CHAPTER 7

The Coronavirus Nucleocapsid Protein

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I. INTRODUCTION

The nucleocapsid (N) proteins of coronaviruses, as with most enveloped viruses, have received less attention than the surface glycoproteins and generally have been perceived to be of lesser concern. Interest in this class of proteins, however, has been stimulated in recent years by such diverse developments as the recognition of the importance of RNA–protein interactions, most notably in the study of spliceosomes and related ribonucleoproteins, as well as the finding in numerous viral systems that internal virion proteins can be major determinants of the cellular immune response.

Coronaviruses represent, together with the recently characterized toroviruses, the only positive-strand enveloped viruses with helically symmetric nucleocapsids. Thus, coronavirus N proteins present a number of intriguing molecular biological problems: What are the geometric parameters of these nucleocapsid structures and how do they compare to their negative strand RNA virus counterparts? What is the nature of the N–RNA and N–N interactions that stabilize the helix? Where are the loci of the multiple phosphorylations in the N molecule and what regulatory role (if any) do these play? How does N interact with the membrane protein (M) during virion assembly and uncoating? How does N participate in the unique mechanism of coronavirus RNA synthesis? Finally, what is the antigenicity of the N molecule? This chapter re-

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views our present state of knowledge about these largely unanswered questions.

II. CORONAVIRUS RIBONUCLEOPROTEIN

The structure of the nucleocapsid (or ribonucleoprotein, RNP) of coronaviruses has not yet been completely elucidated, although electron microscopic studies are in general agreement about the nature of this internal viral component. Ideally, a complete description of the coronaviral RNP would include: the diameter of the helical nucleocapsid as well as the diameter of the central cylindrical hole around which the helix is wound; the number of helical turns per unit length; the stoichiometry of nucleotides of RNA per monomer of N protein; the overall length of the encapsidated genome; and, finally, the superstructure of the helix packed within the virion. The existing studies, carried out by thin sectioning of virion particles (Apostolov *et al.*, 1970) or by negative staining of spontaneously or detergent-disrupted virions (Kennedy and Johnson-Lussenburg, 1975/76; Macnaughton *et al.*, 1978; Caul *et al.*, 1979; Davies *et al.*, 1981), provide some of these parameters for some coronaviruses.

The earlier morphological work described the coronavirus RNP as a thread-like strand 7–8 nm in diameter [avian infectious bronchitis virus (IBV): Apostolov *et al.*, 1970)] or 8–9 nm in diameter [human coronavirus-229E (HCV-229E): Kennedy and Johnson-Lussenburg, 1975/76)]. Subsequent work yielded images of clearly helical structures from HCV-229E (Macnaughton *et al.*, 1978; Caul *et al.*, 1979), murine hepatitis virus-3 (MHV-3) (Macnaughton *et al.*, 1978), and IBV (Davies *et al.*, 1981). These had reported diameters of 9–11 nm, 11–13 nm (Caul *et al.*, 1979), or 14–16 nm (Macnaughton *et al.*, 1978), with 3- to 4-nm diameter hollow cores. The variations in observed dimensions possibly reflect differences in the methods of sample preparation. These helix diameters fall at the most narrow extreme for those observed with nucleocapsids in the paramyxovirus family (i.e., in the pneumovirus genus). RNP structures of IBV were the most difficult to prepare among the viruses studied (Macnaughton *et al.*, 1978; Davies *et al.*, 1981) and fully elongated unwound helices were often observed, the longest of these being 6.7 μm in length.

The long, helical coronavirus RNP apparently must assume a higher-order structure to be packaged into the viral particle. For HCV-229E, a tightly coiled superstructure (approximately 60-nm diameter) was sometimes observed in material from disrupted virions, although more often this appeared as a tangled or knotted mass (Kennedy and Johnson-Lussenburg, 1975/76). Similarly, a coiled or helical superstructure was seen for the RNP of MHV-A59 virions budding into the endoplasmic reticulum of infected cells (Holmes and Behnke, 1981). This coiling suggests that the coronavirus helical nucleocapsid is fairly flexible, another structural similarity it shares with paramyxovirus nucleocapsids.

Several biochemical features of virion-associated N protein, established in early studies, were consistent with its postulated role as the structural protein of the coronavirus RNP. N was found to be the sole virus polypeptide not to be

glycosylated and to be phosphorylated (Pocock and Garwes, 1977; Wege *et al.*, 1979; Stohlman and Lai, 1979; Callebaut and Pensaert, 1980; Macnaughton, 1980; Cavanagh, 1981; Lomniczi and Morser, 1981; Stern *et al.*, 1981; Rottier *et al.*,1981a; Siddell *et al.*, 1981b; King and Brian, 1982). A relatively high content in arginine and glutamine residues was established, consistent with the basic character anticipated for a nucleic acid binding protein (Garwes *et al.*, 1976; Sturman, 1977). Also, protease digestion of virions left the N polypeptide completely unaffected, arguing for its internal location (Sturman, 1977; Callebaut and Pensaert, 1980; Obert *et al.*, 1981; King and Brian, 1982; Schmidt and Kenny, 1982).

Direct experimental support for the assignment of the N polypeptide as the nucleocapsid protein came from the analysis of the composition of subviral components from detergent-solubilized virions separated on density gradients. In several reports, treatment of the viral envelope with a nonionic detergent in low salt concentration failed to liberate true RNP structures. Thus, gel electrophoretic analysis of subviral particles released by NP40 treatment at 20 °C from transmissible gastroenteritis virus (TGEV) virions (buoyant density 1.295 g/ml in cesium sulfate: Garwes *et al.*, 1976), MHV-JHM virions (1.26 g/ml in sucrose: Wege *et al.*, 1979), hemagglutinating encephalitis virus (HEV) virions (Pocock and Garwes, 1977), or from IBV virions treated with Triton X-100 at 37 °C (1.32 g/ml in sucrose: Lancer and Howard, 1980) revealed the presence of two polypeptides, corresponding to the nucleocapsid protein N and the membrane glycoprotein M. The recovery of RNP structures containing essentially only the N polypeptide species was reported following NP40 treatment at 0–4 °C of MHV-A59 virions (Sturman *et al.*, 1980) and of IBV virions (1.27 g/ml in sucrose: Davies *et al.*, 1981). The MHV study recognized a subtle influence of temperature on the interaction of M protein with the RNP: virions disrupted by NP40 at 4 °C released a structure banding at 1.28 g/ml in sucrose and containing only N protein, while subsequent incubation at 37 °C produced an M protein–nucleocapsid complex banding at 1.22 g/ml.

III. N PROTEIN STRUCTURE

Almost all of our present structural knowledge of the N proteins of coronaviruses derives from amino acid sequences deduced from nucleotide sequences of cloned N gene cDNAs. To date, these have been reported for 11 coronaviruses: MHV (Skinner and Siddell, 1983, 1984; Armstrong *et al.*, 1983, 1984; Parker and Masters, 1990; Kunita *et al.*, 1992; Decimo *et al.*, 1993), IBV (Boursnell *et al.*, 1985; Sutou *et al.*, 1988; Williams *et al.*, 1992), TGEV (Kapke and Brian, 1986; Rasschaert *et al.*, 1987; Britton *et al.*, 1988), bovine coronavirus (BCV) (Lapps *et al.*, 1987; Cruciere and Laporte, 1988), HCV-OC43 (Kamahora *et al.*, 1989), HCV-229E (Schreiber *et al.*, 1989; Myint *et al.*, 1990), feline infectious peritonitis virus (FIPV) (Vennema *et al.*, 1991), turkey coronavirus (TCV) (Verbeek and Tijssen, 1991), canine coronavirus (CCV) (Horsburgh *et al.*, 1992; Vennema *et al.*, 1992), porcine epidemic diarrhea virus (PEDV) (Bridgen *et al.*, 1993), and sialodacryoadenitis virus (SDAV) (Kunita *et al.*, 1993).

The general physical properties of the N proteins are markedly similar (Table I). The encoded polypeptides range from 377 to 455 amino acids in length. Notably, MHV, SDAV, BCV, TCV, and HCV-OC43, which belong to the same antigenic cluster, have N proteins roughly 10% larger than those of the remainder of the family. Also striking is the presence in PEDV N protein of an approximately 40-residue-long stretch that has no counterpart in the other members (Bridgen *et al.*, 1993). This unique sequence, particularly rich in serine, arginine, and asparagine residues, is located in the central portion of the molecule (see Fig. 1) and might reflect a recombinational event or a stuttering of the viral polymerase since it exhibits some periodicity. The virion-associated N protein of coronaviruses resolves by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as a single species with molecular weight ranging from 45 to 63 kDa, depending on the virus and on the strain (for references, see Siddell *et al.*, 1982). These apparent sizes are significantly larger than the molecular weights calculated from sequence information, possibly due both to anomalous distributions of amino acid compositions and to phosphorylation of the proteins. Even among five closely related strains of MHV, molecular weight differences of up to 3 to 5 kDa have been observed (Cheley *et al.*, 1981), although the calculated molecular weights for these species are almost identical (Parker and Masters, 1990).

Coronavirus N proteins are highly basic, having an excess of at least 12 lysine and arginine residues over aspartate and glutamate residues. One measure of this is seen in the calculated overall isoelectric points (pIs) of these polypeptides, which fall in the range 10.3–10.7 (Table I). Basic amino acids are not clustered in strings as in proteins such as simian virus 40 (SV40) T antigen or the capsid proteins of alphaviruses, but local densities of positive charge can be found, particularly in two loci in the middle of the N molecule (Fig. 1). In marked contrast to the bulk of the molecule, the carboxy-terminus of each coronavirus N protein is quite acidic, as indicated by pIs ranging from 4.3 to 5.5 calculated for the 45 carboxy-terminal amino acids (Table I). Another noteworthy feature of the N proteins is that they all have a relatively high serine content (7–11%) (Table I). These potential targets for phosphorylation (see Section V) are distributed throughout the molecule, but many are interspersed in the first of the two basic regions mentioned above. It is interesting that most of the above-cited characteristics of coronavirus N proteins are also shared by the nucleocapsid (NP) proteins of influenzaviruses (Table I), perhaps due to an ancestral relationship between these two families.

In spite of the general similarities indicated for the coronavirus N proteins, there exists only a low degree of sequence homology among them. For example, the N protein of BCV shows an overall sequence homology of 29% with the N protein of TGEV or of IBV; the sequence homology of the N proteins of MHV and BCV, which belong to the same antigenic cluster, is 70%, i.e., notably less than between the M proteins (Lapps *et al.*, 1987). An optimal alignment of the 11 sequences reveals about 30 residues common to all, including 8 glycines and 3 prolines; in contrast, the few cysteine residues present in the sequences are not at conserved positions. An exception to this overall lack of N protein homology is a region of roughly 50 amino acids, falling within the amino-terminal third of

TABLE I. Amino Acid Compositions of the Coronavirus N Proteins[a]

	Amino acids	Lys + arg	Asp + glu	Ser	pI total[b]	pI C-terminus[c]	Predicted mol. wt. (kDa)	Reported M_r (kDa)[d]
MHV-A59	454	63	46	41	10.5	5.1	49.7	50–55
SDAV	454	60	42	40	10.6	5.3	49.4	50
BCV	448	59	46	42	10.6	5.3	49.4	52
TCV	448	59	46	43	10.6	5.3	49.4	52
HCV-OC43	448	58	46	42	10.5	5.3	49.3	52
TGEV	382	69	46	42	10.7	5.3	43.4	47
CCV	381	69	47	36	10.6	5.1	43.4	50
FIPV	377	65	45	35	10.7	5.5	42.7	45
HCV-229E	389	60	46	39	10.3	4.5	43.4	47–50
PEDV	441	72	50	34	10.7	4.3	48.9	55–58
IBV	409	72	53	28	10.5	4.9	45.0	50–54
Influenza (A/NT/60/68)	498	70	59	39	10.4	4.5	55.9	50–54

[a]Sources of coronavirus sequences are given in the text. Influenza N protein data was deduced from the sequence data of Huddleston and Brownlee (1982).
[b]Computed isoelectric point of the entire N molecule.
[c]Computed isoelectric point of the C-terminal 45 amino acid residues.
[d]Relative mass, from more recent reports, as determined by SDS-PAGE.

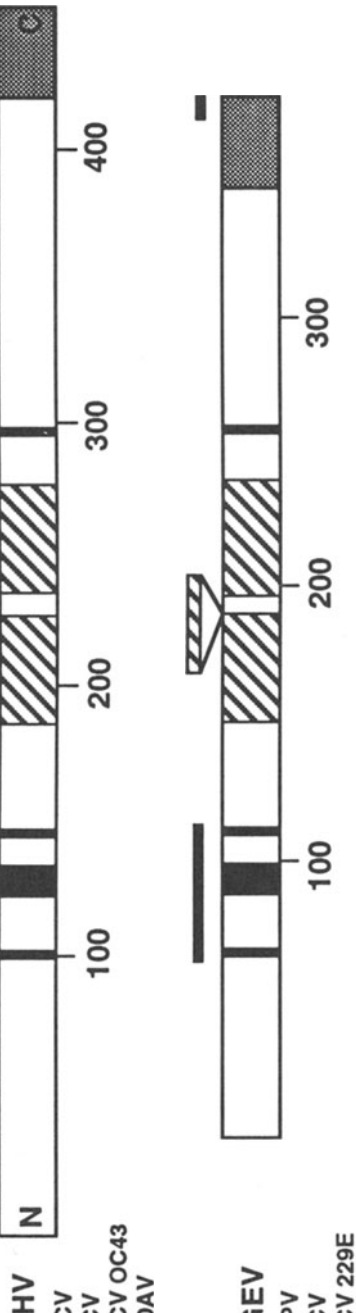

FIGURE 1. Structural organization of the coronavirus N protein. Common structural features and their distribution along the polypeptide chain are shown for two coronaviruses, MHV and TGEV, each being representative of a coronavirus subgroup, as indicated. Regions homologous to all the viruses are indicated by closed boxes (at least 3 consecutive amino acids conserved; the sequence stretch encompassing the amino-most 3 boxes is shown in Fig. 2). The striped boxes indicate two major hydrophylic, basic domains: the first, rich in Arg and Ser residues; the second, rich in Lys residues. The shaded box indicates a markedly acidic domain. The bars above the frames indicate particular domains mapped in the N protein of the relevant virus: open bar, RNA binding domain; striped bar, relative position of the 40 residue insertion in PEDV N; closed bars, antigenic sites. The arrowheads indicate the position of sequence alterations in two *ts* mutants (see text).

each molecule, which contains a relatively high degree of sequence identity (Fig. 2). Such a marked degree of conservation may signal an important function common to the N proteins. The strong proportion of aromatic residues in this segment possibly participate in stabilizing N binding to RNA via ring stacking interactions (Ollis and White, 1987); alternatively, the conserved segment may be involved in other hydrophobic interactions not directly related to RNA binding.

To date, two coronavirus N protein mutants have been described, both in MHV-A59. Each is thermolabile as well as temperature-sensitive (*ts*), forming small plaques at the nonpermissive temperature. The first of these, Albl (Masters *et al.*, 1994), has a single critical amino acid change within the highly conserved region mentioned above, as well as a closely linked amino acid change that is phenotypically silent. The second, Alb4 (Koetzner *et al.*, 1992), contains a 29-amino-acid deletion of a putative spacer region that separates the carboxy-terminal domain of the N protein from the remainder of the molecule. In addition to these, the *ts*209 mutant of MHV-A59 (Koolen *et al.*, 1983) is potentially an N protein mutant (see Section IV) but has not yet been mapped. Further study of these mutants may provide new insights into N protein structure and function.

MHV-A59	102	G	Y	W	Y	R	H	N	R	R	S	F	K	T	P	D	G	Q	Q	K	Q	L	L	P	R	W	Y	F	Y	Y	L	G	T	G
SDAV	102	G	Y	W	Y	R	H	N	R	R	S	F	K	T	P	D	G	Q	Q	K	Q	L	L	P	R	W	Y	F	Y	Y	L	G	T	G
BCV	99	G	Y	W	Y	R	H	N	R	R	S	F	K	T	A	D	G	N	Q	R	Q	L	L	P	R	W	Y	F	Y	Y	L	G	T	G
TCV	99	G	Y	W	Y	R	H	N	R	R	S	F	K	T	A	D	G	N	Q	R	Q	L	L	P	R	W	Y	F	Y	Y	L	G	T	G
HCV-0C43	99	G	Y	W	Y	R	H	N	R	G	S	F	K	T	A	D	G	N	Q	R	Q	L	L	P	R	W	Y	F	Y	Y	L	G	T	G
TGEV	68	G	Y	W	N	R	Q	T	R	-	-	Y	R	M	V	K	G	Q	R	K	E	L	P	E	K	L	H	F	Y	Y	L	G	T	G
CCV	68	G	Y	W	N	R	Q	T	R	-	-	Y	R	M	V	K	G	R	R	K	E	L	P	E	K	L	H	F	Y	Y	L	G	T	G
FIPV	68	G	Y	W	N	R	Q	I	R	-	-	Y	R	I	V	K	G	Q	R	K	E	L	A	E	K	L	H	F	Y	Y	L	G	T	G
HCV-229E	56	G	Y	W	N	V	Q	K	R	-	-	F	R	T	R	K	G	K	R	V	D	L	S	P	N	W	H	F	Y	Y	L	G	T	G
PEDV	51	G	Y	W	N	E	Q	I	R	-	-	W	R	M	R	R	G	E	R	I	E	Q	P	S	N	W	H	F	Y	Y	L	G	T	G
IBV	69	G	Y	W	R	R	Q	A	R	-	-	F	K	P	G	K	G	G	R	K	P	V	P	D	A	W	Y	F	Y	Y	T	G	T	G

P	R	W	Y	F	Y	Y	L	G	T	G	P	H	A	G	A	S	Y	G	D	S	I	E	G	V	F	W	V	A						152
P	R	W	Y	F	Y	Y	L	G	T	G	P	H	A	G	A	S	F	G	D	S	I	E	G	V	F	W	V	A						152
P	R	W	Y	F	Y	Y	L	G	T	G	P	H	A	K	D	Q	Y	G	T	D	I	D	G	V	F	W	V	A						149
P	R	W	Y	F	Y	Y	L	G	T	G	P	H	A	K	D	Q	Y	G	T	D	I	D	G	V	F	W	V	A						149
P	R	W	Y	F	Y	Y	L	G	T	G	P	H	A	K	D	Q	Y	G	T	D	I	D	G	V	Y	W	V	A						149
P	R	W	F	F	Y	Y	L	G	T	G	P	H	A	D	A	K	F	K	D	K	L	D	G	V	V	W	V	A						116
P	R	W	F	F	Y	Y	L	G	T	G	P	H	A	D	A	K	F	K	Q	K	L	D	G	V	V	W	V	A						116
P	K	W	F	F	Y	F	L	G	T	G	P	H	A	D	A	K	F	K	D	K	I	D	G	V	F	W	V	A						116
P	K	L	H	F	Y	Y	L	G	T	G	P	H	K	D	A	K	F	R	E	R	V	E	G	V	V	W	V	A						105
P	N	W	H	F	Y	Y	L	G	T	G	P	H	G	D	L	R	Y	R	T	R	T	E	G	V	F	W	V	A						99
P	A	W	Y	F	Y	Y	T	G	T	G	P	A	A	D	L	N	W	G	D	T	Q	D	G	I	V	W	V	A						117

FIGURE 2. Region of greatest homology among coronavirus N proteins. Numbers indicate the amino acid residues of each N protein shown in the alignment. Boxed residues are those which are identical in at least seven of the eleven sequences. The sources of the sequence data are given in the text.

IV. SYNTHESIS OF N PROTEIN AND NUCLEOCAPSID FORMATION

The nucleocapsid protein appears to be the most abundant viral polypeptide in coronavirus-infected cells during all stages of infection. N protein is readily detected from 3 to 5 hr after infection onward, at the same time or earlier than the other structural proteins, and its synthesis is maintained throughout the virus cycle (Anderson *et al.*, 1979; Bond *et al.*, 1979; Holmes *et al.*, 1981; Siddell *et al.*, 1981b; Stern *et al.*, 1981; Rottier *et al.*, 1981b; Stohlman *et al.*, 1983; Garwes *et al.*, 1984; Talbot *et al.*, 1984; Keck *et al.*, 1988; Dea and Tijssen, 1989; Simkins *et al.*, 1989). In latently infected Schwannoma cells, MHV-JHM N protein was shown to be produced for as long as 7 days and beyond in the absence of infectious virus production (Coulter-Mackie *et al.*, 1985). In cells infected with MHV-JHM defective-interfering RNAs, the level of N synthesis was found to be not significantly altered throughout multiple passages, in contrast to the other viral polypeptides (Makino *et al.*, 1985). Thus, it seems that a strong and permanent expression of N gene product accompanies both productive and nonproductive coronavirus infection.

Coronavirus N protein is synthesized on free polysomes (Niemann *et al.*, 1982), but appears to become rapidly associated to intracellular membranes (Stohlman *et al.*, 1983). Its subcellular location has been examined by immunofluorescence labeling of MHV-, TGEV-, and TCV-infected cells. Anti-N monoclonal antibodies produce a bright particulate fluorescence which tends to distribute evenly in the cytoplasm (Robb and Bond, 1979; Laude *et al.*, 1986; Holmes *et al.*, 1987; Welch and Saif, 1988; Dea and Tijssen, 1989). Intriguingly, two groups of investigators have reported an important concentration of N-antigen within the nuclei of MHV-infected cells (Robb and Bond, 1979; Holmes *et al.*, 1987). The MHV-A59 *ts* mutant Alb4, shown to have a short deletion in its N gene (Koetzner *et al.*, 1992), exhibited an altered distribution of N antigen in the cytoplasm and the nucleus (Holmes *et al.*, 1987). Translocation of N into the nucleus might reflect the ability of the protein to bind to nucleic acids in a nonspecific manner (see Section VI), or it may indicate the fortuitous presence of a nuclear translocation signal in this highly basic protein. However, nuclear translocation cannot be essential for productive infection since MHV is capable of replicating in enucleated cells (Brayton *et al.*, 1981; Wilhelmsen *et al.*, 1981).

Studies on the biosynthesis of coronavirus nucleocapsid proteins in infected cells have revealed the presence of one major intracellular form, designated N, and of several N-related polypeptides. The major N species has been shown to comigrate with virion-associated N protein in denaturing gels (Siddell *et al.*, 1981b; Rottier *et al.*, 1981b; Laude *et al.*, 1986; Dea and Tijssen, 1989) and to be phosphorylated (MHV: Siddell *et al.*, 1981a; Stohlman *et al.*, 1983; TGEV: Garwes *et al.*, 1984), basic (Siddell *et al.*, 1981b), and heterogeneously charged (Bond *et al.*, 1979). The identity between intracellular and virion N has been further established by tryptic peptide fingerprinting in the case of MHV-JHM and MHV-A59 (Siddell *et al.*, 1980; Bond *et al.*, 1984). N polypeptide is a primary gene product, as demonstrated by pulse-chase analysis of cells infected by MHV-JHM and MHV-A59 (Siddell *et al.*, 1981b; Rottier *et al.*, 1981b; Holmes *et*

al., 1981; Perlman *et al.*, 1986/87), MHV-3 (Cheley and Anderson, 1981), TGEV (Laude *et al.*, 1986), and BCV (Deregt *et al.*, 1987). In agreement with this are the results of cell-free translation experiments, where N-assigned mRNA isolated from cells infected by MHV (Rottier *et al.*,1981a; Siddell *et al.*, 1980), TGEV (Jacobs *et al.*, 1986), or FIPV (De Groot *et al.*, 1987) programmed synthesis of a polypeptide species of the same size as the virion-associated protein. The same appears to hold true for recombinant N proteins expressed independently of other viral components in mammalian cells using vaccinia or adenovirus vectors (Pulford and Britton, 1990; Vennema *et al.*, 1991; Stohlman *et al.*, 1992; Wesseling *et al.*, 1993) or in yeast cells (Britton *et al.*, 1988).

Several groups of investigators have mentioned the existence of one or more intracellular polypeptides with a slightly lower molecular weight (2–5 kDa less) than the major N species, sometimes designated N′, N″, etc. These latter have been observed in immunoprecipitated or immunoblotted material, but also in direct lysates from MHV-JHM and MHV-A59, MHV-3, TGEV, BCV, IBV, and TCV, i.e., most of the coronavirus–host cell systems studied (Cheley and Anderson, 1981; Stern *et al.*, 1981; Rottier *et al.*, 1981b; Bond *et al.*, 1984; Garwes *et al.*, 1984; Coulter-Mackie *et al.*, 1985; Robbins *et al.*, 1986; Keck *et al.*, 1988; Welch and Saif, 1988; Dea and Tijssen, 1989). The relationship between N and these faster migrating species has been confirmed by peptide fingerprinting in the case of MHV (Cheley and Anderson, 1981; Bond *et al.*, 1984) and IBV (Stern *et al.*, 1981). Both the N and N′ species found in TGEV grown in LLC-PK1 cells were phosphorylated (Garwes *et al.*, 1984).

While there is general agreement that only the full-length N species is incorporated into virions, it is as yet unclear whether the N subspecies result from a different level of phosphorylation, proteolytic degradation at preferential cleavage sites, premature termination, or translation of deleted transcripts arising from defective particles. It has been proposed that the 57-kDa species of MHV-JHM corresponds to a nonphosphorylated precursor; a phosphorylated species of 60kDa became apparent only after a 10-min chase and the ratio of 57 : 60 kDa decreased as infection proceeded (Stohlman *et al.*, 1983). In contrast, several groups of investigators reported that the N subspecies are made in increasing amounts during the course of infection (Anderson *et al.*, 1979; Holmes *et al.*, 1987; Talbot *et al.*, 1984). Furthermore, pulse-chase experiments with both MHV-JHM and MHV-3 provided strong evidence that the lower molecular weight species are derived from the major N species (Anderson *et al.*, 1979; Cheley and Anderson, 1981). Comparison of immunoblots of MHV-A59- or MHV-JHM-infected cell extracts done with antibody to full-length N or with antibody to a synthetic peptide of the carboxy-terminus led to the conclusion that the intracellular faster-migrating species lacked the carboxy-terminal domain (Holmes *et al.*, 1987). Similarly, the subspecies observed following vaccinia-vectored expression of TGEV N protein in two different cell system were both unreactive toward an anti-N antibody directed to the C-terminal end (Pulford and Britton, 1990).

The virus strain and/or the host cell might determine the number, size, and amount of N subspecies. Thus, cells infected by the MHV-A59 mutant *ts*209 have been found to contain an extra N-related band (Koolen *et al.*, 1983). On the

other hand, an extra band of N was detected in LLC-PK1 but not in thyroid TGEV-infected cells (Garwes *et al.*, 1984), and the N subspecies found in LLC-PK1 and CV1 cells expressing recombinant TGEV N protein differed in size (Pulford and Britton, 1990). In several virus–cell systems, N subspecies were not observed (MHV-JHM: Siddell *et al.*, 1980; Niemann *et al.*, 1982; TGEV: Laude *et al.*, 1986; BCV: Deregt *et al.*, 1987). From the above observations it would appear that the N subspecies most likely represent molecules partially degraded by cellular or serum proteases, although this was not formally proved in any of the published data. Such a phenomenon, which may be of no biological significance, is reminiscent of the limited proteolytic cleavage of the nucleocapsid protein of paramyxoviruses (Mountcastle *et al.*, 1974) and toroviruses (Horzinek *et al.*, 1985). Faster-migrating N species have also been observed during *in vitro* mRNA translation and were assumed to be the result of a proteolytic degradation during immunoprecipitation (MHV: Rottier *et al.*, 1981a; FIPV: De Groot *et al.*, 1987) or of premature termination (MHV: Leibowitz *et al.*, 1982). In addition, various truncated species of N protein have been characterized in the brain tissue from MHV-JHM-infected mice (Talbot *et al.*, 1984) and in immune complexes present in the body fluids of FIPV-infected cats (Horzinek *et al.*, 1986).

There is limited information about the fate of N protein in infected cells. Studies performed with MHV indicated that only a very small fraction of N is chased out of the cell into mature virions even after a 6-hr chase period (Holmes *et al.*, 1981), suggesting that a large pool of free N protein is built up early in the infection. MHV-A59- or MHV-JHM-infected cells appeared to contain a substantial amount of free nucleocapsids; intracellular EDTA-resistant structures of 200–230S, which contained N protein and genomic RNA, were identified (Robb and Bond, 1979; Spaan *et al.*, 1981). Perlman *et al.* (1986/87) determined the rate at which N protein was transferred into such nucleocapsid structures in the absence of *de novo* protein synthesis. The pool of free N protein in cycloheximide-treated cells was found to decay with a half-time of approximately 1 hr, which is, for example, quite slow compared with rhabdoviruses. Nearly all the genomic RNA was found to be incorporated in nucleocapsids (Spaan *et al.*, 1981; Perlman *et al.*, 1986/87).

V. N PHOSPHORYLATION

Phosphorylation is the only known posttranslational modification of coronavirus N proteins (Stohlman and Lai, 1979; Lomniczi and Morser, 1981; King and Brian, 1982; Garwes *et al.*, 1984), and it is also another point of resemblance between the nucleoproteins of coronaviruses and influenza viruses (Kistner *et al.*, 1989). MHV N protein is phosphorylated in the cytoplasm within about 10 min of its synthesis and it concomitantly becomes tightly associated with a cell membrane fraction (Stohlman *et al.*, 1983). N protein represents the major phosphopolypeptide in both MHV- and TGEV-infected cells (Siddell *et al.*, 1981a; Garwes *et al.*, 1984). For MHV, the phosphate linkage has been shown to be exclusively to serine residues (Stohlman and Lai, 1979;

Siddell *et al.*, 1981a). Even though the exact number and location of the phosphoserines are still undefined, the process appears to be highly specific. An high-pressure liquid chromatography (HPLC) analysis of the tryptic phosphopeptides of MHV N revealed that phosphorylation may occur at only two or three sites (Wilbur *et al.*, 1986), a number relatively small in comparison to the 30 to 40 potential target residues in the N proteins. Moreover, the two plaque morphology variants of MHV-JHM examined in this study were found to differ in their phosphorylation patterns. It remains unclear whether the process involves autophosphorylation or a protein kinase activity of either viral or host origin. For MHV, a virion-associated protein kinase activity has been described that can transfer additional phosphate from ATP to the N protein as well as to other substrates *in vitro*. This enzyme appeared to be cyclic AMP-independent and to require divalent cations (Siddell *et al.*, 1981a), features common to those of protein kinases identified in a variety of other enveloped viruses (Tan, 1975). Whether the same enzyme is responsible for the kinase activity *in vivo*, however, has not yet been shown.

The finding that some anti-MHV N monoclonal antibodies recognized the phosphorylated form but not a nonphosphorylated, faster-migrating species assumed to be its precursor, led to the proposal that phosphorylation may induce a drastic conformational change of the protein (Stohlman *et al.*, 1983), possibly relevant to its biological function. However, the question of the role of N protein phosphorylation is largely unanswered. It has been speculated by a number of workers that it may govern the tightness of the association between N and RNA, but there is as yet no direct evidence that bears on this possibility. MHV N proteins derived from purified virions, cellular membranes, and the cytosol of infected cells were reported to have similar phosphopeptide maps (Wilbur *et al.*, 1986), a finding in agreement with the observed rapidity of the phosphorylation process. On this basis, phosphorylation might not be expected to play a critical role in the regulation of virus assembly. Finally, it has been hypothesized that phosphatase-catalyzed nucleocapsid dephosphorylation might control the processes of coronavirus penetration and uncoating, and potentially relevant host enzyme activities have been sought. An acid phosphatase activity associated with the particulate fraction of oligodendrocytes has been shown to convert the MHV N protein to a lower molecular weight product (Beushausen *et al.*, 1987). Also, a neutral, serine-threonine phosphatase activity was detected in the endosomal fraction of L-2 murine fibroblasts and in other susceptible cells, which recognized MHV N protein as a more specific substrate than acid or alkaline phosphatases (Mohandas and Dales, 1991). The relevance of such data would certainly be greater if the site of fusion of entering coronavirus particles were known more precisely.

VI. N PROTEIN BINDING TO RNA

The most noteworthy function of coronavirus N proteins is that they bind to the RNA genomes of their respective viruses. Neither the nature nor the mechanism of this N–RNA complex formation has been well elucidated. Anal-

ogies with other RNA-binding proteins are limited. Coronavirus N proteins do not contain zinc finger motifs (Klug and Rhodes, 1987) or sequences similar to the RNA consensus and RNA-binding motifs found in many cellular RNA-binding proteins such as nucleolin, poly(A)-binding protein, and various hnRNP and snRNP proteins (Dreyfuss *et al.*, 1988). With respect to the properties of other RNA viruses containing helical nucleocapsids, coronaviruses appear to most closely resemble the orthomyxoviruses. The N (or NP) proteins of both of these families form complexes with RNA that tend to dissociate in high concentrations of salt and that provide only limited protection against the action of ribonucleases (Bukrinskraya, 1973; Macnaughton *et al.*, 1978; Robbins *et al.*, 1986; Kingsbury *et al.*, 1987). This contrasts markedly with the N–RNA complexes of the rhabdoviruses and paramyxoviruses, which are stable in high salt and are markedly ribonuclease-resistant (Bukrinskraya, 1973; Leppart *et al.*, 1979).

Efforts to characterize coronavirus N–RNA binding have sought to address two fundamental questions. First, what RNA sequences or structures are specifically bound by N? Second, what parts of the N molecule participate in RNA recognition? The ability of the MHV N protein to bind to nucleic acids has been examined by an RNA overlay protein blot assay (ROPBA) in which electrophoretically separated proteins from purified virions or from MHV-infected cells were transferred to nitrocellulose and probed with labeled RNA (Robbins *et al.*, 1986). Monomeric N as well as a minor multimeric N component, possibly an N trimer, were the only virus-specific RNA-binding proteins detected in this manner. Nucleic acid sequence binding specificity was not demonstrated, however, since N bound equally well to MHV RNA or to single- or double-stranded RNA or DNA of heterologous origin.

Subsequent adaptation of the ROPBA procedure allowed the demonstration of sequence-specific binding of RNA by MHV N protein (Stohlman *et al.*, 1988). The inclusion of a large molar excess of unlabeled (uninfected) cellular RNA with the labeled probe of MHV-infected cellular RNA was found to compete out low-affinity binding of RNA by both N and cellular proteins. Synthetic RNA probes containing defined extents of the 5′ leader RNA adjacent to either mRNA 1 or mRNA 6 were then used to localize a sequence-specific, high-affinity binding site for N to the region of nucleotides 56 to 67 of the MHV genome. This conclusion was reinforced by the observation that an anti-N monoclonal antibody could selectively immunoprecipitate from MHV-infected cells all leader-containing RNA species greater than or equal to 65 nucleotides in length (Baric *et al.*, 1988). Since the leader sequence is contained in all subgenomic positive-strand RNAs, as well as in the genome, this may suggest that some recognition event other than binding by N protein mediates the selective incorporation of genome RNA into assembled virions.

The assignment of the RNA-binding property of N to one or more particular portions of the N molecule is at present incomplete. The detection by ROPBA of the species N′ and N″, presumed to be products of small proteolytic truncations, suggests that at least limited removal of one of the two termini of N does not alter RNA binding (Robbins *et al.*, 1986). Recently, this point has been investigated using a nondenaturing gel assay of MHV N protein translated

in a rabbit reticulocyte lysate programmed with engineered synthetic mRNAs (Masters, 1992). In this system, full-length N protein was shown to bind to an endogenous RNA species in the reticulocyte lysate. Construction of a set of N gene deletions allowed the mapping of this RNA-binding activity to the central one of three domains of N protein proposed previously on the basis of sequence comparisons of different strains of MHV (Parker and Masters, 1990). Deletions entering the central domain abolished this non-sequence-specific RNA-binding activity. Sequence-specific RNA binding, as measured by ROPBA, was also mapped to the central portion of purified MHV N protein following its fragmentation by treatment with formic acid (Nelson and Stohlman, 1993). The isolated RNA-binding domain, comprising amino acids 169 to 308, retained the ability to interact specifically with its target RNA sequence. Considerable work remains to be done to delineate the minimal RNA-binding domain of N and to define the exact nature of the association between this molecule and RNA.

Evidence recently has been presented that MHV N protein binds specifically to cell membranes (Anderson and Wong, 1993). The binding was inhibited by nonviral RNA and DNA, as well as by various membrane phospholipids such as cardiolipin, a property shared by certain DNA binding proteins. This observation led the authors to speculate that membrane lipid association of the N protein may compete for RNA binding sites on the N protein, a mechanism possibly relevant to the processes of nucleocapsid uncoating and assembly.

VII. N–N AND N–M PROTEIN INTERACTIONS

Very little information is presently available about the protein–protein contacts N makes in assembled virions or in infected cells. The structure of the coronavirus nucleocapsid suggests that, at the very least, there must be some form of interactions between N monomers that neighbor each other upon each successive turn of the helix. There may also be an interaction between adjacent N monomers bound along the RNA strand, although these would not necessarily be required for helical encapsidation.

It has been observed for some coronaviruses that SDS-PAGE, under nonreducing conditions, detected an N-related protein of 140–160kDa, present at a level of a few percent compared to N. This species was identified by immunoblotting of BCV, HCV-OC43, and MHV virions (Hogue *et al.*, 1984; Robbins *et al.*, 1986; Deregt *et al.*, 1987), and for MHV it was demonstrated to bind to RNA (Robbins *et al.*, 1986). The N multimer appeared to be the size of a trimer, and it was shown to be held together by disulfide linkages, since it disappeared in the presence of mercaptoethanol. It is possible that this species is a fundamental unit of the nucleocapsid structure. However, since the N protein of each of these three viruses contains just two cysteine residues separated by only four amino acids, it seems likely that other types of N–N binding must also contribute to stabilizing the helix.

The coronavirus M protein is thought to be the principal determinant of virion assembly and budding. Consequently, it might be expected that there exist N–M interactions that play a major role in viral structure, although little

experimental evidence bears on this issue. During the development of techniques to fractionate and purify viral components, it was found that a temperature-dependent binding of M and the nucleocapsid occurred in NP40-disrupted MHV virions (Sturman *et al.*, 1980). At 4 °C, M protein was solubilized by the non-ionic detergent, but at ambient temperature or 37 °C, M remained associated with the nucleocapsid, cosedimenting in sucrose gradients. However, the same study demonstrated that NP40-solubilized M was able to bind to RNA in the absence of N protein; thus, it is not clear whether the M–nucleocapsid binding being examined was due to an N–M interaction. Clearly, it would be desirable to examine the N-binding properties of the cytosolic carboxy-terminal segment of M in isolation from the remainder of the M molecule.

VIII. POTENTIAL ROLE OF N PROTEIN IN RNA SYNTHESIS

Various lines of evidence indicate a possible role for N protein in coronavirus RNA synthesis. An earlier, perplexing observation in this regard was the finding that, after multiple passages, MHV defective-interfering (DI) particles inhibited the synthesis of all normal viral RNA species except RNA7, which was translated abundantly into N protein (Makino *et al.*, 1985). Although the mechanism of this selective resistance is unknown, it may imply that N is required for MHV RNA synthesis, or at least for the synthesis of DI RNA.

More directly, it has been shown that antibodies to N protein almost totally inhibited viral RNA synthesis in an *in vitro* system prepared from MHV-infected cells, whereas, under the same conditions, no significant inhibition was seen with anti-M or anti-S antibodies (Compton *et al.*, 1987). Moreover, the product RNA synthesized in this system, which was mostly of genome length, was found to be encapsidated by N protein. This might suggest that coronavirus RNA synthesis is coupled to the encapsidation of nascent RNA, analogous to the replication of viruses with helical negative-strand RNA nucleocapsids. For coronaviruses, however, the requirement for N may also apply to the synthesis of subgenomic RNAs. The immunoprecipitation of all leader-containing MHV RNAs, as well as replicative intermediate RNA complexes by an anti-N monoclonal antibody, has been taken to mean that the N–leader RNA interaction must be important to the mechanism of leader-primed transcription (Baric *et al.*, 1988).

The analogy with some families of negative-strand RNA viruses may be inexact in another respect. The protein synthesis inhibitor cycloheximide has been shown to prevent or inhibit genomic and subgenomic RNA synthesis in MHV-infected cells (Sawicki and Sawicki, 1986), and this effect was mirrored by puromycin or cycloheximide in the *in vitro* RNA-synthesizing system (Compton *et al.*, 1987). For vesicular stomatitis virus (VSV), a rhabdovirus, the necessity for continuous protein synthesis in order to sustain RNA replication is due solely to a requirement for VSV N protein (Patton *et al.*, 1984). However, for MHV-infected cells it was shown that, even in the presence of cycloheximide, substantial pools of soluble free N protein were available for the encapsidation of nascent RNA (Perlman *et al.*, 1986/87). Thus, synthesis of N may not be rate-

limiting for coronavirus RNA synthesis. Given the complexity and enormity of the putative coronavirus polymerase and the uniqueness of the mechanism of coronavirus RNA synthesis, much needs to be done to clarify how N protein participates in this process.

IX. ANTIGENIC PROPERTIES

The nature of antigenic relatedness between the nucleocapsid proteins of different coronaviruses was first investigated using polyclonal sera raised against complete or subviral particles. The presence of common antigenic determinants on the N protein as well as on the other structural polypeptides of closely related viruses such as TGEV, FIPV, and CCV has been demonstrated. For instance, anti-TGEV sera have been shown to react with FIPV N and CCV N to about the same extent as with TGEV N in immunoblotting or enzyme-linked immunosorbent assay (ELISA) tests (Horzinek *et al.*, 1982; Have *et al.*, 1992). Similarly, antibodies directed against BCV N protein recognize HCV-OC43 N and MHV-A59 N (Hogue *et al.*, 1984). However, it would appear that cross-reactivity at the level of the N protein is not restricted to members belonging to the same antigenic cluster. Thus, HCV-229E RNP could be detected by immunodiffusion using antisera to MHV, TGEV, or HEV (Yassen and Johnson-Lussenburg, 1978). An antigenic relationship was also observed between HCV-229E and MHV-3 using heterologous anti-RNP antisera but not antisera against virion external components (Hasony and Macnaughton, 1982). A two-way cross-reaction between the N proteins of FIPV and PEDV has been evidenced using both blotting and immunoprecipitation tests, whereas the viral envelope proteins exhibited no heterologous reactivity (Yaling *et al.*, 1988). These authors also obtained preliminary evidence of a cross-reactivity between the N proteins of IBV and MHV and of FIPV and HEV. Finally, cross-reactivity has been reported for the N proteins of TCV and IBV (Dea *et al.*, 1990) and of TGEV and PEDV (Have *et al.*, 1992). These observations suggest that a few common determinants may exist on the nucleocapsid antigen that are suitable for the identification of many or all members of the family.

The N protein appears to be a substantially conserved antigen according to studies examining the reactivity of monoclonal antibodies (MAbs) toward different strains of the same virus. The N protein of MHV exhibits a moderate degree of antigenic variation; several determinants common to up to 11 strains or isolates have been identified (Fleming *et al.*, 1983; Talbot and Buchmeier, 1985). In both of these studies, the antigenic polymorphism of N protein was found to be less extensive than that of S protein. The first authors divided the MHV strains into two groups according to the relative binding of MAbs to N and proposed that such variation was correlated with their pathogenesis, whereas the second found that antigenic variation of S protein was a better correlate. The IBV N protein may also be subjected to antigenic variation since no fully conserved epitope could be detected within a panel of Australian field strains (Ignjatovic and McWaters, 1991). By contrast, N protein from different strains of TGEV exhibited little or no significant qualitative or quantitative

antigenic divergence (Laude *et al.*, 1986; Welch and Saif, 1988; Sanchez *et al.*, 1990). Similarly, FIPV N epitopes were generally highly conserved, although a few type-specific epitopes were discriminated (Fiscus and Teramoto, 1987). Thus, the interstrain relatedness is closer for TGEV and FIPV than in the case of MHV, as is also reflected by a lesser variation of S antigen. Finally, a proportion of common epitopes have been identified in viruses showing a close antigenic relationship, like TGEV and FIPV or BCV and TCV (Fiscus and Teramoto, 1987; Sanchez *et al.*, 1990; Dea *et al.*, 1990).

The identification of B cell epitopes as well as their localization on the N primary structure is less documented than for the S protein. Epitope mapping by competitive assays using a small panel of anti-MHV N or anti-TGEV N MAbs delineated at least two nonoverlapping determinants (Talbot *et al.*, 1985). A translation product obtained from an intracellular DI RNA of MHV-JHM and encoding the 89 C-terminal amino acids of N protein was found to be immunoprecipitated by an anti-N MAb (Makino *et al.*, 1988). Four subfragments altogether covering the whole MHV-JHM N protein sequence were individually able to elicit an antibody response and reacted with sera from diseased Lewis rats, thus indicating that B cell epitopes are distributed throughout the entire length of the molecule (Wege *et al.*, 1993). Western blotting immunoscreening of a series of bacterial fusion products has allowed a more detailed antigenic analysis of the TGEV N protein (Martin Alonso *et al.*, 1992). Seven of eleven MAbs recognized the amino-terminal half of the polypeptide chain. Interestingly, one epitope was localized within a 60 amino acid stretch that essentially overlaps the most conserved region of the coronavirus N protein (see Fig. 1). A second antigenic domain was delineated within the carboxy-terminal half, to which the heterogeneity reported for the N protein of PRCV relative to TGEV appears to be restricted. Additionally, an epitope 11 amino acids long has been localized very near the carboxy-terminus; this well-defined epitope has been used as a portable marker allowing detection of various fusion proteins (Parra *et al.*, 1989). Taken together, the above observations suggest that a major epitope situated in the carboxy end might be a common feature of the coronavirus N proteins. Detergent-resistant epitopes have also been detected but not mapped in the case of MHV-JHM, BCV, and IBV N proteins (Talbot *et al.*, 1984; Deregt and Babiuk, 1987; Ignjatovic and McWaters, 1991).

Due to its internal position in the virus particle, the N protein might not be expected to bear neutralization-mediating determinants. Indeed, none of the coronavirus anti-N MAbs isolated thus far exhibited significant neutralizing activity. On the other hand, no B cell epitopes externally exposed at the cell membrane could be demonstrated in the case of MHV-JHM and TGEV N proteins (Collins *et al.*, 1982; Laude *et al.*, 1986; Laviada *et al.*, 1990; Pulford and Britton, 1990). It has been claimed that one MAb specific for MHV-3 N protein reacted with the surface of infected cultured cells and neutralized viral cytopathic effect in the presence of complement, suggesting that at least one epitope of N protein is accessible to antibodies on the cell membrane (Lecomte *et al.*, 1987). Of possible relevance to this point is the apparent protective activity conferred by certain anti-N MAbs when transferred to mice subsequently lethally infected by MHV-2 or MHV-3 (Nakanaga *et al.*, 1986; Lecomte *et al.*,

1987). This paradoxical observation parallels the well-documented protection by anti-N Ab to rabies (Lodmell *et al.*, 1993).

Attention has been drawn only recently to the T-cell determinants potentially expressed on coronavirus antigens. Two MHC class II (I-E^d)-restricted murine T-cell hybridomas generated after immunization with IBV have been shown to be responsive to the N protein (Boots *et al.*, 1991a). This response was strain-specific, thus confirming an antigenic variation of the protein among the different serotypes. Both the antigenic determinants were mapped within the region spanning amino acids 71 to 78 by using recombinant expression products and synthetic peptides. Furthermore, the epitope was shown to prime cellular immune response to IBV in the chicken (Boots *et al.*, 1991b). Studies on MHV-JHM-infected Lewis rats allowed the demonstration of an early and strong T-helper cell response specific for the N protein. N-specific $CD4^+$ T-cell lines were established and shown to confer protection against acute encephalitis upon adoptive transfer to otherwise lethally infected animals (Körner *et al.*, 1991). The carboxy-terminally located, 95-residue-long fragment of N expressed as a bacterial fusion protein was shown to induce the most pronounced response and to mediate protection (Wege *et al.*, 1993). Finally, the MHV-JHM strain has been reported to elicit an immunodominant anti-N protein cytotoxic T lymphocyte (CTL) response in BALB/c mice (Stohlmann *et al.*, 1992). Indeed, among 21 $CD8^+$ T-cell lines derived from animals undergoing an acute demyelinating encephalitis, 17 were found to recognize the N protein as a target (Stohlman *et al.*, 1993). By using a combination of truncated forms of N expressed by vaccinia virus recombinants and a series of overlapping peptides, the response has been mapped to an epitope comprising residues 316 to 330, which contains a described L^d binding motif (Bergmann *et al.*, 1993). Despite a natural sequence variation affecting two residues of the motif, a cross-reactivity of the JHM N-specific CTL with six other MHV strains was observed. Altogether, the above studies lend support to the view that the N protein may be an important antigen with respect to both helper and cytotoxic immune response.

X. CONCLUSION

The N proteins have occupied a more peripheral position thus far in the study of coronaviruses. A substantial fraction of the papers cited in this chapter have been concerned with N as an entity tangential to the principal focus of the study in hand. Thus, our information about this protein is incomplete, and the answers to many important questions remain obscure. An understanding of the organization of the coronaviral nucleocapsid is of utmost importance for a complete elucidation of how the virus expresses its genetic information once it reaches the cytoplasm. The persistent association of N protein with the genome RNA, and possibly with the subgenomic RNAs, suggests that exploring the roles of N in coronavirus transcription and translation will be a fertile area of research for years to come. Techniques recently developed have taken advantage of the high rate of RNA recombination that occurs during coronavirus replication in order to engineer site-specific N gene mutations into the genome

of MHV. It is expected that this approach will greatly facilitate the elucidation of N protein structure function relationships in the future. It should also be possible to extend this methodology to the study of other genes and other members of the coronavirus family.

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