7

Assembly of *Coronaviridae*

I. Introduction

Coronaviridae are enveloped RNA viruses which mature by budding into intracytoplasmic membranes. Coronaviruses cause respiratory and/or enteric infection in humans and many domestic animals (reviewed by Wege *et al.*, 1982). The prototype coronavirus is avian infectious bronchitis virus (IBV, Tyrrell *et al.*, 1978). *Coronaviridae* exhibit rather fastidious requirements for the species and tissue types which they will infect. Because of the difficulty of isolating coronaviruses, most of

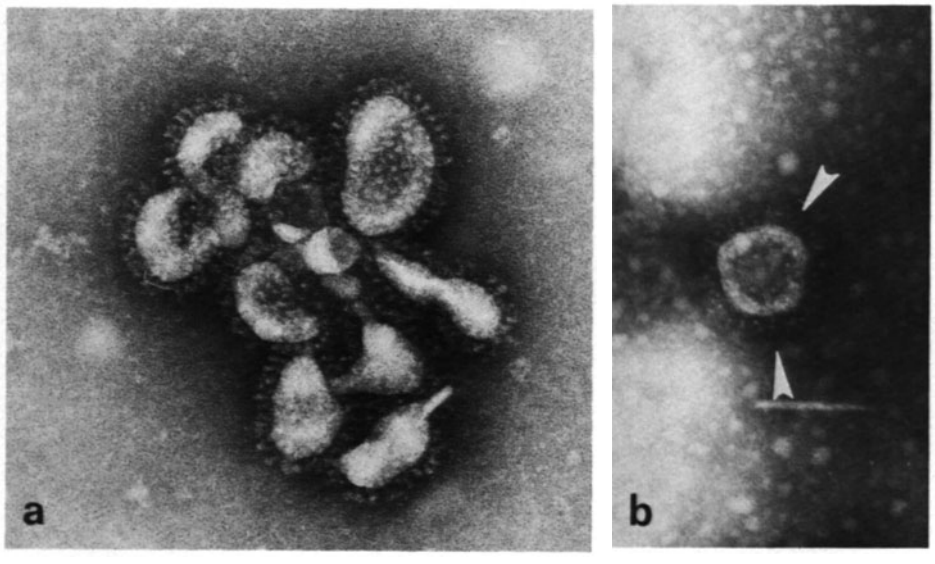

Fig. 7-1. Negatively stained coronavirions. *(a)* shows a cluster of human respiratory coronaviruses with the large, widely-spaced peplomers or spikes that form the "corona" around the virion. *(b)* illustrates a human enteric coronavirus with delicate peplomers (arrowheads). Such viruses have frequently been visualized in preparations of human fecal material, but they are very difficult to propagate *in vitro*. Magnifications: (a) × 110,000, (b) × 90,000. [Courtesy of Dr. L. Oshiro, (a), and Dr. O. Caul, (b)]

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them were first classified by their virion morphology in negatively stained preparations (Tyrrell *et al.*, 1975). The virions are characterized by large, club-shaped peplomers or spikes about 20 nm long and 7 nm wide at their tips (Almeida and Tyrrell, 1967; Tyrrell *et al.*, 1968; Fig. 7-1). Avian coronaviruses vary in diameter from 70 to 120 nm, but murine coronaviruses have more uniform diameters, approximately 90 nm. More recently, common features of virus replication and biochemistry have confirmed the assignment of coronaviruses into a single virus family (Tyrrell *et al.*, 1978; ter Meulen *et al.*, 1981). There are at least 3 subgroups of *Coronaviridae*, based on their mutual lack of antigenic cross-reactivity (Pedersen *et al.*, 1978; Sturman and Holmes, 1983). Avian coronaviruses, such as infectious bronchitis virus (IBV), do not share antigenic determinants with mammalian coronaviruses. Mammalian coronaviruses fall into two distinct groups: one includes mouse hepatitis virus (MHV), bovine coronavirus (BCV), hemagglutinating encephalomyelitis virus of swine (HEV) and human respiratory coronavirus OC43; and a second includes human respiratory coronavirus 229E, transmissible gastroenteritis virus of swine (TGEV), canine coronavirus (CCV), and feline infectious peritonitis virus (FIP). There are also coronaviruses antigenically unrelated to these three major subgroups, such as porcine enteropathic coronavirus, CV777 (Pensaert *et al.*, 1981).

II. Molecular Organization

We will summarize the molecular composition of coronavirions and events in coronavirus replication and then consider the morphology of the virions and their budding mechanisms in detail.

Transcription

The coronavirus genome is a single-stranded molecule of RNA of M.W. 5.4 to 6.9×10^6 which is capped at its 5′ end and polyadenylated at its 3′ end (Lai and Stohlman, 1978; Stern and Kennedy, 1980 a; Siddell *et al.*, 1982; Fig. 7-2). The isolated genomic RNA is infectious and serves as an mRNA within the infected cell. Thus, coronaviruses are positive-stranded RNA viruses. The genomes of IBV and MHV have been mapped by comparing oligonucleotides of genomic RNA with oligonucleotides of 3′ co-terminal, polyadenylated RNAs of different lengths generated by RNAse digestion of virion RNA. These maps have then been compared with the oligonucleotide patterns of the subgenomic mRNAs found in infected cells (Stern and Kennedy, 1980 b, Lai *et al.*, 1981).

There are 5 subgenomic mRNAs for IBV, and 6 for MHV. The mRNAs of coronaviruses are unusual in that they form a nested set of molecules of varying length which share a common, polyadenylated 3′ end (Stern and Kennedy, 1980 a and b; Weiss and Leibowitz, 1981; Cheley *et al.*, 1981; Fig. 7-2). Except for a short sequence of nucleotides at the 5′ end of each mRNA, the oligonucleotides of each mRNA are completely identical to the 3′ end of the next larger mRNA species (Lai *et al.*, 1982 a).

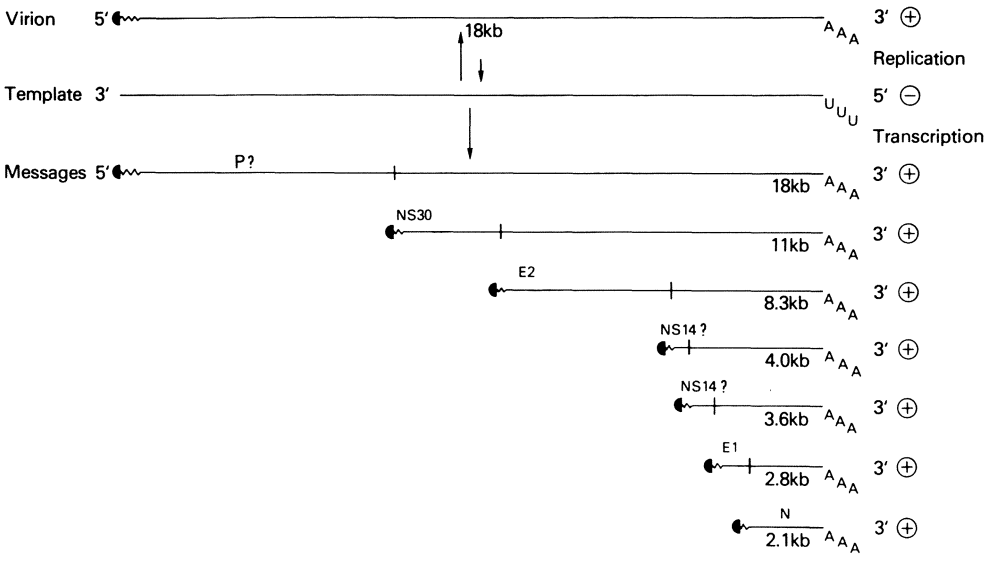


Fig. 7-2. Scheme of the transcription and replication of coronaviruses, using mouse hepatitis virus as a model. The 18 kb positive-stranded genomic (virion) RNA serves as a messenger RNA in the cell to direct the synthesis of RNA-dependent RNA polymerase which uses the genomic RNA as a template to make a full-length negative-stranded RNA. From this template, new 18 kb genomic RNA and 6 subgenomic mRNAs are made; all species form a nested set with common 3′ ends. All of these positive-stranded RNA species are capped (●—) and polyadenylated (AAA). A short leader sequence, indicated by the wavy line, is found on the 5′ end of each mRNA but occurs only once on the genomic strand. For each mRNA, only the gene at the 5′ end (to the short vertical line) is translated; the protein product of each mRNA is indicated by the letters above the 5′ gene. The two smallest mRNAs have been cloned and sequenced. (Adapted from Sturman and Holmes, 1983)

The 5′ end of each mRNA consists of a cap plus a short common nucleotide sequence referred to as the leader sequence (Lai *et al.*, 1983).

In vitro translation of the 6 subgenomic mRNAs of MHV has demonstrated that each species codes for only one polypeptide and that the translated gene is at the 5′ end of the mRNA (Siddell *et al.*, 1980, 1981 c; Leibowitz and Weiss, 1981; Leibowitz *et al.*, 1982; Rottier *et al.*, 1981 a). A tentative map based upon these studies is shown in Fig. 7-2.

The structural proteins of different coronaviruses appear to follow a common pattern, and the terminology for MHV proteins will be used here. There are generally three structural polypeptides: a nucleocapsid protein N, and two envelope glycoproteins, E1 and E2 (Garwes, 1980; Siddell *et al.*, 1982; Sturman and Holmes, 1983 a).

Replication

Coronavirus replication has recently been the subject of several comprehensive reviews (ter Meulen *et al.*, 1980; Siddell *et al.*, 1982 and 1983; Sturman and Holmes, 1983 a; Rottier et al., 1984), and the reader is referred to these for details which are

beyond the scope of this chapter. The major features of coronavirus replication and transcription will be summarized here briefly.

Because of the positive-stranded nature of its genome, the coronavirion contains no RNA-dependent RNA polymerase. This enzyme is synthesized in cells soon after infection and first directs the synthesis of full-length negative strands (Brayton *et al.*, 1982; Dennis and Brian, 1981, 1982; Lai *et al.*, 1982 b). Synthesis of negative strands is complete within 5–6 hours after infection; it is not clear how continued synthesis of negative strands is inhibited. From this template, the RNA-dependent RNA polymerase directs the synthesis of genomic RNA and 5 subgenomic mRNAs (IBV; Stern and Kennedy, 1980 a and b) or 6 subgenomic mRNAs (MHV; Spaan *et al.*, 1981, 1982; Wege *et al.*, 1981). A cap and the same short leader nucleotide sequence are added to the 5′ end of each mRNA, either by RNA splicing or by allowing the common leader sequence to serve as a primer for synthesis of each mRNA (Baric *et al.*, 1983). The primer model is supported by the observation that caps and leaders are found on nascent mRNA strands (Lai *et al.*, 1983 b), but no free leader RNA has yet been isolated from infected cells.

Two distinct peaks of RNA polymerase activity occur during virus replication (Brayton *et al.*, 1982). The first involves primarily synthesis of the full-length negative-stranded template, and the second coincides with synthesis of new genomic RNA and mRNAs. The virus protein species responsible for polymerase activity have not yet been identified.

Throughout the replicative cycle, the ratios of the different subgenomic RNAs remain constant (Stern and Kennedy, 1980 a; Wege *et al.*, 1981; Spaan *et al.*, 1981; Leibowitz *et al.*, 1981). Each of the mRNAs appears to direct the synthesis of a single viral polypeptide (Fig. 7-2).

Virus Proteins

Table 7-1 summarizes the properties of the 3 structural proteins of MHV. The functions of the non-structural proteins of corona-viruses are not known.

1. *N protein* (MW: 50 K). The NC protein, N, is phosphorylated (Stohlman and Lai, 1979). With the positive-stranded viral genomic RNA, the N protein forms a helical NC which is RNAse-sensitive. Regulatory or enzymatic functions of N have not been identified.

2. *E1 glycoprotein* (MW: 23 K). E1 is an unusual virus glycoprotein in several ways. It has 3 domains: external, intramembranous, and cytoplasmic (Sturman and Holmes, 1977, 1983). The short amino-terminal external domain (about 5 K) contains all of the E1 oligosaccharides (Sturman and Holmes, 1977). In MHV, short oligosaccharides are O-glycosidically linked to serine or threonine residues of E1 (Niemann *et al.*, 1982), unlike the complex oligosaccharides N-glycosidically linked to asparagine residues which are found on nearly all other virus glycoproteins (Sharon and Lis, 1981). The E1 glycoprotein of IBV contains only N-linked oligosaccharides (Stern *et al.*, 1982; Stern and Sefton, 1983). The significance of this diversity of glycosylation patterns among coronaviruses is not known.

The E1 molecule is very hydrophobic, particularly when its external domain has been removed. Like a small number of integral membrane proteins, it tends to

Table 7-1. *Proteins of coronavirus MHV*[1]

Name	Coded by mRNA Number	Apparent MW $\times 10^{-3}$	Known or putative function
N	7	50–60	NC protein
E1	6	23	Membrane glycoprotein Determines budding site? Binds to NC
E2	3	180	Peplomeric glycoprotein Binds to cell-surface receptors Causes cell fusion (protease activated) Elicits neutralizing antibody Elicits natural cell-mediated cytotoxicity
NS14	4 or 5	14	?
NS30	2	30–35	?
NS200	1	200	Polymerase?

[1] After Sturman and Holmes (1983).

self-aggregate spontaneously and forms dimers, trimers, and tetramers when boiled with SDS and β-mercaptoethanol (Sturman and Holmes, 1977). Recent evidence from cloning of the E1 genes of MHV and IBV indicates that there are two long stretches of hydrophobic amino acids in E1, suggesting that E1 may traverse the lipid bilayer several times (Armstrong *et al.* and Cavanagh *et al.*, personal communication). Within infected cells, E1 accumulates within the Golgi apparatus (Holmes *et al.*, 1981 a, and 1984). We have suggested that E1 functions as a matrix glycoprotein for coronaviruses (Holmes *et al.*, 1981 a, and 1984; Sturman and Holmes, 1983 a). Thus it interacts with NCs by its cytoplasmic domain(s), determines the intracellular localization of virus budding, and, with the lipid bilayer, forms the virus envelope.

3. *E2 glycoprotein* (MW: 180 K). E2 forms the large peplomers or spikes characteristic of coronavirions. E2-molecules can be removed from virions by protease digestion (Garwes and Pocock, 1975; Sturman and Holmes, 1977), although a small hydrophobic domain is believed to remain embedded in the virus envelope. It is not certain whether this anchor region penetrates the membrane completely to form a cytoplasmic domain. E2 probably interacts with E1 and/or N during virus budding.

E2 contains a large amount of N-linked oligosaccharide (Sturman, 1981; Niemann and Klenk, 1981). E2 is acylated, containing covalently bound palmitic acid (Niemann *et al.*, 1982; Schmidt, 1982 a), presumably located at or near the lipid bilayer (Schmidt, 1982 a and b). E2 is proteolytically cleaved at a late stage in virus maturation (Sturman and Holmes, 1983, and 1984; Holmes *et al.*, 1984). In MHV-A59, cleavage appears to be dependent upon the host cell and yields two molecules, 90 A and 90 B, which comigrate in electrophoresis in SDS-containing polyacrylamide gels with an apparent molecular weight of 90 K. These have different amino

acid compositions and only 90A contains covalently bound palmitic acid. Cleavage of the large peplomeric glycoprotein of IBV in chicken cells may be very efficient, since only the two cleavage products of E2, gp 93 and gp 84, are found on the virion (Cavanagh, 1981; Wadey and Westaway, 1981; Stern *et al.*, 1982).

E2 is a large, multifunctional molecule. It is responsible for virus-induced cell fusion (Holmes *et al.*, 1981 b; Collins *et al.*, 1982) and its cleavage is required for cell fusion (Sturman and Holmes, 1983, and 1984). E2 on the plasma membrane renders cells subject to cell-mediated cytotoxicity (Welsh *et al.*, 1983). It induces neutralizing antibody (Garwes *et al.*, 1976, 1978/79; Hasony and Macnaughton, 1982; Schmidt and Kenny, 1981, 1982) and binds the virion to receptors on the plasma membranes of susceptible cells (Holmes *et al.*, 1981 b). E2 also plays an important role in the pH-dependent thermolability of coronaviruses (Sturman, 1981). The anchoring region of E2 probably interacts with E1 in the virus envelope.

III. Synthesis, Transport and Processing of Virus Proteins

The synthesis of N protein is directed by the smallest mRNA, which is derived from the 3′ end of the viral genome (Siddell *et al.*, 1980; Rottier *et al.*, 1981 a). Synthesis of N occurs on free polysomes, although even in very short pulse-labeling experiments some N is found on RER and smooth membranes isolated from homogenized cells (Frana and Holmes, unpublished observations). Thus, some mRNAs on membrane-bound polysomes may direct the synthesis of N protein. Immunofluorescence studies with anti-N antibody reveal delicate flecks of fluorescence in the cytoplasm by 2 to 3 hours after virus inoculation. These foci increase in size and number during infection.

The N protein is phosphorylated at serine and threonine residues (Stohlman and Lai, 1979). Although protein kinase activity has been demonstrated in the virion (Siddell *et al.*, 1981 a), it is not yet clear whether this activity represents a virus-specific or cellular enzyme. The role (if any) of phosphorylation and dephosphorylation in modulation of the biological functions of N is not known. Pulse-chase studies show that although large amounts of N are synthesized in the infected cell, only a very small fraction of N is chased out of the cell into mature virions (Holmes *et al.*, 1984; Rottier *et al.*, 1981 b; Siddell *et al.*, 1981 b). This suggests that N protein may have several functions during virus replication in addition to formation of NCs. Only one species of N appears in the virion of each coronavirus strain. However, even between closely related strains of MHV the apparent molecular weight of the virion-associated N varies, ranging from 50 K to 60 K (Siddell *et al.*, 1982). Whether this difference is due to phosphorylation has not been determined. Several species of N which migrate more rapidly than the virion N have been detected within infected cells late in the infectious cycle or following immunoprecipitation (Cheley and Anderson, 1981). Their functions are unknown.

The synthesis of the peplomeric glycoprotein E2 (Rottier *et al.*, 1981 a; Siddell *et al.*, 1981 b; Leibowitz *et al.*, 1981), encoded in mRNA 3 occurs on RER membranes (Niemann *et al.*, 1982). By immunofluorescence, E2 can first be detected as fine cyto-

plasmic granules at 4 hours after infection. The glycoprotein then disperses rapidly throughout the cytoplasm, presumably upon intracytoplasmic membranes (Holmes *et al.*, 1981 b). After 7 hours, delicate flecks of fluorescent staining are observed on the plasma membrane. These gradually spread to cover the cell surface.

The first species of E2 to be detected in pulse-label experiments is a 150 K form (Siddell *et al.*, 1981 b) which is cotranslationally glycosylated at asparagine residues by transfer of core oligosaccharides from dolichol-phosphate intermediates. This form of E2 is sensitive to endoglycosidase H. It is transported to the Golgi apparatus where further trimming and glycosylation of the N-linked oligosaccharides and acylation occur under the direction of cellular enzymes (Niemann *et al.*, 1982). Pulse-chase studies show that in the 17 Cl 1 line of BALB/c mouse fibroblasts, synthesis of E2 is well-balanced with its release into virions (Holmes *et al.*, 1981 b); labeled E2 is quantitatively chased into released virions within 2 hours. At least early in infection, there is no large excess of E2 in the cell. At a late stage in the processing of E2, when or just before it reaches the plasma membrane, proteolytic cleavage by a trypsin-like cellular enzyme occurs (Holmes *et al.*, 1984). In tunicamycin-treated cells, E2 is synthesized as a 120 K non-glycosylated polypeptide which is not incorporated into virions (Niemann and Klenk, 1981).

The matrix glycoprotein, E1, of MHV-A 59 is synthesized on membrane bound ribosomes as a non-glycosylated 20 K polypeptide (Niemann *et al.*, 1982). It is transported to the Golgi, where cell fractionation studies show that it is post-translationally glycosylated by the addition of several sugars to serine or threonine residues. This process of O-linked glycosylation, which is probably done by cellular enzymes, is not well understood. Each molecule of E1 receives from 1 to 3 oligosaccharide chains.

Immunofluorescence studies with monospecific or monoclonal antibodies to E1 and E2 have shown that the intracellular transport of the two coronavirus glycoproteins is different. E2, like the peplomeric glycoproteins of most other enveloped viruses, is transported from the RER through the Golgi to the plasma membrane. In contrast, the E1 glycoprotein is transported only as far as the Golgi apparatus, where it accumulates during virus infection (Fig. 7-3A and B). This has been demonstrated by simultaneously staining E1 with immunofluorescent antibody and marking the *trans* cisternae of the Golgi by a histochemical reaction for thiamine pyrophosphatase (Sturman and Holmes, 1983; Doller *et al.*, in preparation). It appears likely that the restricted intracellular transport of the E1 glycoprotein may account for the intracellular budding site of coronaviruses (Holmes *et al.*, 1981 b; Sturman and Holmes, 1983). It is interesting to speculate whether other virus groups which mature by budding from intracellular membranes, such as bunya- and flaviviruses may show a similarly restricted pattern of intracellular transport of a NC-binding protein.

Coronaviruses cause cell fusion *in vitro* and *in vivo*. This cell fusion appears to be mediated by E2 glycoprotein on the plasma membrane of infected cells, since incubation in the presence of anti-E2 antibody prevents virus-induced cell fusion (Holmes *et al.*, 1981 b; Collins *et al.*, 1982). Fluorescent antibody staining and immunoelectron microscopy (Figs. 7-3 C-E) show that the E2 glycoprotein is on the plasma membrane in large amounts late in the infectious cycle, whereas, as noted

above, relatively little E1 migrates to the plasma membrane (Holmes *et al.*, 1981 b). E2 cleavage appears to be dependent upon the host cell, since virions from different cell types show different degrees of cleavage (Holmes *et al.*, 1983).

Proteolytic cleavage of E2 glycoprotein is required for coronavirus-induced cell fusion (Sturman and Holmes, 1983 and in press; Holmes *et al.*, in press). This was shown by experiments in which concentrated, purified MHV did not cause rapid

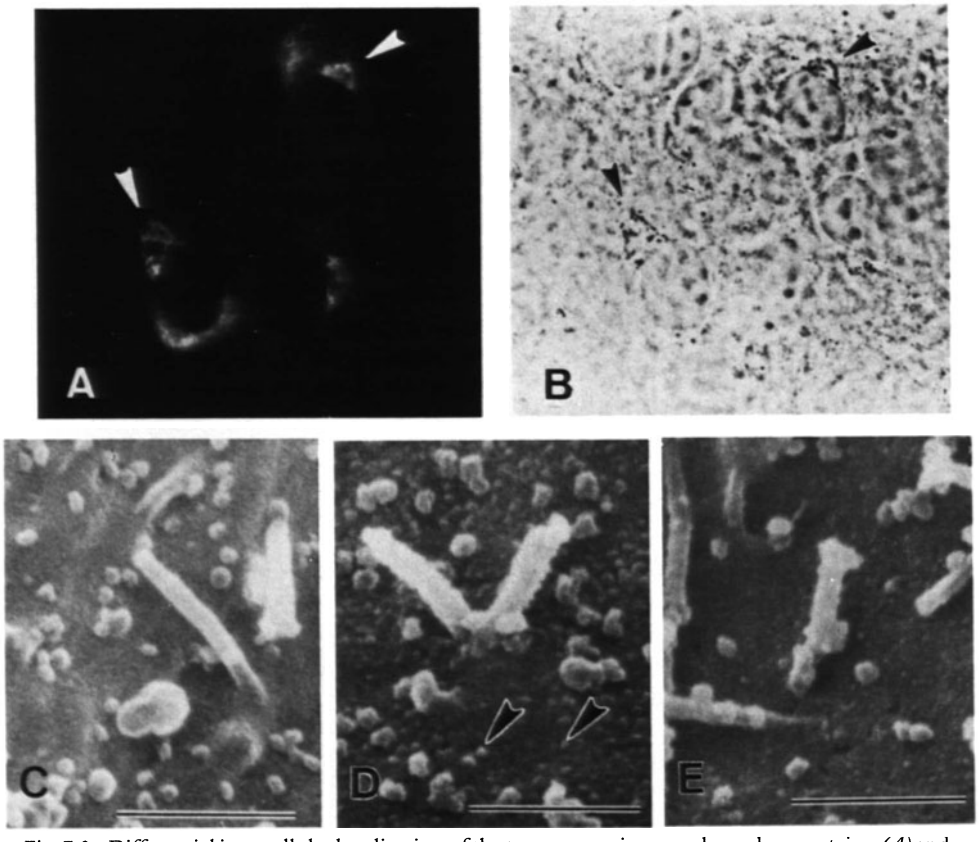


Fig. 7-3. Differential intracellular localization of the two coronavirus envelope glycoproteins. *(A)* and *(B)* show that E1 accumulates within the Golgi apparatus. Cells infected at a low multiplicity with MHV were fixed in formaldehyde at the end of the virus latent period, permeabilized with detergent and labeled with monospecific anti-E1 antibody and fluorescent anti-rabbit IgG (A). The same cells were reacted with a cytochemical marker for the Golgi apparatus, thiamine pyrophosphatase (B). The E1 antigen in the cells of (A) (arrowheads) is located in the Golgi region shown in (B) (arrowheads). E2, the peplomeric glycoprotein, does not accumulate in the Golgi, but is transported to the plasma membrane, as shown in *(C)* to *(E)*. MHV-infected cells 24 hours after infection were fixed and labelled with normal rabbit serum (C), or monospecific anti-E2 antibody (D), or anti-E1 antibody (E) followed by *Staphylococcus aureus* protein A conjugated to peroxidase. The presence of antigen is shown by development of a granular reaction product following reaction with hydrogen peroxide and 3,3′ diaminobenzidine. The cells were coated with metal and examined by scanning electron microscopy. E2 is transported to the plasma membrane as shown by the granular labeling in (D) (arrowheads), but E1 is not transported to the plasma membrane, because panel (E), like the normal rabbit serum control, (C), shows no granular reaction product on the plasma membrane. Magnifications: (A) and (B) × 960; (C) to (E) × 23,000. (Courtesy of Dr. E. Doller)

fusion of susceptible cells in the presence of cycloheximide, whereas trypsin-treated, purified and concentrated MHV caused rapid cell fusion. (The only effect of trypsin on the virions was the quantitative cleavage of E2 180 to E2 90). Activation of plaque formation, hemagglutination and/or virus cytopathic effect by trypsin have been shown for several coronaviruses (reviewed in Sturman and Holmes, 1983). Whether cleavage of the E2 glycoprotein is required for virus infectivity is not yet clear, however, since at present there is no method available to obtain homogeneous populations of virions with uncleaved E2. Some coronaviruses which have been difficult to propagate *in vitro*, such as human enteric coronaviruses (Fig. 7-1b), may undergo a single cycle of infection yielding large numbers of non-infectious virions, as shown in negatively stained preparations (Caul and Egglestone, 1977). It may be necessary to identify a cell type in which cleavage of E2 occurs or to identify a different protease which may activate infectivity in order to propagate these fastidious viruses *in vitro*.

IV. Assembly of Virus Components

In this section, we will review the assembly of the A59 strain of MHV, since it has been studied most extensively. Where other coronaviruses differ from this model, this will be noted in the text.

NC Assembly

The specific interaction of N with genomic RNA has not yet been studied. It is likely that only genomic RNA is encapsidated, since encapsidation of mRNAs would interfere with their translation. If this is true, we postulate that an encapsidation signal is present near the 5′ end of the genomic RNA, since all other regions are shared by subgenomic mRNAs.

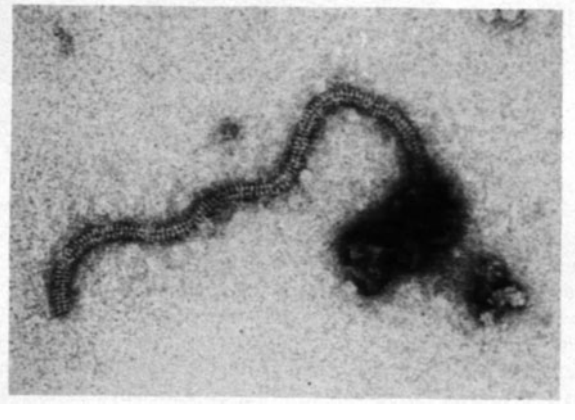


Fig. 7-4. Helical NC from the coronavirion. A long strand of helical NC purified from a human coronavirus by density gradient ultracentrifugation is shown in a negatively stained preparation. Such images have been difficult to obtain with many coronaviruses. Magnification: × 205,000. (Courtesy of Dr. O. Caul, from Sturman and Holmes, 1983, with permission of Academic Press)

The NC of coronaviruses is helical, but the turn-to-turn bonding of the NC strands appears to be very weak. The tubular appearance of the NC is readily observed in thin sections of budding virions (David-Ferriera and Manaker, 1965; Becker *et al.*, 1967; Oshiro *et al.*, 1971), but is not often apparent in released virions, suggesting that it may undergo a conformational change after virus budding and release (Holmes and Behnke, 1981). Intracytoplasmic inclusions of coronavirus NCs were not recognized for many years, perhaps in part due to the great flexibility of the NC strands. Large inclusions of NCs have been observed in coronavirus-infected cells late in the infectious cycle (Caul and Egglestone, 1977). NCs with a density of 1.25 to 1.30 g/cc have been isolated from virions or from infected cells and shown to consist of genomic RNA and N protein with or without E1 (Kennedy and Johnson-Lussenberg, 1975/76; Sturman *et al.*, 1980). Coronavirus NCs are apparently not tightly coiled, so that isolated NCs often appear as thin, kinky strands rather than helical coils. Only when virions are gently or spontaneously disrupted, is it possible to observe or to purify long helices of NC with a diameter of 9 to 11 nm (Macnaughton *et al.*, 1978; Davies *et al.*, 1981; Caul *et al.*, 1979; Fig. 7-4).

Interaction of NC and Envelope Proteins and Virus Budding

Coronaviruses mature by budding into the lumina of RER or Golgi cisternae (Figs. 7-5 and 7-6). Virions form when helical NCs align under regions of intra-

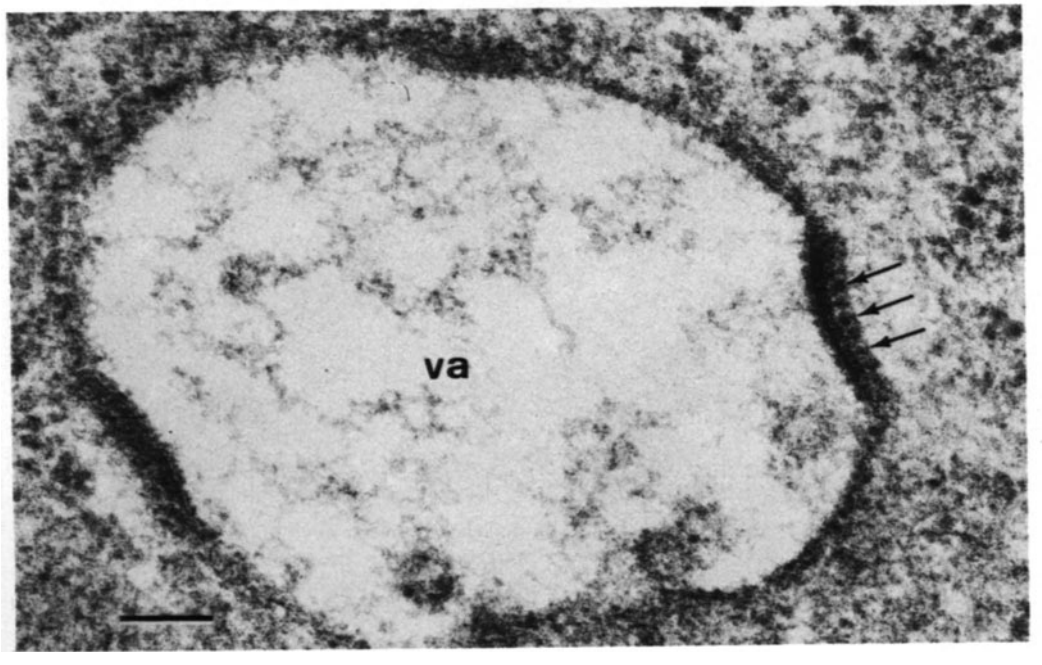


Fig. 7-5. Association of the virus NC with peplomers at smooth-walled intracellular membranes. Regions where virus buds are forming are visible at each side. Arrows indicate cross-sections of the helical NC which has formed a regularly spaced array under the membrane. The fuzzy coat on the lumenal surface probably represents virus peplomers. Magnification: ×130,000; Bar=100 nm. (Courtesy of Dr. S. Dales, from Massalski *et al.*, 1982, with permission from S. Karger AG, Basel)

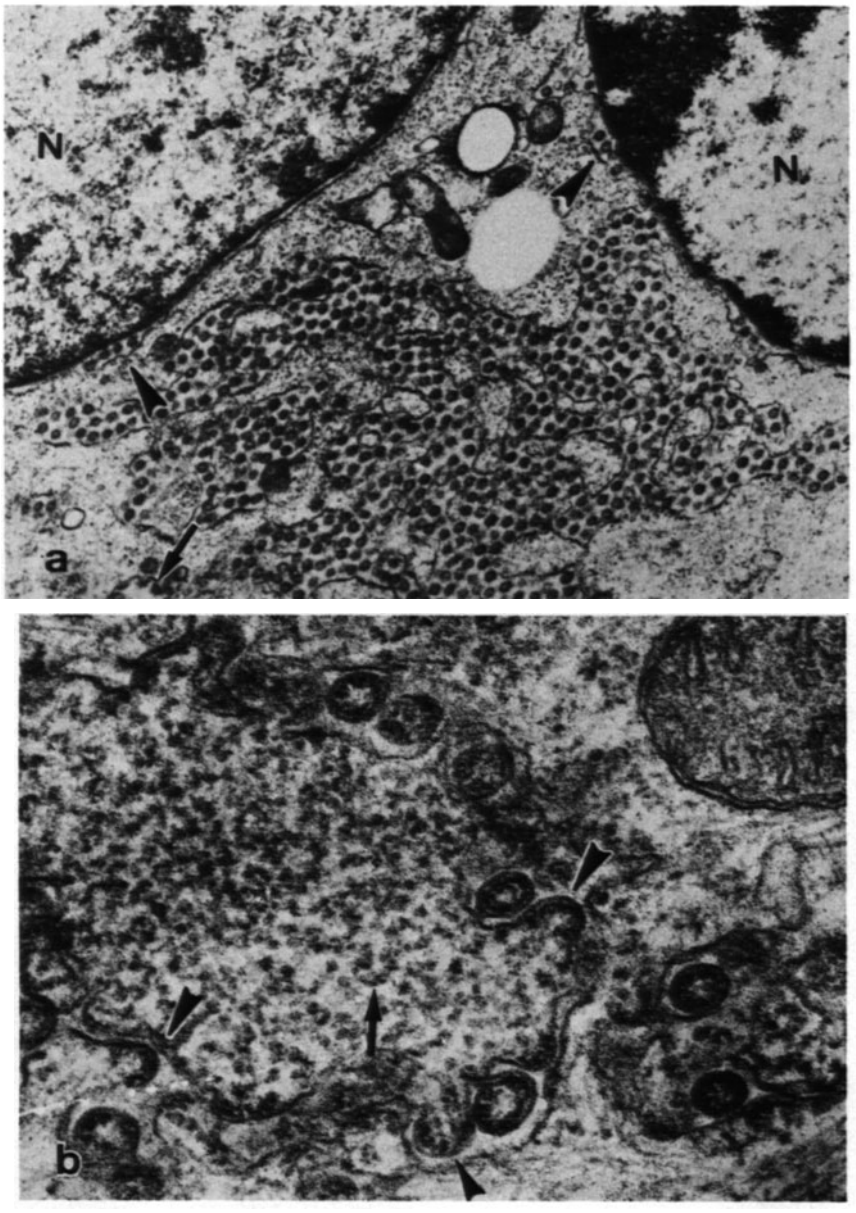


Fig. 7-6. A binucleate cell infected with MHV in which large numbers of virions have accumulated in the lumen of the RER, where a budding virus is seen (arrow). The virions are also commonly seen in the lumen of the nuclear envelope (arrowheads). In *(b),* MHV virions bud (arrowheads) from an area of cytoplasm containing numerous strands of NC (arrow). The centers of the virions are electron-lucent, since the NC is attached to the virus membrane. Magnifications: (a) ×15,000; (b) ×80,000. [(a) Courtesy of J. N. Behnke; (b) from Dubois-Dalcq *et al.,* 1982, with permission from Academic Press]

cellular membranes containing virus glycoproteins (David-Ferriera and Manaker, 1965; Oshiro, 1973; Massalski *et al.*, 1981, 1982; Ducatelle, 1981; Dubois-Dalcq *et al.*, 1982). NCs may associate with the forming envelope by interacting with the cytoplasmic domain of the E1 glycoprotein (Holmes *et al.*, 1981 b). Virions form and are released from cells treated with tunicamycin, although these cells contain little E2 and such virions are completely devoid of E2 (Holmes *et al.*, 1981 a). Thus E2 is evidently not required for the formation of coronavirions. Virions synthesized in the presence of tunicamycin contain normal amounts of N and fully glycosylated E1, but are not infectious, probably because, lacking E2 peplomers, they cannot attach to virus receptors.

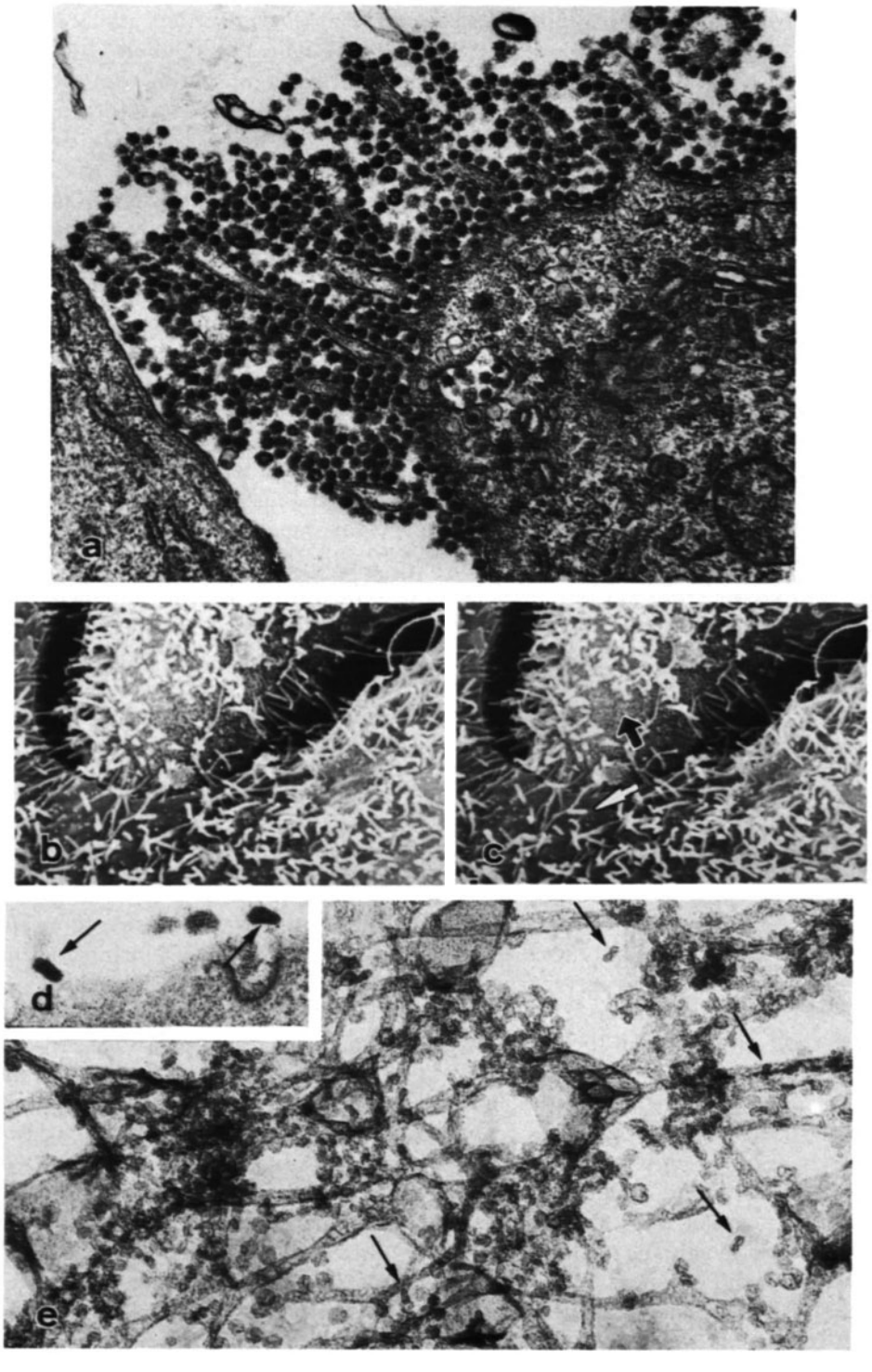
Virions in the RER appear to have large peplomers on their envelopes (Chasey and Alexander, 1976). An important but still unanswered question is whether the glycoproteins E1 and E2 are processed on intact virions as these migrate through the Golgi apparatus, or whether the glycoproteins are fully processed first and then assembled into virions. For the latter to be true, budding of virions from the RER would require migration of glycolysated E2 from the Golgi back to the RER, but such retrograde transport has never been demonstrated. This question can be resolved by isolation of virions from intracellular compartments and characterization of their structural proteins.

Virions are released from membranes by pinching off into the lumina of the RER or Golgi apparatus. Large numbers of virions may accumulate within the lumen of the RER (Fig. 7-6a). In the RER, the virions are spherical particles with electron-lucent centers and NC strands beneath the envelope (Fig. 7-6a and b). Release of virions into the lumen apparently does not involve cellular actin, since actin has not been detected routinely in coronavirions, with the possible exception of IBV (Lomniczi and Morser, 1981).

Release of Virions from the Cell and Post-Release Maturation

Following their release into the RER and Golgi, coronavirions appear to escape from the cell within smooth-walled vesicles that migrate to the cell membrane and fuse with it (Doughri *et al.*, 1976; Oshiro *et al.*, 1971). Time lapse cinematography of MHV-infected cells shows that during the period of maximum virus release, cells are not lysed, although they may undergo fusion (Holmes, unpublished observation). This process of release is similar to secretion of zymogens from pancreatic acinar cells (Holmes *et al.*, 1981 a). Thus, coronaviruses apparently depend on several specialized host cell functions for virus maturation and release, including two mechanisms of protein glycosylation, two pathways of intracellular transport of glycoproteins, and a cellular secretion pathway.

After coronaviruses are released from the cell, many virions remain adsorbed to the plasma membrane (Oshiro *et al.*, 1971; Oshiro, 1973; Doughri and Storz, 1977; Sugiyama and Amano, 1981; Fig. 7-7a–c). It is not clear why such large numbers of virions bind to infected cells. Are they simply virions adherent to the smooth-walled vesicles which have not detached from the membrane following fusion of the vesicle with the plasma membrane? Or were these virions first released into the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medium and then bound to receptors on the plasma membrane? If the latter is true, then why are there very different numbers of virions bound to cells in the same culture (Fig. 7-7b and c)? Do the virions bind to cellular receptors which are differently expressed in different cells, possibly due to cell cycle differences, or do they bind to a virus glycoprotein such as E2, which is present on the plasma membrane?

As virions of the A59 strain of MHV are transported through the Golgi-associated vesicles in some cell types, they become flattened, disc-shaped and electron-dense (Holmes *et al.*, 1981a; Fig. 7-7d and e). Possibly, these changes could be associated with release of the NC from its attachment to the envelope. They might be triggered by changes in pH or ionic concentrations in different intracellular membrane compartments. Alternatively, they may reflect processing of virus components which takes place as virions migrate through different intracellular compartments. For instance, since cleavage of the 180 K E2 to 90 K E2 appears to be an event which occurs immediately prior to release of the virions (Holmes *et al.*, 1984), it is possible that the condensation of virions may be triggered by that event. Since proteolytic cleavage of E2 appears to be dependent upon the host cell, this might explain why post-release condensation of virions is not seen for all coronaviruses or even for MHV-A59 in all cell types.

Many enveloped viruses show polarized budding from epithelial cells (Rodriguez-Boulan and Sabatini, 1978; Chapter 1). Since coronaviruses do not bud from the plasma membrane, variations in the transport of the E2 glycoprotein to apical or basolateral domains of the plasma membrane would be expected to have no effect on virus budding. However, many polarized cells show marked polarity in secretion of cellular proteins and this polarized secretion might affect release of coronaviruses from post-Golgi vacuoles. Although extensive studies on the release of coronaviruses from cells in epithelial tissues have not been done, release of porcine coronavirus from both apical and basal regions of intestinal cells has been demonstrated (Doughri and Storz, 1977). It is clear that MHV virions may be released from the lower surface of mouse fibroblasts, since membrane fragments of cells which remain attached to the substrate after virus-infected cells peel away show large numbers of adherent, flattened, disc-shaped virions (Fig. 7-7e, Holmes and Turner, unpublished observations).

Fig. 7-7. Large numbers of coronavirions adsorbed to the plasma membrane of infected cells. *(a)* shows human respiratory coronaviruses attached to cells *in vitro*. *(b)* and *(c)* are a stereo pair of scanning electronmicrographs of MHV-infected cells demonstrating that adjacent cells may show great differences in the number of adherent virions. On the upper cell, virions (large arrow, c) are tightly packed on the membrane, whereas on the lower cell, virions (small white arrow, c) are widely scattered over the plasma membrane and microvilli. *(d)* and *(e)* show that MHV A59 virions on the plasma membrane often appear flattened and disc-shaped. In sections of infected cells observed by transmission electron microscopy (d), virions (arrows) at the plasma membrane are flattened and electron-dense. In (e), flattened virions are adsorbed to fragments of cell membrane left adhering to the substrate after an MHV-infected cell has detached. The preparation was fixed, dehydrated, dried by the critical point method, and observed with a high voltage electron microscope. Large numbers of virions (arrows) adhere to the membranes beneath the cells. Magnifications: (a) ×35,000; (b) and (c) ×9400; (d) ×52,000; (e) ×33,000. [(a) Courtesy of Dr. L. Oshiro, from Oshiro *et al.*, 1971, with permission of Cambridge University]

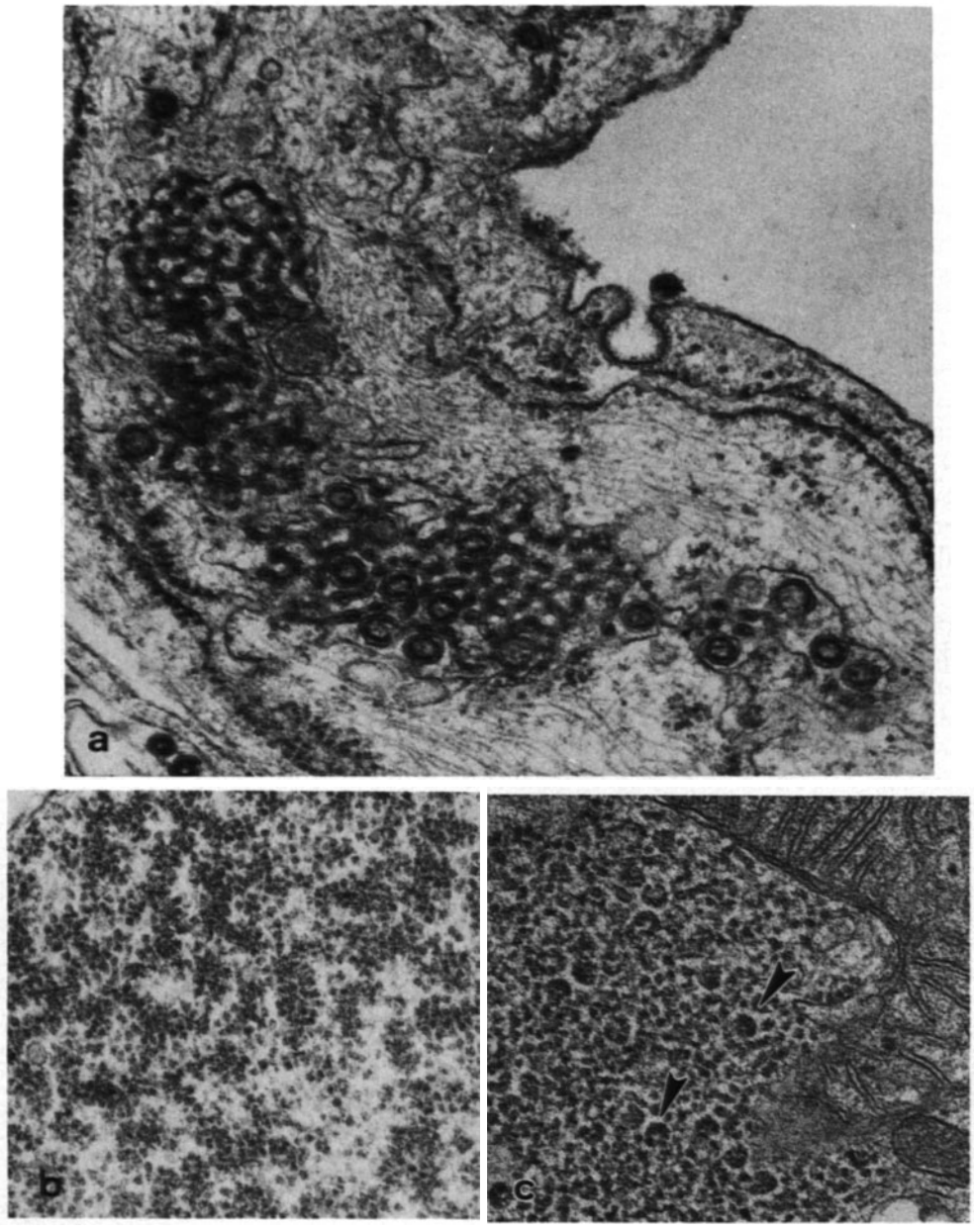


Fig. 7-8. Inclusions characteristic of coronavirus-infected cells. In *(a)*, reticular inclusions formed of specialized infoldings of RER membranes are shown in a cell infected with a human coronavirus. *(b)* and *(c)* show cytoplasmic inclusions of MHV NC seen in differentiated nerve cells *in vitro*. Inclusions formed by the wild Type JHM strain of MHV contain core-like structures composed of twisted NC strands (arrowheads) in *(c)*. Inclusions formed by a ts mutant of the JHM strain are mostly made of granules (b). Magnifications: (a) ×50,000; (b) ×51,000; (c) ×68,200. [(a) from Oshiro *et al.*, 1971, with permission of Cambridge University; (b) and (c) from Dubois-Dalcq *et al.*, 1982; with permission of Academic Press]

Defective Assembly

In this section, we will consider circumstances in which the synthesis of virus components is not synchronized with release of mature virions. Under these conditions, morphological events can be observed which may provide insight into virus assembly. Coronaviruses do not normally bud from the plasma membrane (Oshiro, 1973), although such budding is occasionally seen late in the infectious cycle (Dubois-Dalcq *et al.*, 1982) when some E1 may have been transported to the plasma membrane. After release of virions has ceased, cytoplasmic inclusions of viral NCs have been observed (Caul and Eggleston, 1977; Watanabe, 1969; Massalski *et al.*, 1982; Dubois-Dalcq *et al.*, 1982; Figs. 7-6b and 7-8b and c). These inclusions vary considerably in appearance and their protein composition has not yet been determined. Reticular cytoplasmic inclusions, which consist of a convoluted network of tightly apposed membranes continuous with the RER, are another characteristic of coronavirus infection (David-Ferriera and Manaker, 1965; Oshiro *et al.*, 1971; Fig. 7-8a).

Coronavirus-infected cells often contain spherical inclusions about 100 to 400 nm in diameter, which contain fine fibrils and are bounded by double membranes apparently derived from RER (David-Ferriera and Manaker, 1965; Sturman and Holmes, 1983; Fig. 7-9). These closely resemble structures called type 1 cytopathic vesicles (CPV-I) seen in cells infected with alpha- or flaviviruses (Murphy, 1980; Grimley *et al.*, 1972; see Chapter 8). They may represent aberrent virion formation or be associated with virus RNA synthesis, as has been suggested for alphaviruses (Grimley *et al.*, 1972).

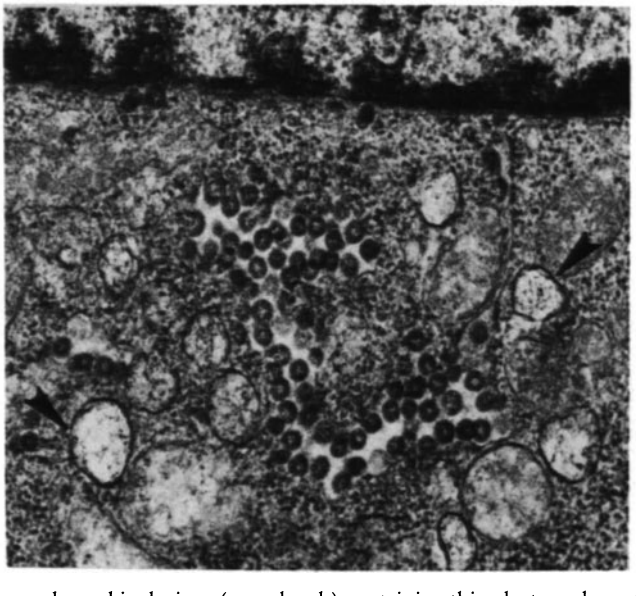
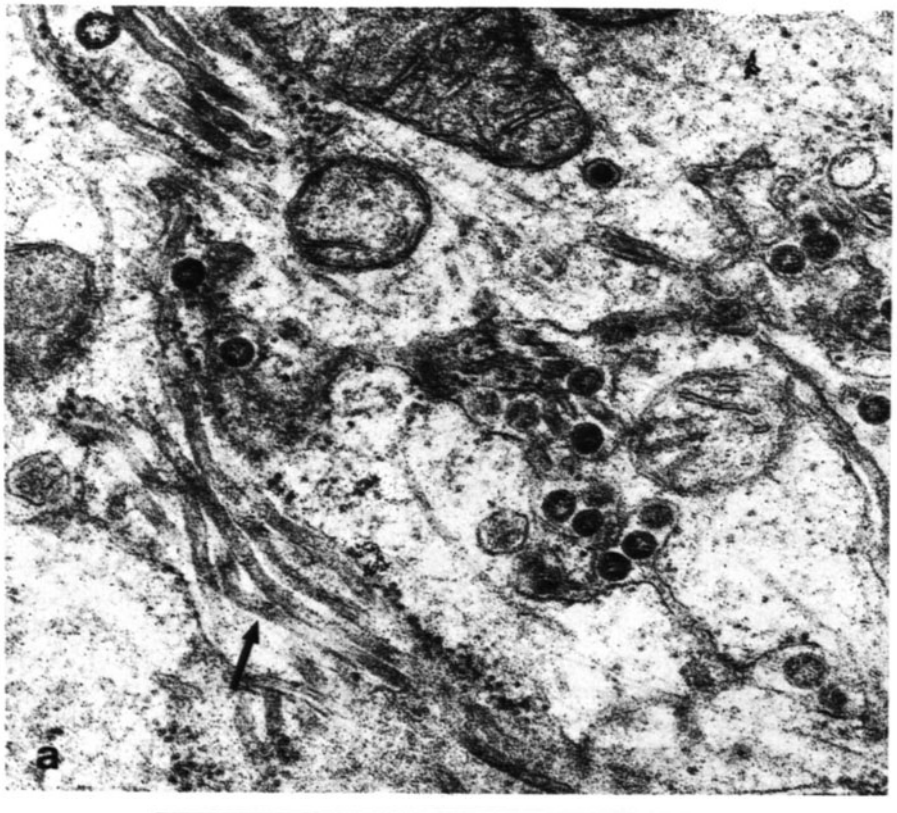
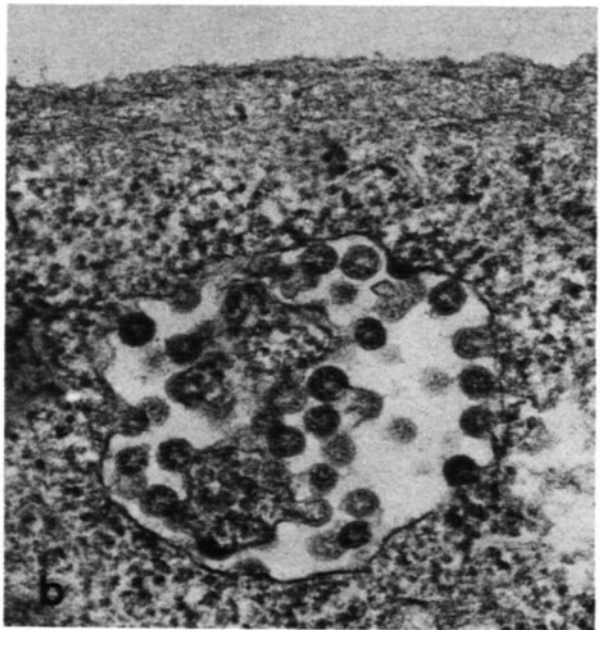


Fig. 7-9. Membrane-bound inclusions (arrowheads) containing thin electron-dense filaments. These inclusions in coronavirus-infected cells resemble the type 1 cytopathic vesicles seen in cells infected with alpha- and flaviviruses (see Chapter 8, Figs. 8-5a and 8-13). They may be involved in virus RNA synthesis. Magnification: ×75,000


a


b

Late in infection with MHV, and in tunicamycin-treated MHV-infected cells, long rigid tubules about 50 nm in diameter are seen in the lumen of the RER and in smooth-walled vesicles (Dubois-Dalcq *et al.*, 1982; Holmes *et al.*, 1981 a; Fig. 7-10a). Similar tubules have been assembled *in vitro* from the matrix protein of a paramyxovirus (Heggeness *et al.*, 1982 a). Since E1 functions as a M-like protein which associates with membranes, it has been suggested that the coronavirus tubules are formed of excess E1 (Sturman and Holmes, 1983).

Empty virus particles have been isolated from coronavirus-infected cells by density gradient ultracentrifugation (Macnaughton and Davies, 1980), but the mechanism of their formation and release is not understood.

To analyze interactions of NCs with virus-specific membrane proteins, investigators of other virus systems have studied the formation of pseudotypes with different viruses. Attempts to isolate pseudotypes of coronaviruses with other virus types have been limited to pseudotypes of MHV with murine leukemia virus (Yoshikura and Taguchi, 1978). Further studies of coronavirus pseudotypes with other viruses that bud into the RER and Golgi may elucidate the assembly of coronaviruses.

Treatment of MHV-infected cells with monensin arrests virus budding (Niemann *et al.*, 1982; Fig. 7-10b), and removal of monensin results in rapid budding of virions into the Golgi apparatus. These effects are probably caused by monensin-induced inhibition of intracellular transport of MHV glycoproteins.

Ts mutants have been used to study the morphogenesis of several groups of enveloped viruses. Although several independent collections of *ts* mutants of coronaviruses have been generated (Robb *et al.*, 1979; Haspel *et al.*, 1978; Koolen *et al.*, 1983), most of these mutants are of the RNA negative (RNA–)phenotype. The few RNA+ mutants which make structural proteins at the non-permissive temperature have not been examined for defects in virus maturation.

V. Organization of the Virion

A summary of the events in coronavirus assembly is shown in Fig. 7-11.

A model of the organization of the structural components of MHV is shown in Fig. 7-12. This general model seems to be valid for most coronaviruses (Sturman and Holmes, 1983), with the possible exception of BCV, which may have an additional glycoprotein (King and Brian, 1982). In negatively stained preparations, coronavirions have only one type of peplomer, with the possible exception of BCV and

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Fig. 7-10. Late in MHV infection, long tubules, 25 to 30 nm in diameter, may accumulate in the lumen of the RER (arrow *a*). These may represent excess E1 glycoprotein in association with membranes. *(b)* shows that monensin may inhibit the maturation of coronaviruses. In monensin-treated cells, partially budded virions are seen in large numbers in smooth-walled vesicles associated with the Golgi. Magnifications: (a) × 53,000; (b) × 60,000. [(a) from Dubois-Dalcq *et al.*, 1982; with permission of Academic Press]

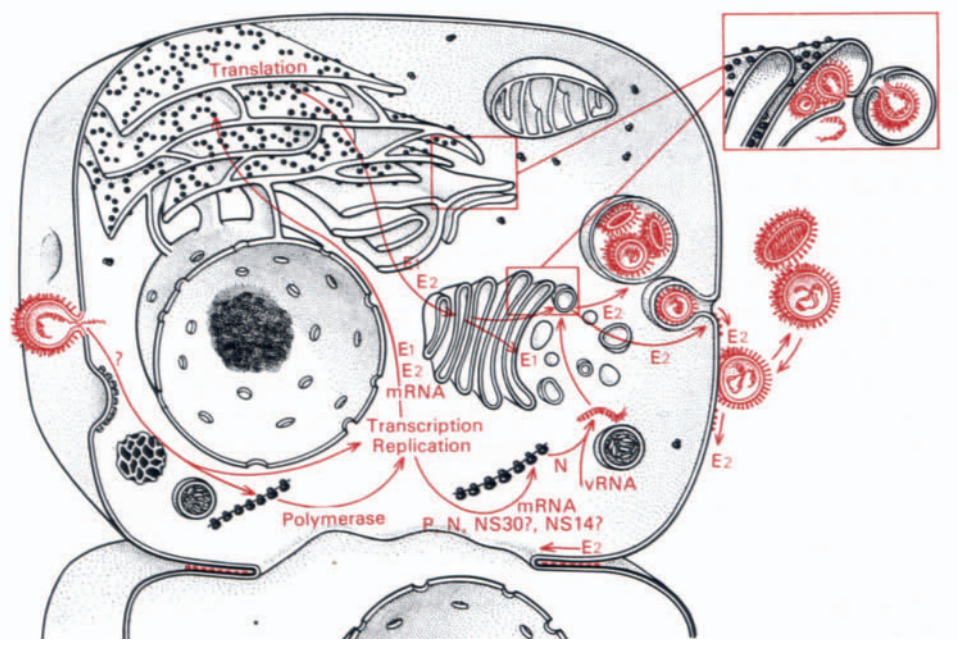

Fig. 7-11. A model summarizing the events in replication, transcription and assembly of mouse hepatitis virus. It is likely that the virus RNA enters the cell by fusion of the virus envelope with the plasma membrane. Primary translation yields the RNA polymerase which copies the genomic RNA to make a full length negative-stranded template. Then, synthesis of the 6 subgenomic mRNAs occurs and the 5′ terminal end of each of these is translated to make a single protein species. The mRNAs for E1 and E2 envelope glycoproteins are translated on membrane-bound ribosomes. E2 is glycosylated with N-linked oligosaccharides and is acylated. In contrast, E1 is glycosylated with O-linked oligosaccharides and is not acylated. E2 is transported through the Golgi to the plasma membrane, but intracellular transport of E1 is terminated at the Golgi. Meanwhile, the other mRNAs appear to be translated on free polysomes to yield the non-structural proteins and the NC protein N. N assembles with new virus genomic RNA to form a helical NC. The NC probably interacts with the cytoplasmic domain of E1 in the membranes of the RER or Golgi at sites where E2 is also present. Budding of virions into the RER or Golgi is followed by their release from the intact cell by a process analogous to protein secretion (see text). The window shows virions budding from the RER and Golgi membranes. Cleavage of the E2 glycoprotein by a host cell protease appears to occur just prior to virus release. E2 is transported to the plasma membrane where it may induce fusion with adjacent cells

one strain of MHV (Bridger *et al.*, 1978; Greig *et al.*, 1971; Sugiyama and Amano, 1981). The smaller type of peplomer may contain the additional glycoprotein. Some glycosaminoglycan, a host cell component, has been detected even in highly purified coronaviruses (Garwes *et al.*, 1976; Sturman, 1981). No function for this has been identified.

The molar ratio of structural proteins within the virion is N : E1 : E2 = 8 : 16 : 1 (Sturman, 1981). This model indicates that the E1 glycoprotein interacts with both the NC and the E2 glycoprotein. The organization of the NC within the released virion is uncertain. Although the NC in the budding virus appears to be a well-formed helix attached to the inside of the virus envelope, in the released virion the NC may be less firmly bound to the membrane and may be partially uncoiled.

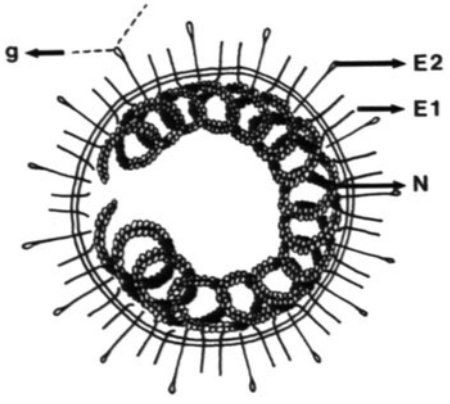

Fig. 7-12. A model of the organization of the MHV virion. The N protein is associated with the virus RNA genome in the helical NC. During budding, the NC probably associates with the cytoplasmic domain of the E1 glycoprotein which acts as a matrix protein. The peplomers are formed of the large glycoprotein E2, which probably interacts with the E1 in the virus envelope. A small amount of host glycosaminoglycan *(g)* is associated with purified coronavirions. (After Sturman and Holmes, 1983)