CHAPTER 10

THE MOLECULAR BIOLOGY OF CORONAVIRUSES

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INTRODUCTION

The family Coronaviridae consists of eleven viruses which infect vertebrates and cause a range of diseases mostly affecting the respiratory or the gastrointestinal tract (1). The name *coronavirus* was coined by Tyrrel *et al.* (2) on the basis of the characteristic *corona* of widely spaced bulbous surface projections, 12-24 nm long, seen in electron micrographs of these viruses (Figure 1). More recently, it has become clear that members of the *Coronaviridae* share a number of molecular biological features which constitute a unique replication strategy and set these viruses apart from other virus groups. These features may underlie the fascinating biological properties of certain murine coronaviruses which provide one of the best available models for human demyelinating neurological diseases (3).

1. BIOLOGICAL PROPERTIES

Coronaviruses affect several species, including chicken, cattle, pigs, dogs, cats, mice, rats and man (Table 1). Although acute respiratory or enteric diseases are common features of coronavirus infections, there are several examples of chronic persistent infections, and these may involve the central nervous system. Mouse hepatitis virus (MHV) exists in four strains, one of which (MHV-4, JHM strain) can induce chronic or recurrent central nervous system demyelination as well as encephalomyelitis in both mice and rats (3-5). The exact outcome of infection depends upon the age and strain of the rodent host as well as the dose and route of inoculation. There is evidence that pathogenicity of JHM virus is genetically controlled, since virus mutants causing demyelination rather than encephalitis can be selected as *ts* mutants (6) or using monoclonal antibodies (7). Perhaps the most interesting rodent model is the infection of 10-15 day old rats with a mutant of JHM

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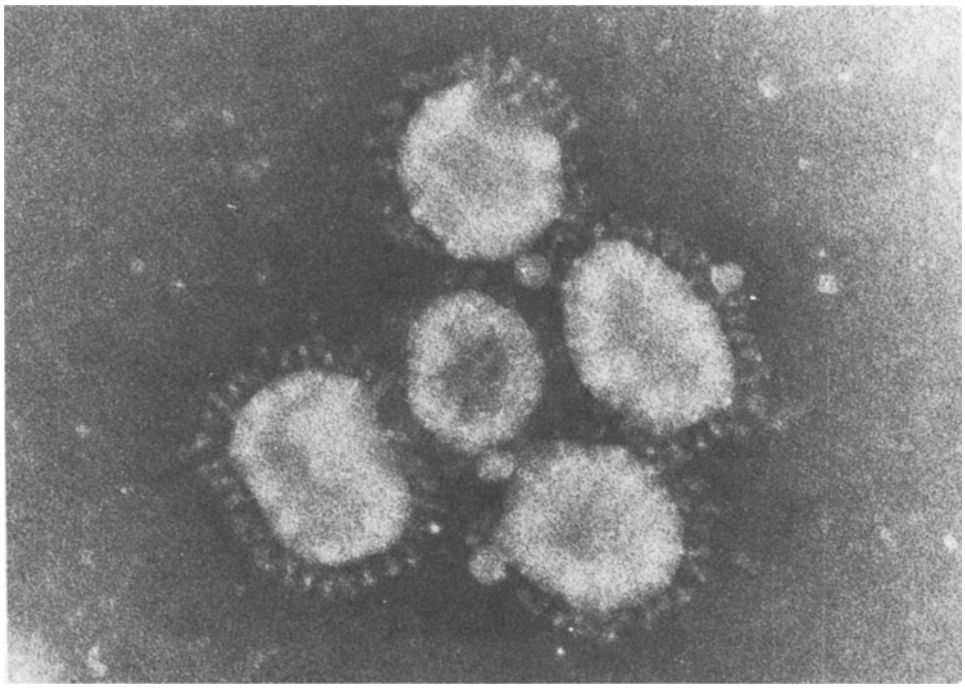

Figure 1. Virions of avian infectious bronchitis virus (electron micrograph courtesy of Dr. Cavanagh and J.K.A. Cook).

virus (*ts* 43), which results in a high proportion of persistently infected animals that develop a subacute relapsing-remitting demyelinating disease with close parallels to multiple sclerosis in man (5). Although the isolation of two human coronaviruses, which may have originated from the brain tissue of two multiple sclerosis cases, was reported some years ago (8), the observation has not been confirmed and is not supported by serological surveys (9). Moreover, owing to the circumstances of their isolation, the viruses in question may be of murine rather than human origin (10). However, the JHM strain of mouse hepatitis virus provides an important virus model for multiple sclerosis (11), despite the fact that clinical epidemiological evidence is more supportive of a paramyxovirus model (12).

In general, human coronavirus infections are found to cause only acute respiratory disease. The avian virus causing acute infectious bronchitis is of considerable economic importance, but may also infect non-respiratory tissues such as lymph node or kidney, to cause long-term persistent infectious even in the presence of high serum antibody wich result eventually in a fatal disease (13,14).

A different type of persistence, involving cells of the reticuloendothelial system, is found in cats with feline infectious peritonitis virus infection. This virus primarily infects macrophages, where a persistent infection becomes established and immune complexes of virus and IgG subsequently develop and become

deposited in the kidney, the severity of the disease being greatest in cats with high antibody titres (15).

The ability of several coronaviruses to establish persistent infections in their hosts can also be studied, using *in vitro* model systems, where it has proved relatively easy to obtain persistently infected cell cultures, particularly in cells of neural origin (11). In a detailed study, Baybutt *et al.* (16) established a persistent infection of Sac(-) cells with JHM virus. The cultures released high levels of virus for more than 100 passages, but the cells were not killed, as in normal JHM infection. It was concluded that a variant JHM virus (JHM-Pi) of reduced cytotoxicity was responsible for the persistent infection (16). Clearly, the molecular events which lead to the production of such a virus variant require further study; comparison of variant and wild-type replication could shed light on how persistent infections become established in the central nervous system of the infected host.

2. CORONAVIRUS STRUCTURE: GENOME RNA AND POLYPEPTIDES

The coronavirus genome is a large, non-segmented, single-stranded RNA molecule which reportedly varies in length in different viruses between 15 kb and 27 kb (5×10^6 to 8×10^8 molecular weight). Recent estimates for the best-studied coronaviruses put the lengths of both avian coronavirus (IBV) and of mouse hepatitis virus RNA at 27 kb (7, S.G. Siddell, personal communication) and of porcine transmissible gastroenteritis virus (TGEV) RNA at 23.6 kb (18). Most

Table 1. – Coronaviruses

Antigenic group	Name	Natural Host	Principal Disease
I	Avian infectious bronchitis (IBV - Many serotypes)	Chicken	Respiratory, Lymphatics, Kidney
II	Turkey coronavirus (TCV)	Turkey	Respiratory, Enteritis
III	Human coronavirus (HCV-229E)	Human	Respiratory
	Transmissible gastroenteritis Virus (TGEV)	Pig	Enteritis
	Feline infectious peritonitis (FIPV)	Cat	Peritonitis, Granulomas in many organs
	Feline enteric coronavirus (FEC)	Cat	Enteritis
	Canine coronavirus	Dog	Enteritis
IV	Mouse hepatitis virus (MHV - Many serotypes)	Mouse	Hepatitis, Enteritis, Encephalomyelitis
	Rabbit coronavirus (RbCv)	Rabbit	Enteritis
	*Bovine coronavirus (BCV)	Cow	Enteritis
	*Haemagglutinating encephalomyelitis virus (HEV)	Pig	Vomiting and wasting
	*Human coronavirus (HCV-OC43)	Human	Respiratory

* These viruses all haemagglutinate.

molecular biological studies have been carried out either on IBV or on MHV, and these form the basis for this review.

The genome RNA is polyadenylated at the 3' end, contains a 7-methyl guanosine cap structure at the 5' end, and is infectious (1). The virion RNA is therefore of positive polarity, and can function directly as mRNA without the requirement for virion enzymes or other proteins.

The genes encoding virion structural polypeptides are located within the 3' half of the genome, and this has facilitated sequence analysis and, hence, predicted their primary structure. For both IBV and MHV, sequences of the major structural proteins have now been determined after molecular cloning of cDNA synthesized from the 3' poly A tail hybridized to oligo dT as the initial primer.

Within the virion, the genome RNA is associated with a phosphoprotein of 50K to 60K molecular weight, termed the nucleocapsid (N) protein, to form a ribonucleoprotein (RNP). This structure is surrounded in the virion by a lipoprotein envelope which consists of host cell-derived lipids and two major virus-specified glycoproteins. One of these is an integral transmembrane glycoprotein which has a variable molecular weight, depending upon the degree of glycosylation, of 23K to 34K (20). The other envelope glycoprotein projects from the virion to form the spikes which make up the *corona* structure seen in electron micrographs of the virus. The nomenclature in current use for these two envelope proteins is confusing: the smaller transmembrane glycoprotein is termed M (matrix) in IBV, but E_1 (envelope 1) in MHV; the larger glycoprotein is termed S (spike) in IBV, but E_2 (envelope 2) in MHV. For reasons which have been discussed previously (10), the terms M and S will be used in this review. A diagrammatic representation of a coronavirus particle is shown in figure 2.

A third envelope glycoprotein, the haemagglutinin (H) protein, has been described in haemagglutinin mammalian coronaviruses, including bovine enteric coronavirus, porcine haemagglutinating encephalomyelitis virus, and human coronavirus OC 43 (21, 22).

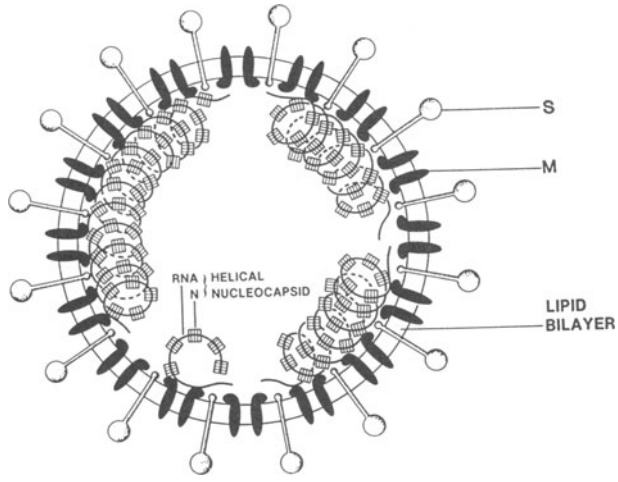

Figure 2. Model of a coronavirus. (Adapted from Holmes, K.V. (87)).

A. The N Polypeptide

The primary structure of the N polypeptide has been determined for several coronaviruses, since this protein is encoded in the genome adjacent to the 3' terminus, which is polyadenylated. In MHV, the nucleotide sequence of this gene revealed a single open reading frame encoding a protein of 50,000 molecular weight which is rich in basic residues (23, 24). The N protein is phosphorylated on serine residues, and a protein kinase activity has been detected in virions of MHV strain JHM which may be responsible for this phosphorylation (25).

Nucleotide sequences of the N protein genes of two strains of IBV (Beaudette and M41) have also been determined and compared with the MHV sequences (26). The predicted molecular weight of the IBV N protein is 45,000 and the two IBV strains differ mainly in that the 3' non-coding region of the Beaudette contains a 184 nucleotide segment not present in M41. Considerable homology was found between the N protein sequences of IBV and MHV, including the positions of clusters of serine residues where phosphorylation occurs (26).

B. The M Polypeptide

The matrix polypeptide is an integral membrane protein which is extremely hydrophobic and has unusual features when compared with most other viral glycoproteins, including the coronavirus S glycoprotein. In MHV and in bovine coronavirus, the carbohydrate moiety lacks mannose and is O-linked to serine and threonine residues (27-29), but in IBV it is mannose-rich and N-glycosidically linked (20, 30, 31).

An elegant series of *in vitro* experiments by Rottier *et al.* (28) showed that integration of M polypeptide into membranes occurs without cleavage of an N-terminal signal peptide sequence, although it requires a signal-recognition particle (32). These features may be important in the accumulation of M polypeptide in the endoplasmic reticulum, where coronavirus budding occurs (rather than at the plasma membrane as with most other enveloped viruses).

With both MHV (28) and IBV (33), studies using proteases have shown that only small portions of M protein at the amino- and carboxy termini are susceptible to digestion and hence lie outside the membrane. The extremely hydrophobic nature of the M protein has been confirmed from its predicted sequence for both IBV and MHV. Although the nucleotide sequences of the M protein gene show little homology between the avian and murine viruses, the amino-acid sequences can be aligned to 27% homology (29), this being concentrated in the amino-terminal half of the protein. From theoretical analyses, a topological model has been suggested according to which the M protein is anchored in the lipid bilayer by three successive membrane-spanning helices present in the amino-terminal half, whereas the carboxy-terminal half is associated with the membrane surface (29). Recently, the complete sequence of the M gene of MHV strain IHM has been obtained; the amino-acid sequence shows only 7 conservative changes as compared to the A59 sequences, and all the potential O-glycosylation sites are conserved (29a).

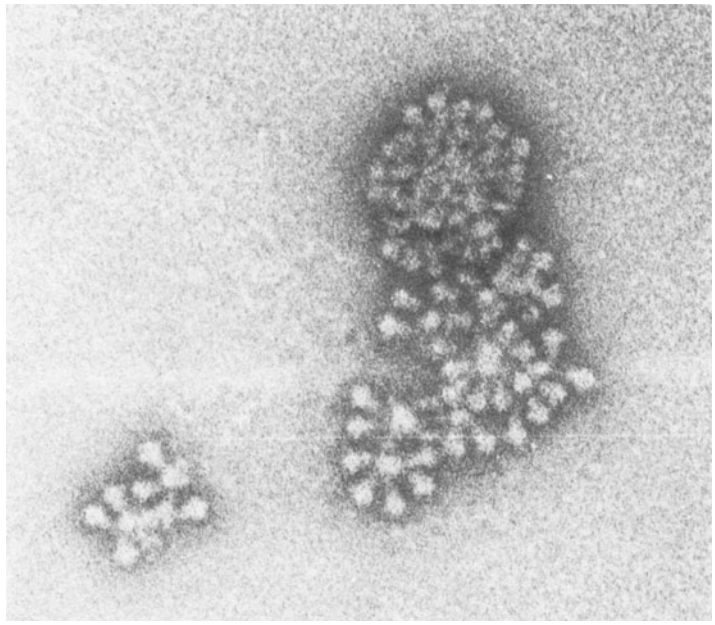

Figure 3. Purified spikes of avian infectious bronchitis virus prepared as described in reference 35 (electron micrograph courtesy of D. Cavanagh).

C. The S Polypeptide

The S polypeptide projects from the virion surface and can be seen in the electron microscope (Figure 3) as bulbous or "tear-drop" shaped in purified preparations (34, 35). Only a small anchor region of the protein is embedded in the envelope lipid bilayer, and the peplomer portion is responsible for attachment to cells as well as mediating cell fusion, since both activities are inhibited by monoclonal antibodies specific for S (36, 37). Clearly the S protein appears analogous to the F protein of paramyxoviruses or the hemagglutinin (HA) protein of influenza virus, and this suggests that proteolytic cleavage of S may be necessary for full biological activity.

Recent structural studies on the S protein of the avian coronavirus, IBV (Beaudette strain), largely support this analogy. The sequence of the S gene predicts a large polypeptide of 127,000 molecular weight (38), which is close to the estimate of 125,000 obtained for S after removal of carbohydrate residues by endoglycosidase H (20). The sequence also predicts a hydrophobic signal sequence which is not present in the mature protein, 28 potential N-glycosylation sites, and a membrane *anchor* region of 44 non-polar amino-acids close to the carboxy-terminus (38).

Stern and Sefton (39) first demonstrated that the S protein of IBV is a precursor to two smaller polypeptides, S1 and S2, which are derived by proteolytic

cleavage. The mature spike protein is an oligomer comprising two or possibly three copies of each of S1 and S_2, anchored in the virus membrane by S_2 (35). It is possible to remove the S_1 portion of the spike selectively using urea, and this abolished infectivity and haemagglutination but not attachment to cells (40). Monoclonal antibodies binding to S_1 alone neutralise infectivity and haemagglutination (37). An arginine-rich sequence has been found at the cleavage site between S_1 and S_2; this consists of 5 residues which after removal by carboxypeptidase activity would leave an S_1 component of 514 residues and an S_2 component of 625 residues (41).

There is evidence from monoclonal antibody binding studies that the S protein of murine viruses undergoes antigenic variation, in marked contrast to the M and N proteins (42). Recently, the sequence of the S protein gene was determined for MHV strain JHM (S.G. Siddell, personal communication) and for a second strain of IBV (M41). The M41 sequence revealed 50 amino-acid differences from the Beaudette strain, and these were clustered in two regions which may be "hot spots" where epitope variations occur naturally (43). The MHV sequence was quite different from the IBV sequences, although DIAGON plots show considerable similarity in the S_2, but not the S_1 region of the polypeptide. Overall, the S polypeptide of MHV has a molecular weight in the unglycosylated form of 137,000, with 21 potential glycosylation sites. The proteolytic cleavage site between S_1 and S_2 has the basic sequence Arg-Arg-Ala-Arg-Arg.

D. Other Proteins

Although most coronaviruses have three major virion structural proteins, N, M and S, a fourth protein (H) has been reported on the haemagglutinating mammalian coronaviruses, including porcine encephalomyelitis virus, bovine enteric coronavirus, and human coronavirus OC43. This glycoprotein has a molecular weight of 130,000 and consists of two disulphide-linked subunits each of 65,000 molecular weight (21, 22). The putative gene encoding this H protein has not been described; a surface glycoprotein of 65,000 molecular weight distinct from S has also been found in MHV strain JHM (44), although haemagglutination has not been described with this virus.

In addition to these major virion proteins, coronavirus-infected cells contain virus-coded polypeptides of 14,000 and 30,000 molecular weight (1, 44-48). A polypeptide of 14,000 molecular weight has occasionally been reported as a minor virion component, but the 30,000 molecular weight proteins seems to be non-structural.

Now that extensive nucleotide sequences are available for both the IBV and MHV genomes, various open reading frames have been described which predict further virus-specified polypeptides not yet identified in infected cells (49-52). The current position regarding the various gene products of IBV and MHV is summarized in Table 2.

3. REPLICATION

Coronaviruses attach to their host cells by the peplomer S protein, but virus from which the S_1 subunit has been selectively removed is still able to attach (40).

Table 2. – Coding assignments of coronavirus RNAs

IBV		RNA	MHV	
mRNA	Product*	Length (Kb)	mRNA	Product
A	Nucleoprotein	2.0	7	Nucleoprotein
B	(B_1 - 9.5K) (B_2 - 7.5K)	2.5	6	Matrix
M	?	3.2	5	(5_1 - 12K) (5_2 - 10K)
C	Matrix	3.6	4	Nonstructural 14K
D	(D_1 - 6.7K) (D_2 - 7.4K) (D_3 - 12.4K**)	4.5		
E	Spike	7.8 10.3	3 2	Spike Nonstructural 30K
F	(Polymerase)	27	1	(Polymerase)

* Products in brackets are predicted from the sequence but have not yet identified in infected cells.

** A membrane associated protein corresponding to the product of the D3 open reading frame has been identified in infected cells (S.C. Inglis, personal communication).

Such virus is non-infective, however, so that entry requires S_1; it is likely, though not yet demonstrated, that receptor-mediated endocytosis is involved in virus entry. Coronavirus RNA alone is infectious, so no virion enzymes are involved.

Replication does not require a functional host cell nucleus (53) and occurs in the cytoplasm even in the presence of inhibitors of DNA-dependent RNA synthesis such as α-amanitin or actinomycin D (1, 46, 47, 54). The virus multiplication cycle lasts about 10 hours and begins with the synthesis, from the virion RNA template, of a genome-length negative strand RNA (55, 56). This negative strand RNA functions in replicative-intermediate structures as the template for the synthesis of a family of positive-strand RNAs; the RNA-dependent RNA polymerases involved in these synthetic events appear to be membrane-bound and to require continuous protein synthesis, but have not been extensively characterized (54-57).

A. Virus-specific RNA in Infected Cells

In coronavirus-infected cells, multiple virus-specific RNA species can be detected by pulse-labelling with ^{3}H-uridine in the presence of actinomycin D. In addition to full-length genome-sized RNA, six smaller, subgenomic RNAs are synthesized in cells infected with MHV, and five in cells infected with IBV. All the intracellular RNAs of both avian and murine coronaviruses are capped and polyadenylated, and can be found in association with polysomes. They also share common sequences, as originally demonstrated by RNase T_1-resistant oligonucleotide mapping studies on RNAs from IBV-infected cells by Stern and Kennedy (58, 59) and subsequently shown for MHV-infected cell RNAs by several groups (60-62). Each RNA contains the sequences present in the next smaller RNA plus a unique 5' terminal sequence, so that the RNAs form a 3' co-terminal

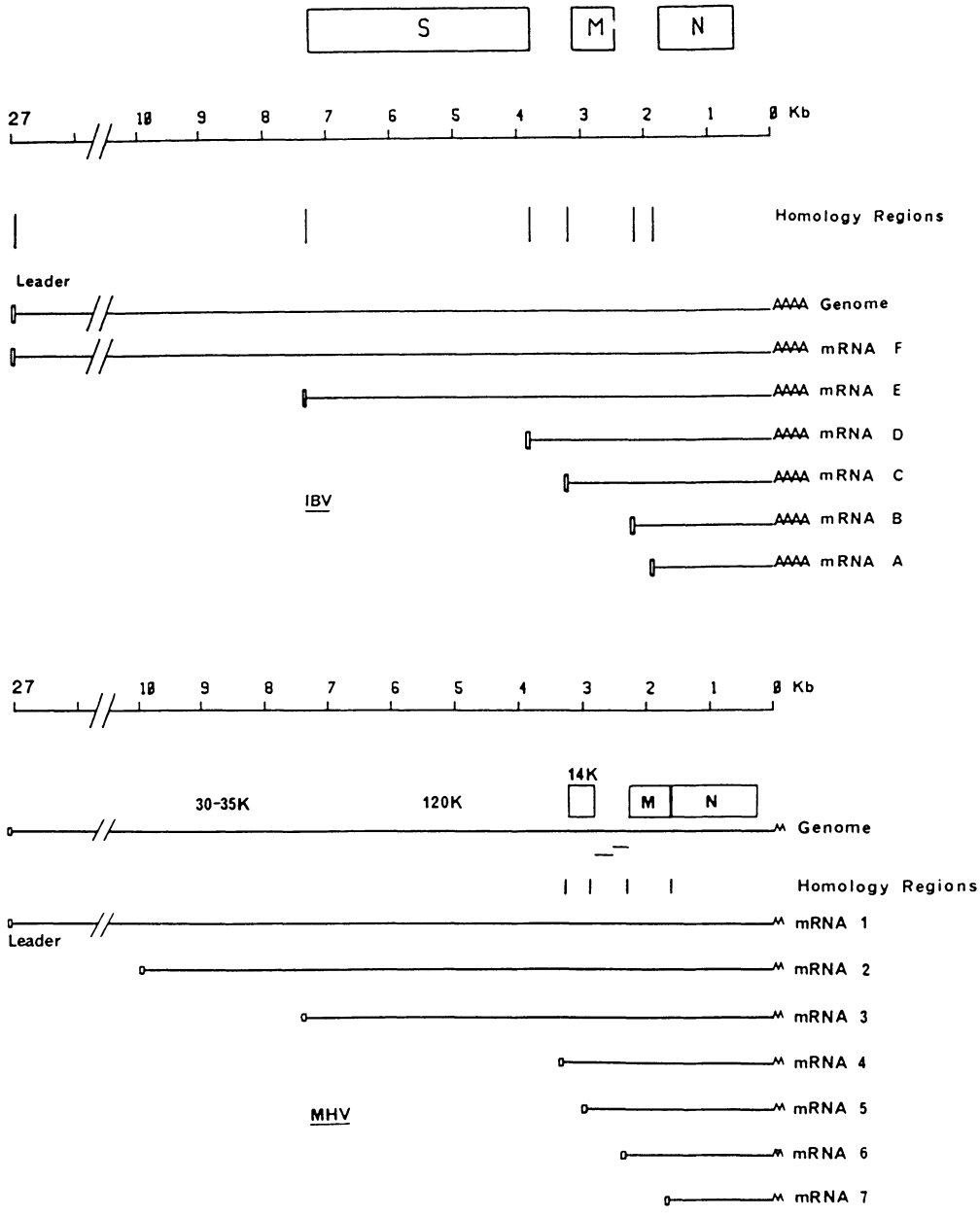

Figure 4. Diagrammatic representation of the "nested set" of coronavirus mRNAs, adapted from Siddell (91).

overlapping 'nested set' (Figure 4). The coding assignment of each RNA species has been determined for both avian and murine coronavirus-infected cells by partial purification of the RNA followed by *in vitro* translation (1, 46, 63, 64). Only the unique sequences at the 5' end are translated, so that each mRNA specifies a single polypeptide even though it may include coding sequences for several polypeptides.

Numerous sequencing studies of the coronavirus genome have established that the order of the genes specifying the major structural polypeptides is 3' N, M, S, 5'. The genome also contains putative genes for non-structural proteins, and it is likely that the very large gene(s) towards the 5' end must specify the RNA polymerase(s). These coding assignments are summarized in Table 2.

B. Mechanism of RNA Synthesis

The first event in coronavirus-infected cells must be the translation of the 5' region of genome RNA to yield an RNA-dependent RNA polymerase. Attempts to reproduce this event by *in vitro* translation of virion-derived genome RNA have met with little success, although Leibowitz *et al* (65) reported the translation of MHV virion RNA into several related high molecular weight polypeptides of around 200,000 molecular weight. An "early" RNA-dependent RNA polymerase activity has been detected in MHV-infected cells one hour post-infection, followed by a second peak of polymerase activity at 6 hours, the latter being coincident with the bulk of virus specific RNA synthesis (66). The early RNA polymerase activity synthesises an RNA copy (negative-strand) of the genome RNA (56), only genome-length negative strand RNA can be detected in infected cells (55) and no sub-genomic negative strand RNAs have been reported. From the foregoing, it can be concluded that the multiple subgenomic plus strand RNAs are synthesised from a full-length negative-strand RNA template.

An important experimental finding concerning the mechanism of subgenomic RNA synthesis was made by Jacobs *et al* (67), who showed that the UV-target sized of MHV-induced subgenomic RNAs corresponded to their physical sizes. A similar result was obtained when IBV-induced subgenomic RNAs were analysed by this method (68), and it was concluded that coronavirus subgenomic RNAs are synthesized independently and not derived by processing from a larger precursor molecule. However, in apparent contradiction to this idea, it was subsequently found that all the subgenomic RNAs share a common 5' sequence (leader sequence), which is 72 nucleotides long in MHV- and 60 nucleotides long in IBV-induced RNAs (69-72). The leader sequences are apparently derived from the 5' end of genome RNA but not by processing a full-length precursor RNA. They appear instead to act as primers for the transcription of individual mRNAs at specific internal initiation sites on the negative strand template RNA (73-74). Small virus RNA species containing leader sequences have been detected in MHV-infected cells, and a *ts* mutant of MHV was isolated which synthesises only small leader RNAs, not mRNAs, at the non-permissive temperature (75). Finally, it was demonstrated by mixed infection of cells with two strains of MHV that leader RNA sequences can reassort. The strains used were recombinant viruses containing either JHM strain sequences at the 5' end and A59 'body' sequences, or the other way

around. The leader sequences were exchanged between the mRNAs of the two co-infecting viruses at high frequency (76).

If the leader RNAs act as primers, they should contain sequences which are complementary to sequences at the initiation site for mRNA synthesis on the negative strand template RNA. Regions of homology between the 3' end of the genomic leader sequence and sequences at the intergenic boundaries have been found in MHV-infected cells (51, 52, 77) and in IBV-infected cells (72). In all the major IBV RNA species the sequence CUUAACAA is present, and this could base-pair with complementary sequences at the intergenic boundaries on the negative strand template RNA. Related sequences containing the trinucleotide AAC are present in the MHV leader. However, the presence of this sequence on free leader RNA has not been formally proven. Alternative hypotheses are that this common intergenic sequence is a recognition site for the RNA polymerase rather than a primer-binding site, or serves both functions. Further evidence is required to decide between these and other possibilities (78).

C. Recombination

In 1986 Lai *et al* (79) reported the isolation of a recombinant coronavirus following mixed infection of DBT cells with two strains (A59 and JHM) of MHV. They used *ts* mutants of each strain, and derived a recombinant (B1) from plaques formed at the non-permissive temperature. From oligonucleotide fingerprint analysis of the B1 virus genome, it was clear that, although most of the genome was derived from A59, some 3 kilobases at the 5' end were derived from JHM virus, so that a cross-over event must have occurred within the 5'-proximal gene. Moreover, analysis of the subgenomic RNA species induced in B1 virus-infected cells showed that they all contained "hybrid" sequences, where the coding sequences of A59 virus were joined to leader sequences of JHM. Subsequently, it was found by co-infecting cells with *ts* mutants of A59 virus and *wt* JHM virus that recombinants could be obtained at high frequency (80).

Analysis of further recombinants (81) revealed some where more than one cross-over site was present, and some where there was a cross-over site within the leader sequence itself. By crossing a *ts* mutant of A59 virus, which synthesises only leader RNA at the restrictive temperature (75), with *wt* JHM virus, recombinants were obtained with up to three cross-over sites clustered close to the 5' end of the genome RNA. These sites correspond to the termination points of three leader-containing RNA species, less than full length (47,50 and 57 nucleotides respectively), which were detected in A59 virus-infected cells (81).

The high recombination frequency of murine coronavirus is remarkable. Lai *et al* (80, 81) have interpreted this as evidence for discontinuous, non-processive RNA species which can dissociate from and reassociate with the negative strand template RNA. Such a model would also provide an explanation for the generation of defective-interfering coronavirus RNAs which contain deletions within the genome. However, high frequency recombination at multiple sites is also observed with another positive strand virus, foot-and-mouth disease virus (see chapter 8 of this volume and ref. 82), and five cross-over sequences have been sequenced in these

recombinants (83). The number of common nucleotides varied from 2 to 32 at the cross-over sites, and there were no common features or recognisably unique sites such as those involved in RNA splicing (84). It will be important to sequence cross-over sites within the coronavirus recombinats to see how general is the recombination event.

D. Virus Assembly and Morphogenesis

One of the early steps in coronavirus assembly is the formation of helical nucleocapsids by interaction of newly synthesised genome RNA with molecules of N protein. Aggregates of nucleocapsids have been visualized in infected cells late in the infectious cycle (85). The nucleocapsid forms a complex with the M protein *in vitro* (86) and it is likely that *in vivo* this complex formation occurs at the maturation site where budding occurs from intracellular membranes in the rough endoplasmic reticulum (RER). Although the S protein may accumulate at the plasma membrane, virions never bud there, and it seems that the M protein alone determines the site of budding. Virions are released into the lumen of the RER and migrate through the Golgi apparatus, where they are transported into smooth-walled vesicles. Release from the cell presumably involves fusion of these vesicles with the plasma membrane (87).

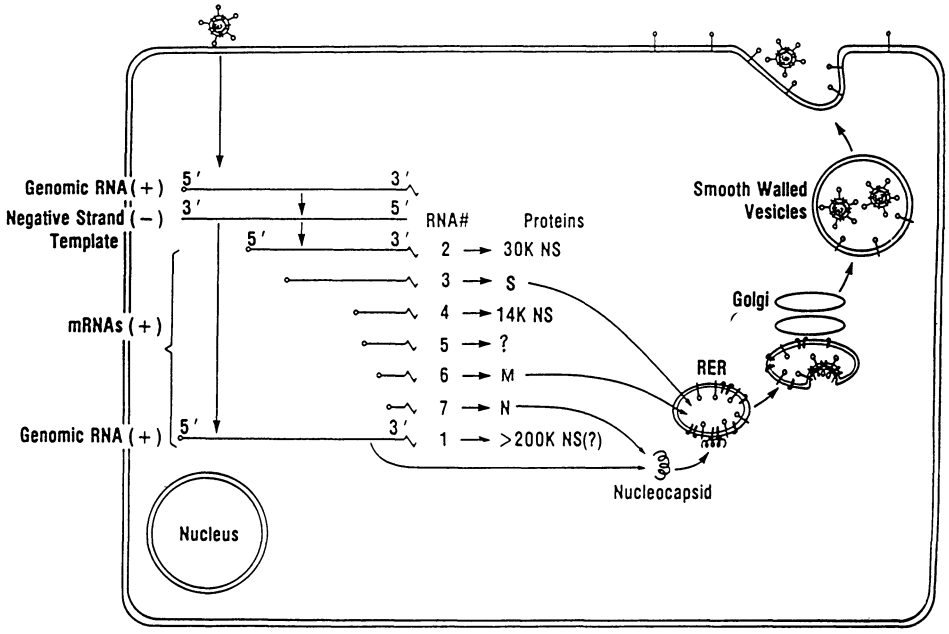

Figure 5. Steps in the replication and assembly of coronaviruses. Adapted from Holmes K.V. (87).

Studies using inhibitors support the importance of the M protein in determining the budding process. In MHV-infected cells, treatment with tunycamycin blocks S protein synthesis but allows synthesis of glycosylated M, since O-linked glycosylation is resistant to the drug. In such cells, virions lacking spikes still form in the RER and the Golgi apparatus (88). The production of IBV (47) and MHV (89) was also inhibited by monensin, an ionophore which inhibits transport of secretory glycoproteins at the level of the Golgi complex (90). In MHV-infected cells, virions accumulated in large amounts within the RER in the presence of monensin, which also blocked the O-linked glycosylation of the M polypeptide (89). An outline of the steps in replication and assembly of coronaviruses is given in Figure 5.

4. CONCLUDING REMARKS

When first discovered, the coronaviruses were thought to be negative-strand RNA viruses akin to influenza or paramyxoviruses on the basis of their general morphology including the helical structure of the ribonucleoprotein. It is now clear that they form a unique group of positive strand viruses with unusual molecular biological properties. At the time of writing, the complete nucleotide sequence of one coronavirus, IBV, is virtually completed (M.E.G. Boursnell, personal communication). This shows the enormous information content of the coronavirus genome, which is the largest infectious RNA molecule yet described. Unravelling the way in which this information is expressed in the infected cell is a fascinating challenge for the future.

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