 3′ end of the (positive-strand) genome template (Fig. 28.10). A pause in RNA synthesis occurs when the RdRp crosses a TRS-B. At this point, the RdRp may continue to elongate the growing negative strand. Alternatively, it may switch to the leader at the 5′ end of the genome template, guided by the complementarity between the 3′ end of the nascent negative strand and the TRS-L of the genome. The resulting negative-strand sgRNA, in partial duplex with positive-strand gRNA, then serves as the template for synthesis of multiple copies of the corresponding positive-strand sgRNA.

Leader-to-body fusion during negative-strand synthesis is amply supported by accumulated experimental results with coronaviruses and the closely related arteriviruses. First, as necessitated by the model, negative-strand sgRNAs contain antileaders at their 3′ ends.[508] Second, in infected cells, there exist transcription intermediates containing negative-strand sgRNAs in association with the genome. These complexes actively participate in transcription[24,497] and can be biochemically separated from replication intermediates containing genome-length negative-strand RNAs.[500] Finally, as would be predicted for discontinuous negative-strand synthesis, engineered (or naturally occurring) variant nucleotides incorporated into the TRS-B, rather than the TRS-L, end up in the leader–body junction of the resulting sgRNA.[238,434,579,664] There remains, however, considerable further work to be done to elucidate the details of the model.[433,498] It is not clear how the transcribing RdRp might continuously monitor the ability of its nascent product to base pair to the TRS-L. Additionally, the synthesis of genome-length negative strands would require the RdRp to bypass all of the TRS-B sites in the genome template. This may come about through a stochastic process, or it may be actively promoted by some RTC component under certain conditions. These and other questions will need to be addressed, possibly with the aid of a robust *in vitro* viral RNA synthesizing system.[578] Such a system may also be decisive in assessing the potential roles of host factors in transcription and replication. Several cellular proteins, including hnRNP A1,[327,512,513] polypyrimidine tract-binding protein,[326,526] mitochondrial aconitase,[404] and polyadenylate-binding protein,[532] have been proposed to take part in coronavirus RNA synthesis, mainly based on their ability to bind *in vitro* to genomic RNA segments. Because many putative host factors also play critical or essential roles in normal cellular functions, it has been difficult to convincingly demonstrate their specific involvement in viral processes. As yet, only a single candidate host factor has been shown to be required for *in vitro* viral RNA synthesis.[578]

In addition to its central role in sgRNA formation, template switching is also at the heart of RNA recombination—another prominent feature of coronavirus RNA synthesis. Significant rates of both homologous and nonhomologous RNA recombination have been found among selected and unselected markers during the course of infection.[266,267,268,370] It is presumed, but remains to be formally demonstrated, that coronavirus RNA recombination results from a copy-choice mechanism, as originally established for poliovirus.[273] In MHV, recombination takes place at an estimated frequency of 1% per 1.3 kb (almost 25% over the entire genome)—the

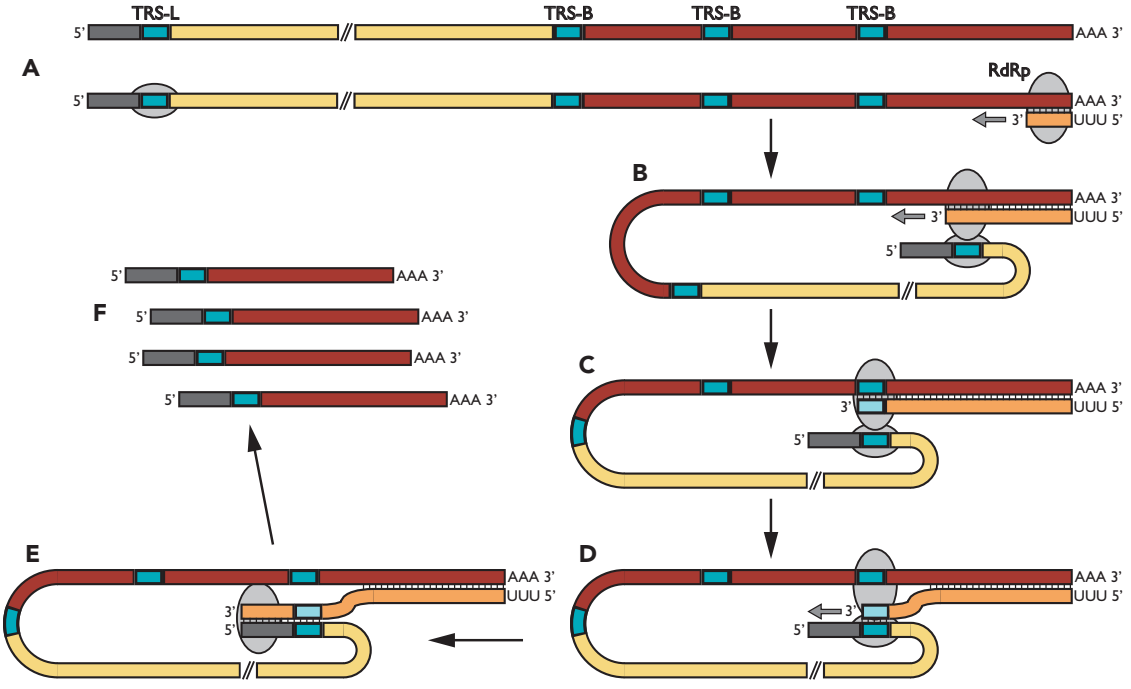



**FIGURE 28.10. Coronavirus transcription through discontinuous extension of negative-strand RNA synthesis.**[496,498,664] **A, B:** Negative-strand sgRNA synthesis initiates at the 3′ end of the positive-strand genomic RNA template. In the version of the model shown here, the genomic template loops out in such a way as to allow a component of the RTC to constantly monitor the potential complementarity of the 3′ end of the nascent negative-strand RNA with the TRS-L. **C:** Transcription pauses at a TRS-B. At this point, elongation may resume, thereby bypassing the TRS-B. **D:** Alternatively, the nascent negative strand may switch templates, binding to the TRS-L. **E:** Resumption of elongation results in completion of synthesis of an antileader-containing negative-strand sgRNA. **F:** The resulting complex of genome and negative-strand sgRNA acts as template for the synthesis of multiple copies of the corresponding positive-strand sgRNA. sgRNA, subgenomic RNA; RTC, replicase-transcriptase complex; TRS, transcription-regulating sequence. (Adapted from Zúñiga S, Sola I, Alonso S, et al. Sequence motifs involved in the regulation of discontinuous coronavirus subgenomic RNA synthesis. *J Virol* 2004;78:980–994.)

highest rate observed for any RNA virus.[22] On a fine scale, the sites of crossover are random,[19] although selective pressures can generate the appearance of local clustering of recombinational hot spots.[18] This facility for RdRp strand switching may make a major contribution to the ability of the huge coronavirus genome to evolve and to circumvent the accumulation of deleterious mutations. It also serves as the basis for targeted RNA recombination (see the Coronavirus Genetics section).

## Assembly and Release of Virions

The immediate outcome of transcription is to enable translation of the proteins that build progeny viruses. The membrane-bound proteins M, S, and E are initially inserted into the ER; from there, they transit to the site of virion assembly, the endoplasmic reticulum–Golgi intermediate compartment (ERGIC).[275,285,563] Here, nucleocapsids composed of progeny genomes encapsidated by N protein coalesce with the envelope components to form virions, which bud into the ERGIC[117,222,378] (see Fig. 28.6).

Coronavirus assembly occurs through a network of cooperative interactions, most of which involve M protein. However, despite its central role, M is not assembly competent by itself. Expression of M protein alone does not result in virion-like structures, and M traverses the secretory pathway beyond the budding site, as far as the *trans*-Golgi.[275,362,363,489] The first virus-like particle (VLP) systems developed for coronaviruses led to the key finding that co-expression of E protein with M protein is sufficient to yield the formation of particles that are released from cells and appear morphologically identical to coronavirus envelopes.[37,582] More recently, it has been shown that the additional co-expression of N protein substantially increases the efficiency of VLP formation[38,519] and can even compensate for mutational defects in M.[15] Other viral structural proteins, in particular S protein, are gathered into virions but are not specifically required for the assembly process. Because virions and VLPs contain very little E protein, this indicates that lateral interactions between M molecules provide the driving force for envelope morphogenesis. Investigations of the ability of M protein mutants to support VLP assembly concluded that M–M interactions occur via multiple contacts throughout the molecule, especially between the transmembrane domains.[114,120] Recent cryo-electron tomographic reconstructions of whole virions suggest that the M protein forms dimers that are maintained through multiple monomer–monomer contacts, while dimer–dimer interactions occur among the globular endodomains.[415]

It remains enigmatic how E protein critically assists M in envelope formation. Like M, E protein by itself moves to a compartment past the ERGIC[93,100]; however, co-expression or infection somehow secures localization of M and E at the

budding site. Some evidence suggests that E protein promotes assembly by inducing membrane curvature.[100,166,465] Other work indicates a role for E in maintaining M protein in an assembly-competent state by preventing its nonproductive aggregation—a function that crucially depends on palmitoylation of E.[38] Such a chaperone-like role would be consistent with demonstrations that diverse heterologous E proteins, and even truncated versions of M protein, can functionally replace E protein in MHV.[293,294] Finally, there are reports that point to a need for E protein to facilitate the release of assembled virions from infected cells.[364,427] These roles are not mutually exclusive, and some recent studies have begun to assign individual functions to various regions of the E molecule. The C-terminal endodomain of the IBV E protein governs Golgi localization[100,101] and when linked to a heterologous transmembrane domain can support VLP and virion assembly.[364,491] Conversely, the transmembrane domain of E alters the host secretory pathway in a way that promotes virus release.[491] This latter effect is potentially a consequence of the putative ion channel properties of the E transmembrane domain[449,609,610,638]; however, it is unresolved whether native E protein acts as an ion channel at intracellular membranes *in vivo*.[420]

The dispensability of S protein for VLP formation is consistent with earlier observations that spikeless (noninfectious) virions were formed by infected cells treated with the glycosylation inhibitor tunicamycin[224,487] or by cells infected with particular S mutants.[360,474] S protein thus appears to play a passive role in assembly; however, during its passage through the secretory pathway, it is captured by M protein for virion incorporation.[387,426] For some S proteins, localization at or near the budding compartment is abetted by targeting signals contained in the endodomain.[353,386,611] The S endodomain is also the region of the protein that interacts with M during assembly.[39,636] Conversely, the ability of M protein to interact with S maps to a locus close to the C-terminus of the M endodomain[118] (see Fig. 28.3).

Virion assembly is completed by condensation of the nucleocapsid with the envelope components. This is brought about principally by N and M protein interactions, which have been mapped to domain 3 of N[236,585] and the extreme C-terminus of the M endodomain[162,295] (see Fig. 28.3). These interacting regions likely account for the thread-like connections that have been visualized between the M protein endodomain and the nucleocapsid in virion reconstructions.[21,415] Nucleocapsid formation is presumed to be concomitant with genome replication; however, the details of how the nucleocapsid traffics to the budding compartment are not known. It is also not well understood how coronaviruses selectively package genomic RNA from among the many positive- and negative-strand viral RNA species that are synthesized during infection. DI RNA analyses have mapped the genomic packaging signal of MHV to a small span of RNA sequence embedded in the region of the *replicase* gene that encodes nsp15[169,373,575] (see Fig. 28.7C). Highly homologous structures exist in the genomes of BCoV and HCoV-HKU1.[81,96] However, for most coronaviruses, including SARS-CoV,[256] packaging signals are clearly not found at the same locus, and the relevant structures for these viruses may occur at a large distance, near the 5′ ends of their respective genomes.[80,161] The mechanism by which the MHV packaging signal operates is undetermined. Some studies have shown that it is specifically bound by N protein,[96,396] although other work demonstrates that M protein, in the absence of N, acts as the discriminatory factor for packaging signal recognition.[406,409]

Following assembly and budding, progeny virions are exported from infected cells by transport to the plasma membrane in smooth-walled vesicles and are released by exocytosis. It remains to be more clearly defined whether coronaviruses follow the constitutive pathway for post-Golgi transport of large cargo or, alternatively, if specialized cellular machinery must be diverted for their exit.[222] For some coronaviruses, but not others, a fraction of S protein that has not been assembled into virions transits to the plasma membrane, where it can mediate fusion between infected cells and adjacent, uninfected cells. This leads to the formation of large, multinucleate syncytia, enabling the spread of infection by a means not subject to neutralization by antibody. For MHV, cell–cell fusion depends on S1-S2 cleavage carried out by a furin-like protease late in infection.[119] However, this form of proteolytic activation of S does not appear to affect virus–cell fusion that occurs at the initiation of infection. Similarly, the SARS-CoV S protein has different proteolytic requirements for cell–cell and virus–cell fusion.[168,516] On the opposite side of the membrane from the cleaved ectodomain, the cysteine-rich region of the S protein endodomain also plays a critical role in cell–cell fusion[36,68,636]; specifically, this has been shown to depend on the palmitoylation of a subset of endodomain cysteine residues.[388]

## PATHOGENESIS AND PATHOLOGY OF CORONAVIRUS INFECTIONS

### General Principles

Most coronaviruses spread to susceptible hosts by respiratory or fecal–oral routes of infection, with replication first occurring in epithelial cells (Table 28.3). Some, including HCoV-OC43, HCoV-229E, and porcine respiratory coronavirus (PRCoV), replicate principally in respiratory epithelial cells, where they produce virus and cause local respiratory symptoms. Other coronaviruses, including TGEV, BCoV, porcine hemagglutinating encephalomyelitis virus (PHEV), CCoV, FeCoV, and enteric strains of MHV, infect epithelial cells of the enteric tract. Some of these viruses, such as TGEV, cause diarrhea that is particularly severe, and sometimes fatal, in young animals.[492] Inapparent enteric infection of adult animals maintains the virus in the population.[98] In addition to local infection of the respiratory or enteric tracts, several coronaviruses cause severe disease. For example, SARS-CoV spreads from the upper airway to cause a severe lower respiratory tract infection, whereas FIPV spreads systemically to cause a generalized wasting disease in felines.[439,448] Rat coronavirus strains cause respiratory infection or sialodacryoadenitis owing to infection of the salivary and lacrimal glands[446] but can also interfere with reproduction by infecting the female urogenital tract.[571] PHEV of swine predominantly causes enteric infection but is also neurotropic.[389] Infection spreads to nerves that innervate the stomach of infected piglets and prevents gastric emptying, resulting in vomiting and wasting disease. The ability to cause localized versus systemic disease is mirrored in polarized tissue culture cells. Thus, coronaviruses such as MHV, which can cause systemic disease, enter the apical side of cells and exit the basolateral side, whereas others, such as HCoV-229E, which causes only a localized infection, enter and

**TABLE 28.3 Representative Coronaviruses and Associated Diseases**

| Virus | Host species | Sites of infection | Clinical disease |
|---|---|---|---|
| **Alphacoronaviruses** | | | |
| CCoV | Canine | GI tract | Gastroenteritis |
| FeCoV | Felidae | GI tract, respiratory | Gastroenteritis |
| FIPV | Felidae | Systemic disease | Peritonitis, wasting disease |
| HCoV-229E | Human | Respiratory | Upper respiratory tract infection |
| HCoV-NL63 | Human | Respiratory | Upper respiratory tract infection, croup |
| PEDV | Pig | GI tract | Gastroenteritis |
| TGEV | Pig | GI tract, respiratory | Gastroenteritis |
| BatCoV | Bat | GI tract, respiratory | Unknown |
| Rabbit CoV | Rabbit | Heart, GI tract, respiratory | Enteritis, myocarditis |
| **Betacoronaviruses** | | | |
| BCoV | Bovine, ruminants | GI tract, respiratory | Enteritis, upper and lower respiratory tract infection |
| HCoV-OC43 | Human | Respiratory | Upper respiratory tract infection |
| HCoV-HKU1 | Human | Respiratory | Upper and lower respiratory tract infection |
| MHV | Mouse, rat | GI tract, liver, brain, lungs | Gastroenteritis, hepatitis, encephalitis, chronic demyelination |
| PHEV | Pig | Respiratory, brain | Vomiting, wasting, encephalomyelitis |
| RCoV | Rat | Respiratory, salivary and lachrymal glands, urogenital tract | Respiratory tract infection, metritis, sialodacryoadenitis |
| SARS-CoV | Human | Respiratory, GI tract | Pneumonia (SARS) |
| BatCoV | Bat | GI tract, respiratory | Unknown |
| **Gammacoronaviruses** | | | |
| IBV | Chicken | Respiratory, kidney | Bronchitis, nephritis |
| TuCoV | Turkey | GI tract | Gastroenteritis |

CCoV, canine coronavirus; GI, gastrointestinal; FeCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; HCoV, human coronavirus; PEDV, porcine epidemic diarrhea virus; TGEV, transmissible gastroenteritis virus; BatCoV, bat coronavirus; CoV, coronavirus; BCoV, bovine coronavirus; MHV, mouse hepatitis virus; PHEV, porcine hemagglutinating encephalomyelitis virus; RCoV, rat coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome; IBV, infectious bronchitis virus; TuCoV, turkey coronavirus.

exit the cell apically.[481,482,595] Specific examples are described in more detail later.

## Animal Coronavirus Infections

Several coronavirus infections have been extensively studied in their natural hosts. Here, we will focus on murine and feline coronavirus infections.

### Mouse Hepatitis Virus

MHV, which until the advent of SARS was the most widely studied coronavirus, causes enteric, hepatic, and neurologic infections of susceptible strains of rodents. Remarkably, closely related strains of MHV, all of which use the same host cell receptor for entry,[606] infect different organs. Enteric strains, such as MHV-Y and MHV-RI, are a major problem in animal research facilities.[98] These viruses spread within infected colonies to young, uninfected animals. They do not generally cause symptomatic disease but may subtly impair the host immune response to other pathogens and immunological stimuli.[98,540] Studies of MHV pathogenesis predominantly use the neurotropic JHM and A59 strains of virus (JHM virus [JHMV] and MHV-A59), in part because they cause a demyelinating encephalomyelitis with similarities to the human disease multiple sclerosis (MS). Originally isolated from a mouse with hindlimb paralysis, JHMV became progressively more virulent on passage in mice.[16,75] The most virulent strains of JHMV cause rapidly fatal acute encephalitis with widespread neuronal infection.[600] Subsequently, most studies have used either attenuated JHMV variants or the mildly neurovirulent MHV-A59 strain for studies of demyelination. Infection with these viruses results in minimal infection of neurons, with oligodendrocytes, microglia, and astrocytes commonly infected.[167,276,313] Myelin destruction occurs during the process of virus clearance from infected glia.[594] Initial studies suggested that demyelination resulted from virus-mediated lysis of oligodendrocytes.[304,600] However, more recent studies show that demyelination is largely immune mediated. In support of this, irradiated mice or congenitally immunodeficient mice (mice with severe combined immunodeficiency [SCID]) or with a disrupted recombination activation gene [$RAG^{-/-}$]) do not develop demyelination after infection with JHMV. When these mice, which lack T and B cells, are reconstituted with virus-specific T cells, demyelination rapidly develops[593,620] (Fig. 28.11). Demyelination is accompanied by infiltration of macrophages and activated microglia into the white matter of the spinal cord.[621] Little is known, however, about how macrophages and microglia are actually attracted to the spinal cord or about the nature of the signals that cause these cells to phagocytose infected myelin. Both CD4 and CD8 T cells are required for virus clearance from the central nervous system (CNS), with CD8 T cells considered most important in

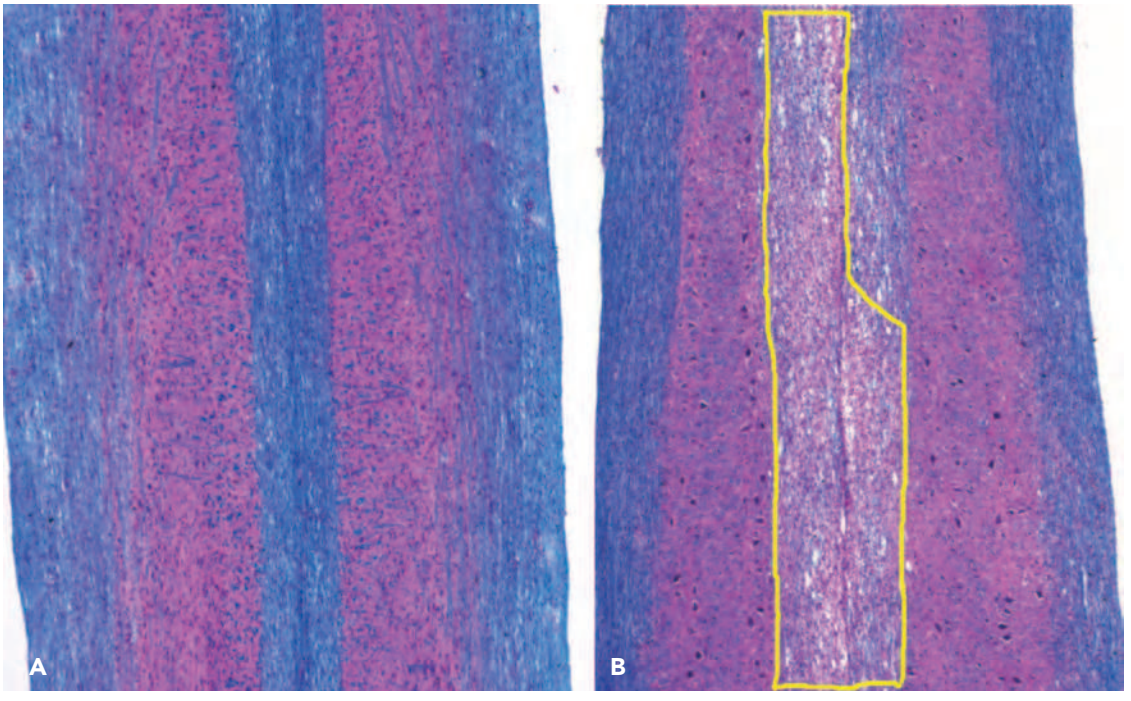



**FIGURE 28.11. Immune-mediated demyelination in mice infected with a neurotropic MHV.** RAG1$^{-/-}$ mice, lacking T and B cells, were infected with a neurotropic coronavirus as described.[620] Four days later, some mice received adoptively transferred spleen cells from a wild-type C57Bl/6 mouse that was previously immunized intraperitoneally with MHV **(B)**. All mice were sacrificed 8 days later and analyzed for demyelination (marked with a *yellow line* in **B**). Demyelination was observed only in mice that received adoptively transferred MHV-immune cells **(B)** and not in those that did not **(A),** showing that myelin destruction is largely mediated by T cells during the process of virus clearance. MHV, mouse hepatitis virus.

this process.[608] CD8 T cells eliminate virus from infected astrocytes and microglia by perforin-dependent pathways, whereas clearance from oligodendrocytes is IFN-$\gamma$ dependent.[340,432] However, T-cell–mediated virus clearance is not complete, and antivirus antibody is required to prevent virus recrudescence.[338] Virus persistence in neonatal mice occurs, in part, because virus variants mutated in an immunodominant CD8 T-cell epitope are selected in specific strains of mice, with subsequent evasion of the cytototoxic T-cell immune response.[451] However, this mechanism of immune evasion has not been detected in older mice that are persistently infected with JHMV. The antivirus CD4 T-cell response, while critical for virus clearance, is also pathogenic. Partial diminution of this response decreases morbidity and mortality, whereas enhancement of the antivirus CD4 T-cell response increases disease severity.[11]

Other strains of MHV, including MHV-A59, MHV-2, and MHV-3, infect both the liver and the CNS. Most notably, MHV-3 causes a fulminant hepatitis in susceptible strains of mice and chronic neurologic infections in semisusceptible strains.[649] In susceptible strains, MHV-3 infects macrophages, resulting in up-regulation of several proinflammatory cytokines, including fibrinogen-like protein 2 (FGL2), a transmembrane procoagulant molecule.[431] FGL2 is also expressed by Foxp3$^{+}$ regulatory T cells.[511] Expression of this molecule results in prothrombin cleavage, with consequent disseminated intravascular coagulation (DIC), hepatic hypoperfusion, and necrosis.[375] Levels of FGL2 are better predictors of a fatal outcome than virus titers. It is known that the propensity to develop severe disease occurs at a postentry step because the MHV-3 receptor, CEACAM1, is expressed in both resistant and susceptible strains of mice. Like JHMV, MHV-3 also infects the CNS; however, infection of this organ occurs only in strains that do not develop a fulminant hepatitis. MHV-3 does not cause a demyelinating disease but rather ependymitis, hydrocephalus, encephalitis, and thrombotic vasculitis.[315,589] The pathogenesis of these entities is not well studied but appears to be immune mediated. Unlike most other strains of MHV, MHV-3 directly infects T and B cells, resulting in lymphocyte apoptosis and lymphopenia.[303] Lymphopenia, with consequent immunosuppression, facilitates virus persistence and its immunopathologic consequences.

## Feline Enteric Coronavirus and Feline Infectious Peritonitis Virus

Feline enteric coronavirus (FeCoV) commonly causes mild or asymptomatic infection in domestic cats and other felines. Two serotypes of FeCoV are recognized, with serotype II strains arising by recombination of serotype I FeCoV with CCoV in dually infected animals.[216] In some cats infected persistently with FeCoV, mutations in the virus occur, resulting in the development of a lethal disease called *feline infectious peritonitis* (FIP); FIPV is the virulent strain of FeCoV. Virulence correlates with the ability of the virus to replicate in macrophages.[110] The nature of the mutations required for transition from FeCoV to FIPV is not well understood, although, at least for serotype II viruses, virulence maps in part to the surface glycoprotein.[488] This was shown using reverse genetics, in which S proteins from virulent and avirulent strains were swapped and tested for their ability to cause severe disease in cats. FIPV causes a multiphasic

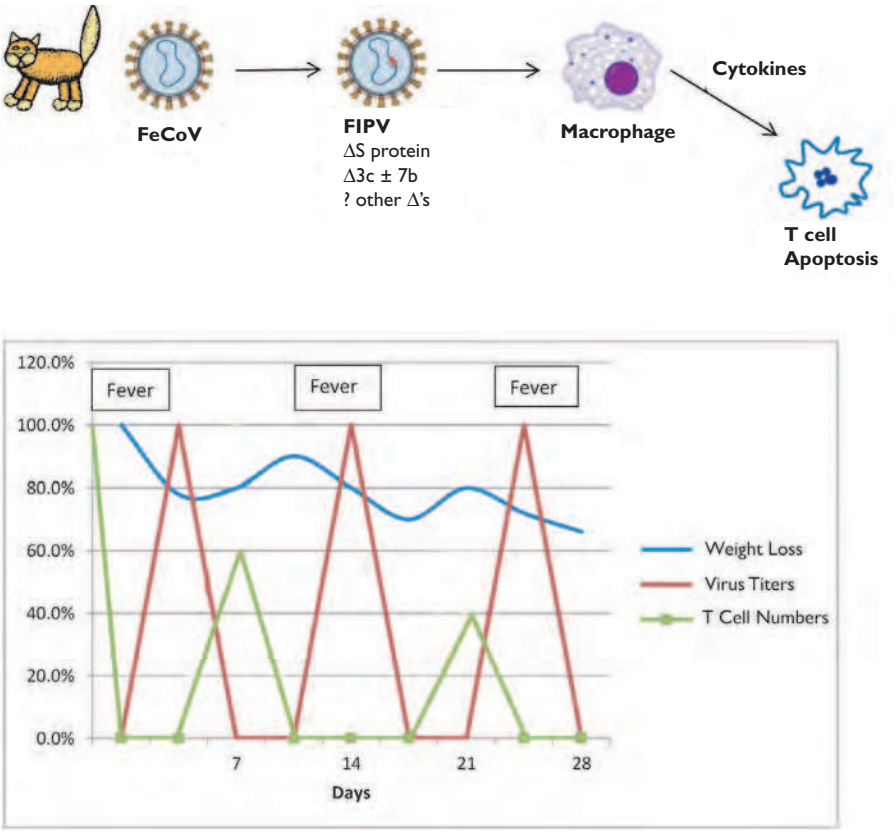



**FIGURE 28.12. Recurrent feline infectious peritonitis (FIP).** FIP virus—the etiologic agent of FIP—occurs in felines persistently infected with feline coronaviruses. **Upper panels:** Mutations in the S glycoprotein and the ORF3b and 7b proteins occur as virus gains the ability to replicate in macrophages. Infected macrophages serve to transport the virus to sites in the host distant from the initial infection. These infected cells also express several cytokines that are believed to contribute to T-cell apoptosis. **Lower panel:** Clinical disease is characterized by recurrent bouts of virus replication accompanied by fever and clinical disease. Lymphopenia subsequently occurs as disease progresses. The pattern of disease shown in the figure is representative of progressive disease; however, the rate and extent of recurrence of virus replication, as well as the rate of weight loss and of development of lymphopenia, are variable from animal to animal. (Based on De Groot-Mijnes JD, van Dun JM, van der Most RG, et al. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. *J Virol* 2005;79:1036–1044.)

disease with relapses that result, ultimately, in immunosuppression, weight loss, and death (Fig. 28.12). Each episode is characterized by increased virus replication, fever, and lymphopenia.[112] FIPV does not directly infect lymphocytes. Rather, lymphopenia is believed to be a consequence of infection and activation of macrophages and dendritic cells. Subsequent lymphocyte depletion occurs when cells are exposed to high levels of proinflammatory cytokines, such as tumor necrosis factor, released by these infected cells.[205] Virus dissemination occurs when infected macrophages traffic throughout the body and are deposited in the vasculature. Infected macrophages provoke a pyogranulamatous reaction, which is responsible for many disease manifestations of FIP, such as peritonitis and serositis. Another consequence of immune dysregulation is hypergammaglobulinemia. Antibody-antigen complex formation commonly occurs in FIPV-infected cats and may contribute to vascular injury.[252] However, its precise role in pathogenesis remains uncertain because it is a late manifestation of disease and may make only a minor contribution to disease progression. Neutralizing antibody against the S glycoprotein enhances FIPV infection of macrophages. Enhanced macrophage infection is mediated by virus entry through Fcγ receptors, although virus binding to fAPN—the specific FIPV host cell receptor—is also likely required.[110] This phenomenon has been demonstrated *in vitro* using isolated macrophages and also occurs in cats that have been previously immunized with vectors that express the S glycoprotein.[581] FIPV, but not FeCoV, uptake is augmented by neutralizing antibody that contributes to the propensity of FIPV strains to replicate in macrophages. Although the potential occurrence of antibody-enhanced disease has hindered vaccine development and was raised as a potential difficulty in development of a live attenuated SARS-CoV vaccine, it has never been demonstrated in the natural infection. In fact, cats infected with FeCoV often develop only low antivirus neutralizing antibody titers.[226]

## Human Coronavirus Infections

### Human Coronaviruses, Other Than Severe Acute Respiratory Syndrome Coronavirus, Associated with Respiratory and Enteric Disease

Prior to 2003, HCoVs were primarily considered to be agents of upper respiratory tract disease and to cause little mortality. In general, whereas coronaviruses were readily isolated from infected birds and other animal species, and serially propagated in continuous cell lines, isolation of HCoVs from infected individuals was only rarely achieved.[389] HCoV-229E and HCoV-OC43 were isolated from patients with upper respiratory tract infections in the 1960s.[210,390,570] There are striking differences in extent of genetic variability when isolates of HCoV-OC43 and HCoV-229E are compared. HCoV-229E isolated at geographically distinct locations show little evidence of variability.[87] In contrast, isolates of HCoV-OC43 isolated from the United States and from France differ in sequence, and virus from the same geographic area but isolated in different years show considerable sequence variations.[587] The ability of HCoV-OC43 to tolerate mutations probably accounts for its ability to grow in mouse cells and infect the mouse brain[389] as well as its ability to cross species (see the Epidemiology section). In contrast, HCoV-229E does not readily cross species

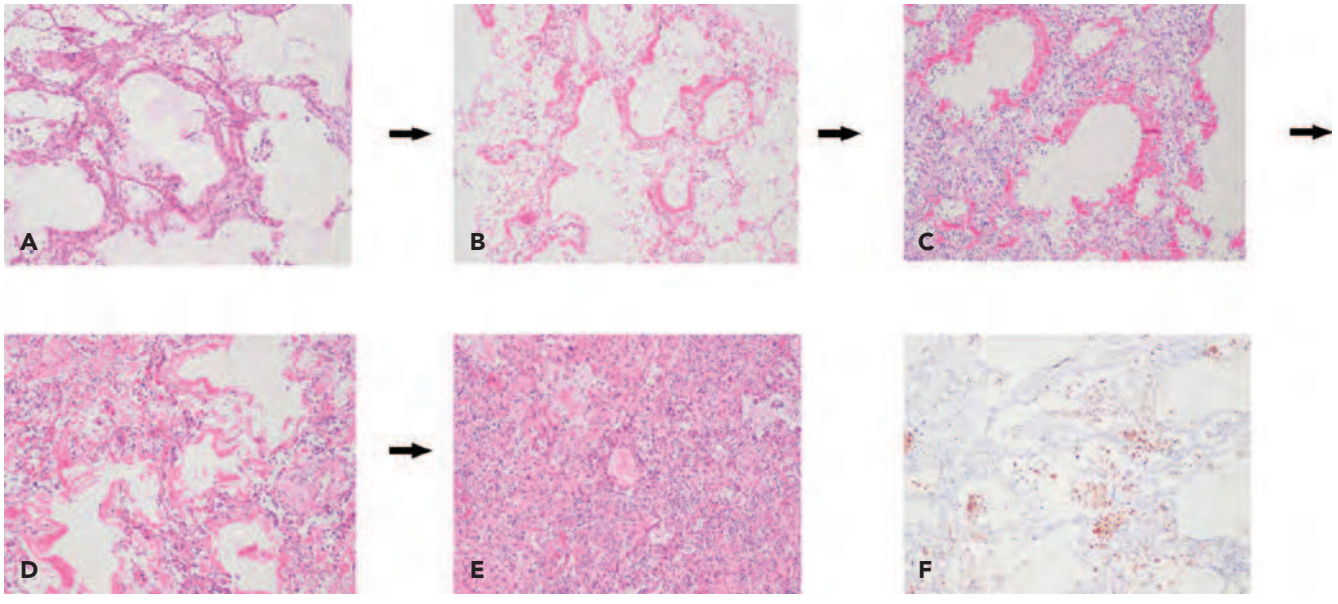



**FIGURE 28.13. Pathologic changes in lungs of patients with SARS.** Lung samples obtained on autopsy were examined for pathologic changes following SARS-CoV infection. **A–E:** Hematoxylin and eosin stain showing the progression of SARS pneumonia. Early stages of the SARS infection show edema and early hyaline membrane formation **(A)**, hyaline membrane formation **(B)**, and increased inflammatory cell infiltration and pneumocyte hyperplasia **(C)**. As the disease progresses, fibrotic changes become apparent **(D)**. Late manifestations include obliteration of the alveolar volume by fibrous tissue, reactive pneumocytes, and inflammatory cells **(E)**. **F:** Viral antigen is detected most prominently during early stages of the infection in macrophages and alveolar pneumocytes. Magnification, ×100. SARS, severe acute respiratory syndrome; SARS-CoV, severe acute respiratory syndrome coronavirus. (Courtesy of Dr. John Nicholls, University of Hong Kong.)

and does not infect mice. Even in mice that are transgenic for expression of the HCoV-229E host cell receptor (human aminopeptidase N [hAPN]), the virus does not grow unless mice are also rendered immunodeficient by genetic disruption of the *STAT1* gene.[306]

Several new HCoVs were isolated from the respiratory tracts of patients in the post-SARS era. HCoV-NL63, which causes mild respiratory disease, displays homology with HCoV-229E.[460] Phylogenetic analyses suggest that HCoV-NL63 and HCoV-229E diverged approximately 1,000 years ago.[461] A novel feature of HCoV-NL63 is that unlike HCoV-229E, HCoV-NL63 does not use hAPN as a receptor. Rather, infection of cells is mediated by ACE2, the same molecule that is used by SARS-CoV, an unrelated betacoronavirus.[219,331] However, unlike SARS-CoV, HCoV-NL63 does not use cathepsin L or require endosomal acidification to infect ACE2-expressing cells[232] and does not cause severe respiratory disease. HCoV-HKU1, isolated from an adult patient in Hong Kong with pneumonia,[615] also generally causes mild respiratory disease.

A role for HCoVs in the etiology of the human disease MS was postulated based on the ability of murine coronaviruses to cause chronic demyelinating diseases. Coronavirus-like particles have occasionally been detected in the CNS of patients with MS and have also been isolated from the brains of patients after passage in mice or murine cell lines. HCoV-229E RNA was detected in about 44% (40 of 90) of human brains tested, with similar frequencies in brains from MS patients and patients who died from other neurologic diseases or normal control subjects.[13] HCoV-OC43 sequences were detected in 23% (21 of 90) of brains tested, with 36% incidence in brains from MS patients and 14% in that of controls. Although these results are suggestive, the role of non-SARS-CoV HCoVs in diseases outside the respiratory tract, especially in those involving the CNS, is not proven and requires further investigation.

## Severe Acute Respiratory Syndrome Coronavirus Infections

SARS-CoV causes the most severe disease of any HCoV.[79,310,439,448,602] The virus infects both upper airway and alveolar epithelial cells, resulting in mild to severe lung injury. Virus or viral products are also detected in other organs, such as the kidney, liver, and small intestine, and in stool. Although the lung is recognized as the organ most severely affected by SARS-CoV, the exact mechanism of lung injury is controversial. Levels of infectious virus appear to diminish as clinical disease worsens, consistent with an immunopathologic mechanism.[437] However, this conclusion must be tempered because patient samples were obtained from nasopharyngeal aspirates, not from the lungs or other organs. Thus, it is not known whether virus titers in the lung also decrease as virus is cleared. Furthermore, virus titers obtained from patients at autopsy do not provide longitudinal information about the relationship between viral load and disease. The SARS-CoV spike protein may also contribute to disease severity. Administration of the SARS-CoV S protein to mice with pre-existing lung injury enhanced disease severity.[239,289] ACE2 appears to have a protective role in animals with lung injury, and S protein may exacerbate disease by causing its down-regulation.[289]

Pathologic findings are nonspecific in patients who died from SARS. Cells in the upper airway were initially infected, resulting in cell sloughing but relatively little epithelial cell damage. However, virus rapidly spread to the alveoli, causing diffuse alveolar damage. This was characterized by pneumocyte desquamation, alveolar edema, inflammatory cell infiltration, and hyaline membrane formation (Fig. 28.13). Over time, alveolar damage progressed, eventually resulting in pathologic signs of acute lung injury (ALI) and, in the most severe cases, acute respiratory distress syndrome (ARDS). Most notably, multinucleated giant cells, originating either from macrophages or respiratory epithelial cells, were detected in autopsy specimens. Although virus could be cultured from infected patients for several weeks, viral antigen was rarely detected in lung autopsy samples after 10 days postinfection.[137,172,318,417]

Like other coronaviruses, such as MHV and FIPV, SARS-CoV infects macrophages and dendritic cells; however, unlike these two animal coronaviruses, it causes an abortive infection in these cells.[314,436,533] Several proinflammatory cytokines and chemokines, such as interferon-inducible protein (IP)-10 (CXCL10), monocyte chemoattractant protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1$\alpha$ (CCL3), RANTES (regulated on activation normal T cell expressed and secreted) (CCL5), MCP-2 (CCL8), tumor necrosis factor (TNF), and interleukin (IL)-6, are expressed by infected dendritic cells; many of these molecules are also elevated in the serum of SARS-CoV–infected patients.[310] Lymphopenia and neutrophilia were detected in infected patients and were likely to be primarily cytokine driven.[613] A potentially confounding factor is that many patients with SARS in the 2003 epidemic were treated with corticosteroids,[538] and steroid treatment is a well-known cause of lymphopenia.

An important unresolved issue is how SARS-CoV causes severe respiratory disease in humans. This question is virtually impossible to address in patients, because SARS has not recurred in humans since 2004. SARS-CoV infects several species of animals, including mice, ferrets, hamsters, cats, and monkeys,[549] although most of these animals develop either mild or no clinical disease, making them not useful for studies of lethal SARS. However, serial passage of SARS-CoV in mice or rats resulted in the isolation of several rodent-adapted strains that cause severe disease in some strains of young mice and rats.[400,401,478] Most importantly, these strains cause a fatal disease in all aged rodents, paralleling the age-dependent severity observed in infected patients.[140] An age-dependent increase in disease severity is also observed in aged animals experimentally infected with the original human isolates, although disease severity is less than that observed with the mouse-adapted strains.[479] Animals with severe disease, whether infected with human isolates of SARS-CoV or rodent-adapted strains, show pathologic signs of ALI, increased levels of proinflammatory chemokines and cytokines, and diminished T-cell responses. These observations suggest that immune dysregulation contributes to severe disease in these animals, paralleling pathologic changes observed in infected humans.

## Immune Response and Viral Evasion of the Immune Response

As in most viral infections, both the innate and adaptive arms of the immune response are required for successful virus clearance and must be appropriately controlled to minimize bystander immunopathologic damage. One of the first steps in the host immune response to a coronavirus infection is the production of type I IFN (IFN-$\alpha/\beta$). Plasmacytoid dendritic cells (pDCs) are the source for most IFN-$\alpha/\beta$ produced in coronavirus-infected hosts, although other cells, such as macrophages, also express IFN.[63,484,657] pDC expression of IFN is mediated by signaling through a toll-like receptor (TLR) 7- and interferon regulatory factor (IRF) 7-dependent pathway. The importance of IFN signaling in the initial immune response to coronaviruses was shown using mice that are defective in expression of the IFN-$\alpha/\beta$ receptor (IFNAR$^{-/-}$).[63,244] Infection of IFNAR$^{-/-}$ mice with mildly virulent strains of MHV results in rapid and uniformly fatal diseases. Additionally, the importance of the IFN response is also evidenced by the multiple IFN evasive mechanisms that coronaviruses employ, as described later. Although the importance of the IFN response is well established, little is known about which specific IFN-induced proteins are most critical for protection. Ribonuclease L (RNase L) appears to have a role in the immune response to neurotropic strains of MHV[243]; however, whether this molecule is also important in the immune response to nonneurotropic strains of coronavirus remains to be determined.

Once the initial IFN response is induced, virus clearance requires expression of proinflammatory cytokines and chemokines and their receptors, such as CCL2, CXCL9, CXCL10, CCL3, to mediate T-cell and macrophage trafficking to sites of infection.[31] Infection of the CNS also requires breakdown of the blood–brain barrier, which is partially neutrophil dependent. In the absence of neutrophils or of neutrophil chemoattractants, such as CXCL1 and CXCL2, breakdown does not occur, resulting in more severe disease.[658] A robust T-cell response is required for destruction of infected cells and clearance of infectious virus. T-cell responses are poor in felines with progressive FIP (see Fig. 28.12) and in some strains of mice with severe SARS-CoV infections[112,653] Virus is not cleared in MHV- or SARS-CoV–infected mice that lack T cells, again demonstrating the importance of the response in clearance.[621] Both CD4 and CD8 T-cell epitopes have been identified in mice infected with MHV or SARS-CoV and in patients with SARS. Most epitopes are located on the N, M, and S proteins.[78,345,444,447] Once virus has been cleared, the proinflammatory response must be controlled to prevent immunopathology. In MHV-infected mice, regulatory CD4 T cells, characterized by Foxp3 expression, are important for dampening a potentially pathogenic immune response.[565] IL-10, another anti-inflammatory factor important for minimizing immunopathologic changes in MHV-infected mice, is expressed predominantly by virus-specific CD4 and CD8 T cells in the infected brain.[339,566] As described earlier for MHV-infected mice, T cells are responsible for initial virus clearance; however, an effective antivirus antibody response is required to prevent virus recrudescence.[338] Similarly, a robust neutralizing antibody response was detected in survivors during the 2002–2003 SARS outbreak.[56]

Coronaviruses use several approaches, both active and passive, to evade the host IFN response and thereby establish a productive infection (Table 28.4). Coronaviruses replicate in DMVs (see Fig. 28.8), which may shield viral RNA from recognition by intracellular sensor molecules, such as RIG-I, MDA5, and TLR3. Thus, in fibroblasts or conventional DCs infected with MHV or SARS-CoV, no IFN is induced.[173,586,656] However,

**TABLE 28.4 Coronavirus Proteins with Immunoevasive Properties**

| Protein | Virus source | Function | References |
|---|---|---|---|
| nsp1 | MHV, SARS-CoV, SARSr-BatCoV Rp3, BatCoV HKU4, BatCoV HKU9, TGEV | a. Suppresses host protein expression through direct inhibition of translation or by promoting degradation of host mRNA, including IFN mRNA | 230,258,259 |
| | | b. Inhibits IFN induction and signaling | 598,666 |
| nsp3 ($PL^{pro}$) | SARS-CoV, HCoV-NL63, MHV | Blocks IRF3 activation and NF-κB signaling | 91,136,174,654 |
| nsp3 (ADRP) | SARS-CoV, HCoV-229E, MHV | a. Interferes with IFN-induced antiviral activity | 158,297 |
| | | b. Enhances host proinflammatory cytokine expression | |
| nsp16 | MHV | Evades MDA5 activation, evades IFIT recognition | 106,665 |
| ORF 3b protein | SARS-CoV | Inhibits IFN synthesis and signaling | 283 |
| ORF 5a protein | MHV | Interferes with IFN-induced antiviral activity | 278 |
| ORF 6 protein | SARS-CoV | Inhibits STAT1 nuclear translocation | 175 |
| ORF 7 protein | TGEV | Interferes with PKR and 2′–5′ OAS/RNase L activities | 103 |
| N protein | MHV, SARS-CoV | Inhibits IFN induction; interferes with 2′–5′ OAS/RNase L activity | 283,637 |
| M protein | SARS-CoV | Inhibits IRF3 activation | 518 |

nsp, nonstructural protein; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARSr, severe acute respiratory syndrome–related; BatCoV, bat coronavirus; TGEV, transmissible gastroenteritis virus; mRNA, messenger RNA; IFN, interferon; $PL^{pro}$, papain-like protease; HCoV, human coronavirus; IRF, interferon regulatory factor; NF-κB, nuclear factor-kappaB; ADRP, adenosine diphosphate-ribose-1″-phosphatase; MDA5, melanoma differentiation-associated gene 5; IFIT, IFN-induced proteins with tetratricopeptide repeats; ORF, open reading frame; STAT, signal transducers and activators of transcription; PKR, double stranded RNA-dependent protein kinase; OAS/RNase L, oligoadenylate synthetase/ribonuclease L.

the IFN response does not appear to be actively blocked in these cells, because infection with Sendai virus or exposure to poly I-C induces IFN. In some cells, such as macrophages, microglia, and oligodendrocytes, coronaviruses induce an IFN response by signaling through MDA5, and in oligodendrocytes, RIG-I.[328,484] To counter IFN induction through activation of MDA5, all coronaviruses express a 2′-*O*-methyltransferase (nsp16; see the Expression of the Replicase-Transcriptase Complex section). In the absence of 2′-*O*-methylation, viral RNA induces a potent MDA5-dependent IFN response, which limits replication in wild-type animals but not in those deficient in IFNAR expression[665] (see Table 28.4). Additionally, SARS-CoV, but not MHV nsp3, inhibits IFN induction by antagonizing IRF3 and NF-κB function.[136,174]

Once IFNs are expressed, they bind to IFNAR, resulting in the up-regulation of a large number of interferon-stimulated genes (ISGs). Several coronaviral proteins inhibit either IFN signaling or specific ISGs (see Table 28.4). In addition to inhibiting IFN induction, the nsp16 2′-*O*-methyltransferase counters the ability of IFN-induced proteins IFIT1 and IFIT2 (also referred to as ISG56 and ISG54) to inhibit translation of viral mRNA.[106] N protein inhibits IFN signaling, as do SARS-CoV, MHV and TGEV nsp1, and SARS-CoV ORF3b and ORF6 proteins.[173] The mechanism of action of some of these proteins has been elucidated. The N protein interferes with 2′,5′-oligoadenylate synthase-associated RNase L activity.[637] Nsp1 appears to enhance host cell mRNA degradation and inhibit host cell protein synthesis, with specific effects on IFN signaling.[259,598,666] The karyopherin complex is required for nuclear import of STAT1, a critical component of the IFN signaling pathway, as well as the import of many other host proteins. SARS-CoV ORF6, by binding karyopherin $\alpha 2$, sequesters karyopherin $\beta 1$ in the cytoplasm, indirectly inhibiting nuclear translocation of STAT1.[175]

## EPIDEMIOLOGY

### Human Coronaviruses Other Than Severe Acute Respiratory Syndrome Coronavirus

Four known coronaviruses—HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1—are endemic in human populations. HCoV-OC43 and HCoV-229E cause up to 30% of all upper respiratory tract infections, based on several prospective studies.[245,389] The variable range of detection reflects year-to-year variability, detection methods, season, and age of subjects. These studies also suggest that peak activity occurs every 2 to 4 years.[184,264,397] In temperate climates, infections occur predominantly in the winter and early spring. HCoV-OC43 and HCoV-229E have also been associated with severe pneumonia in neonates and aged populations, especially those with underlying illnesses, such as chronic obstructive pulmonary disease, or those requiring intensive care.[163,198] The high rate of HCoV infections early in life and the pattern of infections during outbreaks demonstrate that HCoVs are efficiently transmitted in human populations, most likely via large and, to a lesser extent, small droplets. Serologic studies suggest that infection with HCoV-229E and HCoV-OC43 frequently occurs in young children and then repeatedly throughout life.[245,264,556,557] Neutralizing antibodies against HCoV-OC43 or HCoV-229E have been detected in about 50% of school-age children and up to 80% of adults.[264,389,458]

HCoV-NL63 and HCoV-HKU1 also have worldwide distributions, causing up to 10% of respiratory tract infections.[1,460] Initial reports suggested that HCoV-NL63 was associated with severe respiratory disease; however, subsequent population-based studies showed that most patients developed mild disease, similar to those infected with HCoV-229E or HCoV-OC43. HCoV-NL63 is also an important etiologic agent of acute laryngotracheitis (croup).[1] HCoV-HKU1 was initially identified in an elderly patient with severe pneumonia,


**FIGURE 28.14. SARS-CoV spread from infected bats to infect humans in wet markets in Guangdong Province, China.** SARS-related coronaviruses were detected in Chinese horseshoe bats and other bat species in China. The virus spread to human populations, likely animal handlers, in wet markets in Guangdong Province. Spread occurred either indirectly, via infection of exotic animals such as Himalayan palm civets, or directly, with subsequent human transmission to Himalayan palm civets and other exotic animals. This transmission occurred more than once, because a fraction of the animal handlers were positive for anti–SARS-CoV antibody.[203] In one episode, a physician taking care of an animal handler became infected. He then flew to Hong Kong and stayed at Hotel M, where he inadvertently infected several other people staying at the hotel, probably via superspreading events. These infected individuals then flew to other countries, resulting in the international outbreak. SARS-CoV, severe acute respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome.

although more recent studies suggest that it is associated with both mild and severe respiratory infections.[460,615]

## Severe Acute Respiratory Syndrome

During the 2002–2003 epidemic, SARS-CoV was isolated from several exotic animals, including Himalyan palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*), in wet markets in Guangdong Province in China[203] (Fig. 28.14). Subsequent investigations showed that SARS-CoV could not be detected in these animals in the wild but that severe acute respiratory syndrome–related coronaviruses (SARSr-CoV) could be isolated from wild bats in China[308,332] (see Table 28.1). Bats are now considered to be the ultimate source for SARS-CoV, with probable infection of human populations occurring after initial adaptation to animals in Chinese wet markets. Sequences from several distinct SARSr-CoVs have been amplified from Chinese horseshoe bats from Hong Kong and several provinces in China, and 30% to 85% of bats of this genus (*Rhinolophus*) had serologic evidence of infection with a SARSr-CoV. *N* gene sequences for three SARSr bat coronaviruses (BatCoVs) differed by 3% to 6%, similar to the level of difference between the N proteins

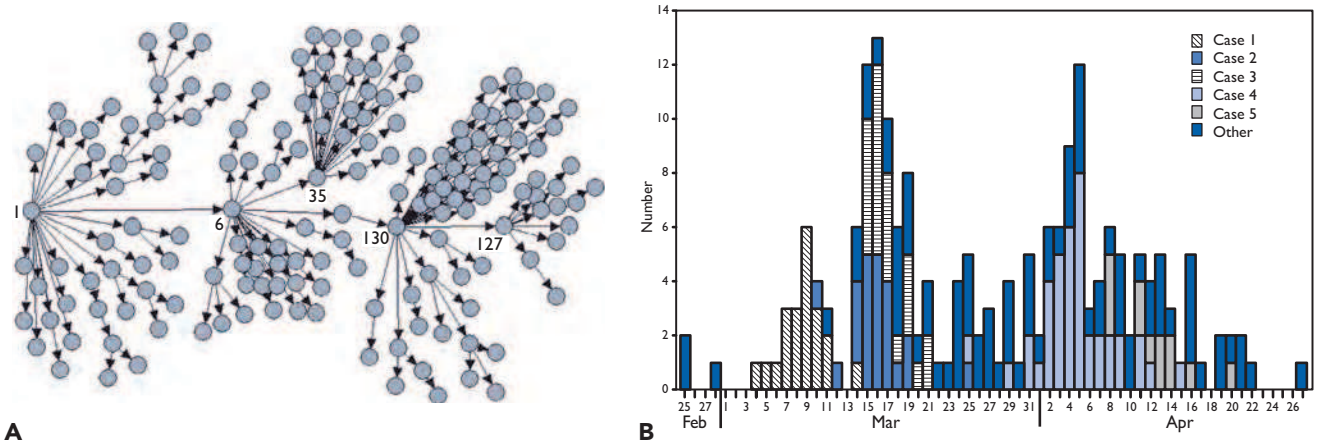



**FIGURE 28.15. Role of superspreading events in SARS-CoV epidemics.** SARS-CoV spread in Singapore in 2003, illustrated here, via superspreading and non-superspreading events. Most infected persons transmitted virus to fewer than five susceptible contacts. However, in a few instances, infected individuals were highly contagious, resulting in infection of larger numbers of contacts. The basis for superspreading events is not known but likely is a manifestation of larger virus burdens in a few infected patients. **A:** Probable cases of SARS by reported source of infection. **B:** Number of probable cases of SARS, by date of onset of fever and probable source of infection. SARS-CoV, severe acute respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome. (From Leo YS, Chen M, Heng BH, et al. Severe Acute Respiratory Syndrome — Singapore, 2003. *Morb Mortal Wkly Rep* 2003;52:405–411.)

of each of these viruses and that of SARS-CoV. This degree of difference between SARS-CoV and the various SARSr-BatCoVs indicates that the precise source of the 2002–2003 SARS outbreak viruses remains unknown. Neither SARS-CoV nor reconstructed BatCoVs can use the Chinese horseshoe bat ACE2 protein to enter target cells, raising the possibility that the bat host receptor is unrelated to ACE2[26]; alternatively, the virus that was the actual progenitor for SARS-CoV may have originated from a BatCoV distantly related to the SARSr-CoVs identified thus far.[225]

Serologic studies demonstrated that SARS-CoV had not circulated to a significant extent in humans prior to the outbreak in 2002–2003.[64,320] However, some persons working in wild animal wet markets in China had serologic evidence of a SARS-CoV–like infection acquired before the 2003 outbreak but reported no SARS-like respiratory illness.[203] Thus, virus may have circulated in these wild animal markets for a few years, with the SARS outbreak occurring only when a confluence of factors facilitated spread into larger populations. Although animals were the original source of SARS, its global spread occurred by human-to-human transmission. Transmission appeared to occur through close contact—that is, direct person-to-person contact, fomites, or infectious droplets and probably aerosols in some instances.[438] Because transmission usually only occurred after onset of illness and most efficiently after the patient was sufficiently ill to be hospitalized, most spread occurred in household and healthcare settings but infrequently in other settings.[440] There was also substantial patient-to-patient variation in efficiency of transmission, which, in part, was associated with the degree of illness severity. Many susceptible persons were infected in superspreading events; however, fortunately, only a minority of infected individuals were involved in this type of spread[342,475] (Fig. 28.15). Superspreading events, which occurred when a single individual infected multiple susceptible contacts, may have resulted from high virus burdens or a tendency for these individuals to aerosolize virus more efficiently than most infected persons. Most infected individuals spread the virus to only one or a few susceptible persons, suggesting that virus spread was relatively inefficient.[342,475] The outbreak was partly controlled using quarantining, and the lack of efficient spread contributed to the success of this approach. Because the SARS outbreak was controlled in June 2003, only 17 cases of SARS were subsequently confirmed, and none of these occurred after June 2004. Thirteen of these 17 cases resulted from laboratory exposures, including 7 secondary cases associated with one of the cases.[336] The other 4 cases occurred in southern China and resulted from exposure in the community, presumably to SARS-CoV–infected animals from wild animal markets.[334]

## Genetic Diversity of Coronaviruses

The SARS outbreak demonstrated the ability of coronaviruses to cross species, as the virus, naturally a bat virus, was able to infect small mammals, such as the Himalayan palm civet, and humans. Initially predicted from studies of coronavirus-infected cultured cells,[23] the ability of coronaviruses to cross species was also demonstrated when the betacoronaviruses HCoV-OC43, PHEV, and BCoV were analyzed[588] (Fig. 28.16). It is estimated that PHEV diverged from HCoV-OC43 and BCoV 100 to 200 years ago, whereas HCoV-OC43 and BCoV diverged about 100 years ago. Whether the common ancestor of HCoV-OC43 and BCoV was a human or bovine virus is not known. More recently, BCoV has crossed species to infect many ruminants, including elk, giraffe, and antelope,[4] and also canines.[159,160] Other phylogenetic studies suggest that the porcine alphacoronavirus TGEV resulted from cross-species transmission of a CCoV.[355]

In addition to their ability to cross species, coronaviruses readily undergo recombination (see the Viral RNA Synthesis section). Recombination events between canine (CCoV-I) and feline (FeCoV-I) coronaviruses and an unknown coronavirus resulted in the appearance of two novel viruses (CCoV-II and FeCoV-II).[355] In another illustration, new strains of IBV

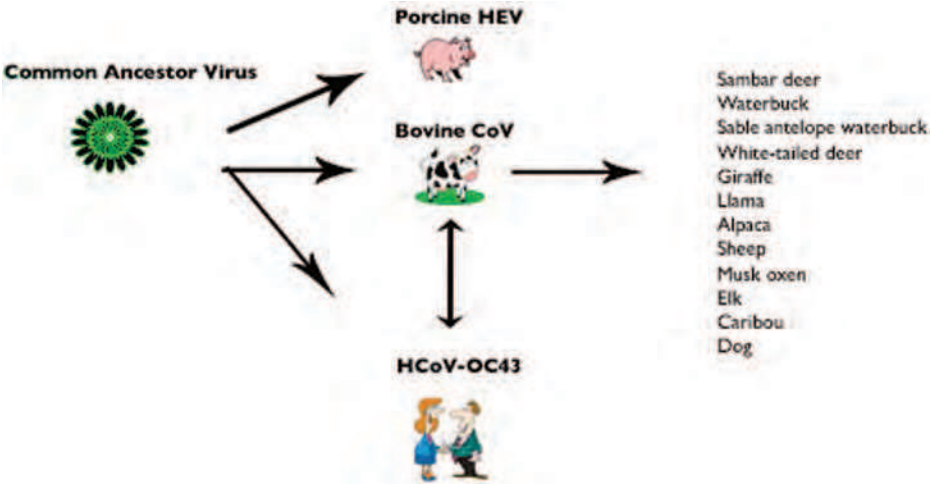



**FIGURE 28.16. Coronaviruses mutate and recombine to cross species barriers.** Phylogenetic analyses indicate that HCoV-OC43, BCoV, and PHEV shared a common ancestor and diverged about 200 years ago. More recently (100–130 years ago), HCoV-OC43 and BCoV diverged; however, it is not known whether BCoV infected human populations or HCoV-OC43 crossed species barriers to infect bovids. BCoV then spread to many ruminants and to dogs, probably via contact with infected domesticated cows. HCoV, human coronavirus; BCoV, bovine coronavirus; PHEV, porcine hemagglutinating encephalomyelitis virus.

have been detected in chicken populations and appear to have resulted from recombination between circulating vaccine and wild-type IBV strains.[284] This propensity for recombination has raised concerns about the use of live attenuated coronavirus vaccines (see the Prevention section).

## CLINICAL FEATURES

### Human Coronaviruses Other Than Severe Acute Respiratory Syndrome Coronavirus

In humans, coronaviruses have been clearly shown to cause respiratory disease, including its most severe manifestation—SARS. HCoVs have occasionally been implicated in enteric disease, particularly in newborns, using electron microscopy.[185,270,365] Electron microscopy has been used in these studies, because efforts to propagate human enteric coronaviruses in tissue culture cells have thus far been unsuccessful, hindering further studies. Because other particles in stool specimens (e.g., cellular membranes) can have similar morphology to coronaviruses, electron microscopic detection of coronavirus particles in stools is not considered diagnostic of infection. However, polymerase chain reaction (PCR) assays designed to detect coronavirus RNA sequences in pathologic specimens will now make it possible to determine whether these viruses play a role in enteric diseases. It seems likely that coronaviruses will be the etiologic agent in a fraction of patients with gastroenteritis, given the ability of these viruses to cause enteritis in a variety of domestic and companion animals.

Clinical features of infections in humans follow two distinct patterns: one for the non–SARS-CoV coronaviruses (i.e., HCoV-229E, -NL63, -OC43, -HKU1), and one for the zoonotic coronavirus SARS-CoV. Among the HCoVs, HCoV-229E and HCoV-OC43 were extensively characterized in volunteer studies in the 1960s.[389] Human volunteers inoculated intranasally with respiratory coronaviruses developed symptoms that included fever, headache, malaise, chills, rhinorrhea, sore throat, and cough, with peak infection observed 3 to 4 days following infection. About half of the volunteers challenged with virus developed illness, and approximately 30% were asymptomatically infected, as indicated by detection of virus in the upper respiratory tract. Symptoms lasted for a mean of 7 days, with a range of 3 to 18 days. Natural infection in both adults and children is also usually associated with a common cold–like illness.[44,389] Natural infection is probably acquired in a fashion similar to that for many other respiratory viruses (i.e., inoculation of infectious secretions from infected persons or fomites onto mucous membranes of the upper respiratory tract or inhalation of infectious droplets), with primary infection of ciliated epithelial cells in the nasopharynx.[3] Destruction of these cells, combined with exuberant production of chemokines and cytokines by resident and infiltrating cells, results in signs and symptoms of clinical illness.

HCoV infections are also occasionally associated with lower respiratory tract disease in children and adults. Coronaviruses have been detected in children hospitalized with lower respiratory tract disease at varying rates, although usually less than 8% of patients.[88,155,179,556,557,572] One caveat is that coronaviruses are also sometimes detected in well, control patients; thus, the presence of virus may not be etiologically related to the illness.[108] Coronavirus infection has also been detected in adults with acute respiratory tract illness, including about 5% of those hospitalized with lower respiratory tract disease.[108,155,163,182,198] Studies using PCR to detect viral RNA in middle ear fluids suggest that coronaviruses, like other respiratory viruses, can cause otitis media.[454,455] In addition, HCoVs have been associated with wheezing and exacerbations of asthma.[245,556] HCoV-NL63 and HCoV-HKU1 have also been detected in persons with acute upper and lower respiratory tract illness,[1,108,182,556,557] and as described earlier, HCoV-NL63 is associated with croup in children younger than 3 years.[574] Studies of natural infection and volunteer studies have shown that reinfection with coronaviruses is common, demonstrating that infection does not induce stable protective immunity.[245,264,389] For example, previously infected volunteers developed symptomatic disease if infected 1 year later with the same strain of HCoV-229E.[470]

### Severe Acute Respiratory Syndrome Coronavirus Infections

In contrast to the mild illness usually associated with HCoV infections, SARS-CoV have nearly always resulted in a serious lower respiratory tract illness that required hospitalization, often in an intensive care unit (up to 20% of infections)[438] (Fig. 28.17). In the 2002–2003 epidemic, approximately 8,000 individuals were infected, with an overall mortality rate of 10%. Disease severity increased proportionally with age. Thus, no mortality occurred in patients younger than

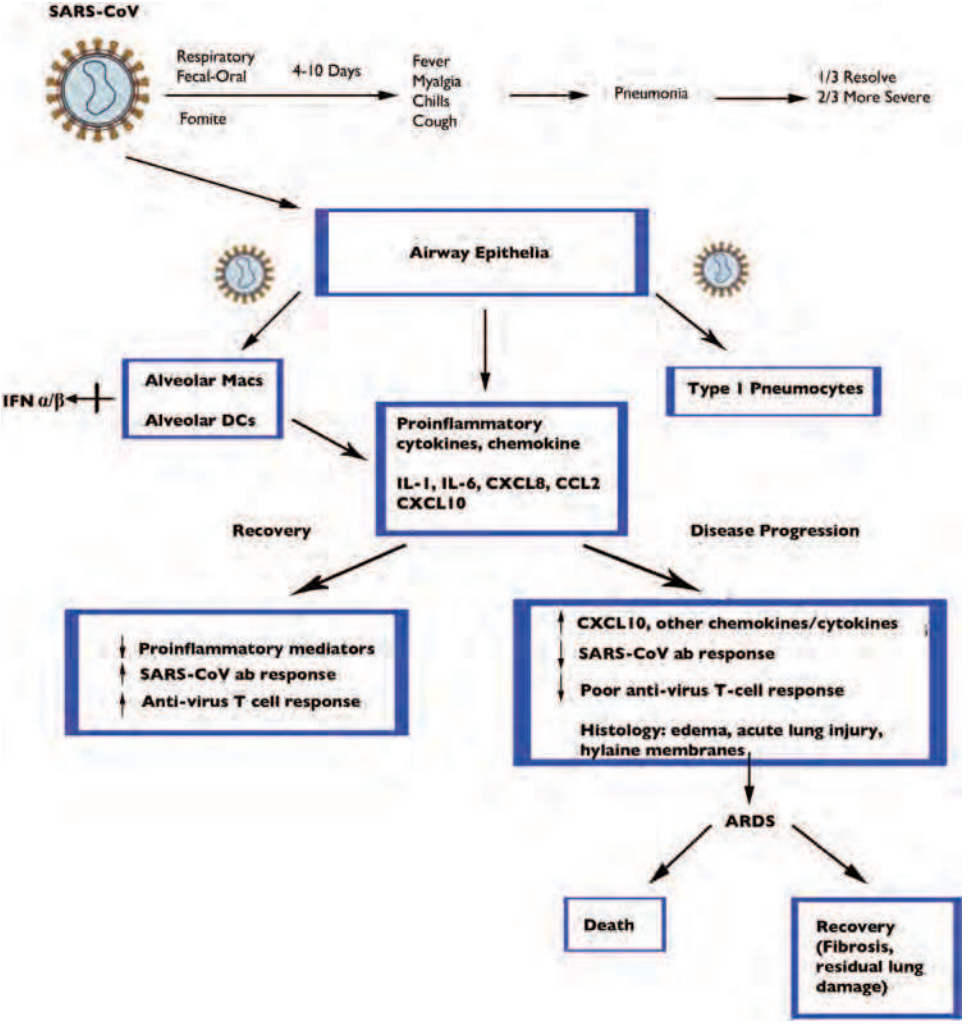



**FIGURE 28.17. Clinical disease in patients infected with SARS-CoV.** SARS-CoV spread to susceptible individuals via respiratory and fecal–oral routes and, less commonly, if at all, via fomites. Virus replication was initiated in the upper airway epithelial cells, based primarily on animal studies and *in vitro* studies using primary cultures of airway epithelial cells. Virus subsequently spread to the lower respiratory tract, with infection of type 1 pneumocytes and macrophages and dendritic cells most prominent. The infection of the latter two cell types was abortive, resulting in production of proinflammatory cytokines and chemokines such as CXCL10 and CXCL8 but not type 1 IFN. In patients who recovered, expression of proinflammatory cytokines diminished, and robust antivirus antibody responses were detected. In patients who developed progressively more severe disease, cytokine production continued and patients remained lymphopenic without developing an effective anti-SARS-CoV antibody response. Some of these patients died, and significant long-term morbidity was found in many of the survivors. SARS-CoV, severe acute respiratory syndrome coronavirus; IFN, interferon.

24 years, although about 50% of infected individuals older than 60 years succumbed to the infection. Mortality was also greater in patients with underlying disease. Clinical disease in patients with SARS was not diagnostic; however, some features were more common in SARS patients compared to those infected with other pathogens.[35,318,437,438] Illness usually had an onset of 4 to 7 days, although occasionally an incubation period of as little as 2 days or as long as 10 to 14 days was observed. Disease was characterized by systemic symptoms such as fever, malaise, and myalgias. Unlike many other respiratory tract infections, upper respiratory tract signs and symptoms such as rhinorrhea, sore throat, and nasal congestion were not common, although they still occurred in a minority of patients. The first lower respiratory tract symptoms (usually a nonproductive cough and shortness of breath) developed several days after onset of systemic symptoms. Respiratory symptoms were often accompanied by evidence of involvement of other organ systems. Thus, whereas diarrhea occurred at disease onset in fewer than 25% of patients, up to 70% developed gastrointestinal disease during the course of the illness. Most patients developed abnormal liver function tests (70%–90%) and lymphopenia (70%–95%), with a substantial drop in both CD4 and CD8 T-cell numbers.[104,438] Patients who failed to resolve their illness often had progressive respiratory failure leading to ARDS and death weeks to months

after illness onset.[56,171,321] In these patients, lymphocyte and platelet counts remained abnormally low, whereas neutrophilia and elevated titers of virus or viral RNA in clinical specimens for prolonged periods of time were common features. Asymptomatic or mild illness was uncommon, as illustrated by studies of exposed healthcare workers. In these studies, fewer than 1% of those without a SARS-like illness had serologic evidence of infection.[70,218,466] Most survivors of SARS-CoV infection achieved full recovery, although pulmonary function abnormalities sometimes took months to subside.[86,626] Some, however, had persistently abnormal pulmonary function. Curiously, a fraction of survivors showed more evidence of neurologic or psychiatric disease than expected based on the degree of respiratory illness or steroid use. Although brains were not commonly studied during the 2002–2003 epidemic, a few studies did demonstrate SARS-CoV infection of the brain, suggesting that CNS infection may have occurred in some cases.[84,201,307,316]

## DIAGNOSIS

Most HCoV infections, other than SARS-CoV, are not diagnosed because they cause mild, self-limited upper respiratory disease, and no specific therapy is available. Diagnosis is laboratory-based because coronavirus infections cannot be distinguished clinically from other causes of upper respiratory tract infections, such as rhinoviruses. However, in some clinical settings, such as in hospitalized patients with pneumonia and in epidemiologic studies, specific diagnosis is important. Coronavirus infections in animals and humans were initially diagnosed by isolation of infectious virus, by electron microscopy, and in serologic assays, with the caveat that some coronaviruses, especially those in the stool, are not easily cultured. HCoV-229E and related alphacoronaviruses have sometimes been isolated in human diploid cell lines. Other HCoVs, most notably HCoV-OC43, initially required cell organ culture systems for isolation,[389] although this virus can now be grown in tissue culture cells. HCoV-NL63 can infect monkey kidney LLC-MK2 cells or Vero cells,[170,460,573] whereas HCoV-HKU1 has been grown only in primary human airway epithelial cells.[463] RT-PCR–based methods and immunofluorescence assays (IFA) for virus antigen have largely replaced these other methods for the diagnosis of respiratory coronavirus infections.[108,156,163,198,392] PCR primers can be designed to be broadly reactive or strain specific, based on primer location and design. With a sensitive system to detect the PCR amplicon (e.g., a real-time assay), fewer than five RNA copies in the reaction mixture can be consistently detected.[156] A multiplex real-time RT-PCR assay has also been described that is able to detect all four respiratory coronaviruses and may become the diagnostic method of choice.[184]

Electron microscopic examination of clinical material, although laborious, contributed to the identification and characterization of many coronaviruses, including SARS-CoV.[143,288,389,439] At present, electron microscopy is used most commonly to identify coronaviruses in patients with enteritis,[270] because none of these coronaviruses have been cultured; however, because other particles in clinical specimens can resemble coronaviruses and coronaviruses may be present without causing disease, identification of such particles does not confirm infection.

Various serologic assays have been used to detect coronavirus infections, including complement fixation, hemagglutination inhibition (HI) for viruses with an HE protein (i.e., some betacoronaviruses), neutralization, IFAs, and enzyme-linked immunoassays (EIAs). Initially, these assays used virus lysates or inactivated whole virus; more recently, cloned expressed proteins, synthesized peptides, and pseudoviruses have been used as antigens for serologic assays.[319,357,389,418,458,558]

SARS or another coronavirus infection of equivalent severity presents a different diagnostic situation. A specific diagnosis is critical because a positive result will guide clinical management and have public health implications. However, testing should only be considered when, based on the likelihood of an exposure and clinical features of the illness, infection is plausible. SARS-CoV was initially isolated in fetal rhesus kidney cells and Vero cells; however, during the 2002–2003 epidemic, a combination of serologic and RT-PCR assays, not virus culture, were used to detect and confirm SARS-CoV infection.[440] With very sensitive PCR assays (e.g., a nested or real-time PCR assay) and RNA extraction procedures that increased the amount of specimen available for the assay, the positivity rate in respiratory specimens obtained during the second and third days of illness increased from less than 40% to more than 80% as the epidemic progressed.[459] SARS N protein EIA was positive in 50% to 80% of serum specimens collected during the first week of illness[74] and in more than 50% of respiratory and stool specimens collected during the second and third weeks of illness.[309] SARS-CoV–specific antibodies were usually detected by 14 days into the illness, although sometimes not until 4 weeks after infection.[229,233] Whereas RT-PCR provided the best way to make an early diagnosis, serologic assays were important in confirming or ruling out SARS-CoV as the cause of infection. Because serum specimens from persons not infected with SARS during the 2002–2003 outbreak have rarely tested positive for SARS-CoV antibodies,[320] a single serum specimen positive for SARS-CoV antibodies was usually considered diagnostic; a negative test on a serum specimen collected late in the illness (28 days or later after onset of illness) could be used to rule out SARS-CoV infection.

## TREATMENT

At present, there are no antiviral drugs for HCoV infections, and therapy is supportive. During the major part of the SARS epidemic, most patients were treated with ribavirin or high-dose steroids, based on the idea that the virus would be susceptible to ribavirin and steroids might diminish immune-mediated bystander damage.[538] Late in the outbreak, based on their ability to inhibit SARS-CoV replication *in vitro* and/or in experimental animals, IFN-$\alpha$, SARS convalescent-phase immune globulin, and lopinavir plus ritonavir (two protease inhibitors licensed for the treatment of HIV) were used to treat patients.[65,85,89,356,530] However, a large-scale review of all of these therapies concluded that whereas some showed efficacy in inhibiting SARS-CoV replication in tissue culture cells, none showed a beneficial effect in patients.[538] The molecular biology of coronavirus infection suggests several potential targets for antiviral drugs, including the viral RdRp, virus-encoded proteases, host cell receptors used by the virus for entry, and the viral S glycoprotein. Subsequent to the outbreak, several antiviral drugs targeting these

viral proteins or processes have been developed and evaluated for their ability to inhibit SARS-CoV replication *in vitro.* These include specific coronavirus protease inhibitors,[468] monoclonal antibodies that inhibit SARS-CoV binding to cells,[551] peptides from the heptad repeat regions of the S protein or from ACE2 that inhibit receptor binding or fusion,[40,211] and small interfering RNAs.[71] If SARS or another severe coronavirus-mediated disease emerges, *in vitro* and animal model studies of antiviral drugs will be used to guide treatment.

## PREVENTION

No vaccines are available to prevent HCoV infection; however, vaccines against common veterinary coronaviruses, such as IBV and CCoV, are routinely used to prevent serious disease in young animals. Efforts are ongoing to improve these vaccines and to enhance safety and efficacy while minimizing the likelihood of reversion to a virulent strain.[492] In addition, various SARS-CoV vaccines have been developed, including inactivated whole virus, live virus vectors expressing single viral proteins and recombinant proteins, and DNA vaccines.[10,480] Nearly all of these vaccines express the surface glycoprotein and are designed to induce SARS-CoV neutralizing antibodies. For some of these vaccines, efficacy has been demonstrated in animal models. Large stocks of anti–SARS-CoV neutralizing antibody have been prepared and will be used for passive immunization of healthcare workers and other high-risk personnel if SARS recurs.

In general, live attenuated vaccines are likely to be most effective in inducing protective immune responses against coronaviruses. This has been illustrated elegantly in the case of TGEV—an important cause of neonatal diarrhea and death in swine. In the mid-1980s, a naturally occurring, attenuated variant of TGEV—PRCoV—was identified in pig populations. This virus, which causes mild disease and no enteritis, induces an immune response in pigs that is protective against TGEV and largely eliminated it from dually infected populations.[312] Live attenuated vaccines induce not only neutralizing antibodies but also antivirus T-cell responses, which are required for virus clearance from infected cells in SARS and other coronavirus infections. However, the development of live coronavirus vaccines is challenging.[492] First, in many instances, natural infection does not prevent either subsequent infection or disease, therefore an effective vaccine would need to be superior to immunity induced naturally. Second, the genetic and antigenic variability of coronaviruses and their ability to readily recombine hinder vaccine development. Thus, a vaccine may not provide equal protection from all antigenic variants, and subsequent recombination with vaccine strains could increase the number of different strains circulating in the wild. As an example, recombinants of IBV vaccine strains with virulent wild-type strains have caused disease outbreaks in chicken flocks.[249,596] In addition, the finding that immunization with an S protein–expressing FIPV vaccine led to more severe disease after subsequent natural infection raises the concern that other coronavirus vaccines might also enhance, rather than protect, from disease.[581] Several strategies to minimize the likelihood of recombination and to attenuate candidate vaccines without compromising efficacy have been recently described. These include engineering viruses with deletions in nsp1, important for the anti-IFN response,[666] or in E protein, important for virus assembly.[124] In other approaches to minimizing the likelihood of recombination of vaccine viruses, the coronavirus genome has been reconstructed, changing the order of structural genes at the 3′ end[121] or modifying the leader and body TRSs (see the Viral RNA Synthesis section) to eliminate homology with natural virus sequences.[644]

In the absence of effective vaccines and antiviral drugs, the most important ways to prevent coronavirus infections are a highly active public health surveillance system and good infection control practices. This was demonstrated unequivocally during the SARS outbreak in 2002–2003, in which sharing of information by national public health agencies and governments and involvement of international agencies such as the World Health Organization resulted in the rapid identification of a coronavirus as the cause of SARS and implementation of measures that minimized spread. At the local level, strict attention to good isolation and infection control practices and identification and management of exposed persons (contacts) minimized human-to-human spread of the virus within a few months of its global spread. The low risk of SARS-CoV transmission before hospitalization and the low rate of asymptomatic infection facilitated the efficacy of these public health measures.[70,218,466] The identification of cases of laboratory-acquired SARS-CoV, with subsequent transmission to others after one of these cases,[334,336] reinforces the importance of strict attention to safe laboratory practices. These practices include handling the virus in the appropriate type of facility, using standardized operating procedures, and providing appropriate training and medical surveillance programs for staff.

## PERSPECTIVES

Many important problems remain to be resolved by future studies of coronaviruses. One critical task will be to broaden our picture of how coronaviruses jump between species. We need to know whether cross-species viral trafficking events, both abortive and successful, are rare or common. Although there has been a recent expansion of our knowledge of spike protein interactions with receptors and associated proteases, we cannot yet fully gauge the height of the barrier preventing productive adaptation by a spike protein to new receptors and proteases. Such information will be directly relevant to forestalling or coping with the re-emergence of a SARS-related (or other) coronavirus from ubiquitous bat reservoirs. Related to this is the challenge of developing *in vitro* culture systems for virus species that are currently only known through their genomic sequences. A second area of crucial importance will be to further develop our understanding of the immunopathogenesis of the more severe human and animal coronaviruses and to more precisely delineate the correlates of immune protection. This will better inform the effective design and evaluation of vaccines for control of these agents. Finally, one of the most exciting areas of future research will be to address the many gaps in our basic knowledge of the intricacies of the coronavirus RTC—the largest and most complicated machinery of RNA synthesis found in any RNA virus. The past few years have seen tremendous advances in this field, particularly in structural and biochemical studies, and it is likely that progress will continue apace. A long-term goal will be the total *in vitro* reconstitution of coronavirus RNA synthesis, which would definitively demonstrate the roles

of the many viral replicase subunits as well as those of putative host factors. It can be expected that studies of this type will reveal fundamental principles common to all RNA-dependent RNA synthesis, in addition to mechanisms unique to the order *Nidovirales*. Knowledge derived from this enterprise will be critical for the design of antiviral drugs to combat diseases caused by existing and emerging coronaviruses.

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