Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Identification of a Specific Interaction between the Coronavirus Mouse Hepatitis Virus A59 Nucleocapsid Protein and Packaging Signal

Richard Molenkamp and Willy J. M. Spaan

Department of Virology, Institute of Medical Microbiology, Leiden University, AZL-L4-Q, P.O. Box 9600, 2300 RC Leiden, the Netherlands

Received July 21, 1997; returned to author for revision August 20, 1997; accepted September 25, 1997

The coronavirus mouse hepatitis virus (MHV) is an enveloped positive stranded RNA virus. In infected cells MHV produces a 3′ coterminal nested set of subgenomic messenger RNAs. Only the genomic RNA, however, is encapsidated by the nucleocapsid protein and incorporated in infectious MHV virions. It is believed that an RNA packaging signal (Ps), present only in the genomic RNA, is responsible for this selectivity. Earlier studies mapped this signal to a 69-nt stem–loop structure positioned in the 3′ end of ORF1b. The selective encapsidation mechanism probably initiates by specific interaction of the packaging signal with the nucleocapsid protein. In this study we demonstrate the in vitro interaction of the MHV-A59 nucleocapsid protein with the packaging signal of MHV using gel retardation and UV cross-linking assays. This interaction was observed not only with the nucleocapsid protein from infected cells but also with that from purified virions and from cells expressing a recombinant nucleocapsid protein. The specificity of the interaction was demonstrated by competition experiments with nonlabeled Ps containing RNAs, tRNA, and total cytoplasmic RNA. The results indicated that no virus specific modification of the N-protein or the presence of other viral proteins are required for this in vitro interaction. The assays described in this report provide us with a powerful tool for studying encapsidation (initiation) in more detail.

INTRODUCTION

The murine coronavirus mouse hepatitis virus (MHV) is an enveloped virus containing a positive stranded RNA genome of about 31 kb (Holmes, 1991; Spaan et al., 1988). The virion envelope is composed of a lipid bilayer derived from an internal compartment of the host cell and three or four virus-encoded structural membrane proteins (Luytjes, 1995): the spike protein (S), the membrane protein (M), the small membrane protein (E), and the optional hemagglutinin-esterase protein (HE). The viral envelope surrounds a nucleocapsid with helical symmetry composed of the genomic RNA and multiple copies of the nucleocapsid protein (N). Evidence for the presence of a fifth structural envelope protein translated from an internal open reading frame (ORF) within the N gene has been published recently (Fischer et al., 1997).

In infected cells MHV produces a 3′ coterminal nested set of subgenomic mRNAs (sgRNAs) which possess an identical 5′ leader sequence derived from the 5′ end of the genome (Lai et al., 1984; Spaan et al., 1983, 1988). Only genomic length RNA is packaged into virus particles; however, trace amounts of sg RNAs are sometimes detected in purified virus (Makino et al., 1988). Earlier studies (Fosmire et al., 1992; Most et al., 1991) have mapped a region in the 3′ end of ORF1b that is essential for encapsidation of defective genomes. Within this region a domain (from here on called Ps) of 69 nt could be identified that is probably required for the encapsidation of defective genomes (Fosmire et al., 1992). This signal is present in genomic RNA, but not in sgRNAs and it is likely that it has also a similar function in the encapsidation of genomic RNA. Recently, it was demonstrated that the encapsidation of a heterologous RNA by MHV was fully dependent on the presence of this Ps (Woo et al., 1997). Furthermore, it was shown by Bos et al. (1997) that transferring the Ps to a sgRNA resulted in the specific encapsidation of this sgRNA, though with reduced efficiency.

The nucleocapsid protein of MHV is a basic phosphoprotein of 454 amino acids and has an apparent molecular weight of approximately 55 kDa (Armstrong et al., 1983; Parker and Masters, 1990; Laude and Masters, 1995). It is phosphorylated exclusively on serine residues (Stohlman and Lai, 1979). The N protein contains 41 of these potential phosphorylation sites, but the exact number and location of phosphoserines have not been identified yet. The basic amino acids are not clustered in strings, but local densities of positive charge can be found, particularly in two regions in the middle of the N protein (Laude and Masters, 1995). In contrast, the C-terminus is quite acidic. The MHV N protein does not contain known RNA binding motifs, like the arginine-rich motif (ARM) or zinc fingers (Draper, 1995; Holmes and Behlke, 1981; Burd and Dreyfuss, 1994).

Interaction of the MHV nucleocapsid protein with the
leader RNA has been reported although there is some discrepancy about the specificity of this interaction (Stohlman et al., 1988; Bredenbeek, 1990). A leader-RNA binding domain in the N protein was mapped and comprises the two basic regions mentioned above (Nelson and Stohlman, 1993; Masters, 1992). Furthermore, specific interaction of the coronavirus infectious bronchitis virus (IBV) nucleocapsid protein with the 3′ terminus of the genome has recently been reported (Zhou et al., 1997).

The 69-nt Ps is able to form a stable secondary structure and the integrity of this structure is essential for the encapsidation of defective genomes (Fosmire et al., 1992). It has been postulated (Fosmire et al., 1992) that the Ps functions as an encapsidation initiation site, probably by interacting specifically with the N protein. Initiation of encapsidation by packaging signal/(nucleo)capsid interactions has been observed for several other RNA viruses, including alphaviruses, retroviruses, and Escherichia coli bacteriophages. (Owen and Kuhn, 1996; Berkowitz et al., 1995; Zhang and Barklis, 1995; Schlesinger et al., 1994; Dupraz and Spahr, 1992; Witherell et al., 1991; Aldovini and Young, 1990; Weis et al., 1989). However, a specific interaction of the MHV N protein with the Ps has not been demonstrated yet.

In this report we have used gel retardation and UV cross-linking assays to study the in vitro interaction of MHV-A59 nucleocapsid protein and a small RNA containing the Ps domain. We observed specific interaction between in vitro transcripts containing the Ps and N protein isolated from infected cells, but also with N protein extracted from purified viruses. Furthermore, we were able to identify a similar interaction with recombinant nucleocapsid protein expressed in the vaccinia T7 expression system. These experiments underline the possibility of studying the encapsidation of MHV-A59 RNA at a molecular basis and allow us to map important domains in both the nucleocapsid protein as well as the RNA packaging signal.

**MATERIALS AND METHODS**

**Cells and viruses**

Mouse L cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 8% fetal calf serum. MHV-A59 stocks were grown as described (Spaan et al., 1981). Vaccinia virus vTF7.3 (kindly provided by Dr. B. Moss) stocks were grown on RK13 cells.

**Recombinant DNA techniques**

Standard recombinant DNA procedures were used (Sambrook et al., 1989). Restriction enzymes, T4 DNA ligase, and T7 RNA polymerase were obtained from Gibco BRL. The T7 sequencing kit of Pharmacia and [α-33P]dATP of NEN-Dupont were used for DNA sequence analysis. All enzyme incubations and biochemical reactions were performed according to the instructions of the manufacturers.

**Construction of plasmids**

(i) pPs290. A 204-nt fragment containing Ps was obtained by polymerase chain reaction (PCR) using pMIDI-C as a template (Most et al., 1991) and oligonucleotide primers C060 and C061 (Table 1). To obtain pPs290 this fragment was cloned in pCRII using the TA cloning kit (Invitrogen) according to the instructions of the manufacturer.

(ii) pEMCV-N. A Nco I restriction site was created at the position of the AUG start codon of the N gene by Ps functions as an encapsidation initiation site, and the second amino acid of the N protein was changed from a Ser to an Ala. The reconstituted N gene was then exchanged with the NcoI–BamHI fragment of pL1asinger et al., 1994; Dupraz and Spahr, 1992; Witherell et al., 1991; Aldovini and Young, 1990; Weis et al., 1989). However, a specific interaction of the MHV N protein with the Ps has not been demonstrated yet.

In this report we have used gel retardation and UV cross-linking assays to study the in vitro interaction of MHV-A59 nucleocapsid protein and a small RNA containing the Ps domain. We observed specific interaction between in vitro transcripts containing the Ps and N protein isolated from infected cells, but also with N protein extracted from purified viruses. Furthermore, we were able to identify a similar interaction with recombinant nucleocapsid protein expressed in the vaccinia T7 expression system. These experiments underline the possibility of studying the encapsidation of MHV-A59 RNA at a molecular basis and allow us to map important domains in both the nucleocapsid protein as well as the RNA packaging signal.

**MATERIALS AND METHODS**

**Cells and viruses**

Mouse L cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 8% fetal calf serum. MHV-A59 stocks were grown as described (Spaan et al., 1981). Vaccinia virus vTF7.3 (kindly provided by Dr. B. Moss) stocks were grown on RK13 cells.

**Recombinant DNA techniques**

Standard recombinant DNA procedures were used (Sambrook et al., 1989). Restriction enzymes, T4 DNA ligase, and T7 RNA polymerase were obtained from Gibco BRL. The T7 sequencing kit of Pharmacia and [α-33P]dATP of NEN-Dupont were used for DNA sequence analysis. All enzyme incubations and biochemical reactions were performed according to the instructions of the manufacturers.

**Construction of plasmids**

(i) pPs290. A 204-nt fragment containing Ps was obtained by polymerase chain reaction (PCR) using pMIDI-C as a template (Most et al., 1991) and oligonucleotide primers C060 and C061 (Table 1). To obtain pPs290 this fragment was cloned in pCRII using the TA cloning kit (Invitrogen) according to the instructions of the manufacturer.

(ii) pEMCV-N. A Nco I restriction site was created at the position of the AUG start codon of the N gene by Ps functions as an encapsidation initiation site, and the second amino acid of the N protein was changed from a Ser to an Ala. The reconstituted N gene was then exchanged with the NcoI–BamHI fragment of pL1asinger et al., 1994; Dupraz and Spahr, 1992; Witherell et al., 1991; Aldovini and Young, 1990; Weis et al., 1989). However, a specific interaction of the MHV N protein with the Ps has not been demonstrated yet.

In this report we have used gel retardation and UV cross-linking assays to study the in vitro interaction of MHV-A59 nucleocapsid protein and a small RNA containing the Ps domain. We observed specific interaction between in vitro transcripts containing the Ps and N protein isolated from infected cells, but also with N protein extracted from purified viruses. Furthermore, we were able to identify a similar interaction with recombinant nucleocapsid protein expressed in the vaccinia T7 expression system. These experiments underline the possibility of studying the encapsidation of MHV-A59 RNA at a molecular basis and allow us to map important domains in both the nucleocapsid protein as well as the RNA packaging signal.
TABLE 1
Oligonucleotide Sequences and Purposes

| Primer | Sequence 5' → 3' | Purpose |
|--------|------------------|---------|
| C060   | GTCCCAAGCTTmGAAGTTGGAGATTC 5' | 5' PCR ppS290 |
| C061   | TCCGACGCGTAGAGCTTCATTACC 3' | 3' PCR ppS290 |
| C258   | TAATACGACTCATATA mGAAGTTGGAGATTC 5' | 3' PCR Ps180 template |
| C268   | TAGAGCTTCGGCTCGGTTGAAGGC 3' | 5' PCR PsΔHP template |
| C261   | ATAGCCATGGCTTTTGTTCCTGGGC 5' | 3' PCR PsΔHP template |
| C224   | TGTAGGATCCA CTGCACCACCATCCTTCTGG 3' | Oligonucleotide for introduction of a Nco I restriction site at the ATG start codon of the nucleocapsid gene |

Note. Non-MHV sequences are indicated in italics.

phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 mM binding buffer (5 mM HEPES (pH 7.9), 50 mM KAc, 2.4 mM MgAc₂, 0.1 mM EDTA, 0.01 mM DTT, 1 mM ATP, 0.4 mM GTP). Where necessary specific or nonspecific competitor RNA was added as indicated in the figure legends. In supershift experiments, 1 μl of the N-specific monoclonal antibody 5B188.2 (Talbot and Buchmeier, 1985) or β-galactosidase monoclonal antibody (Boehringer Mannheim) and 18 units of RNAGuard (Pharmacia) were added. The mixtures were incubated at room temperature for 20 min, after which 5 μl of 50% glycerol was added to each reaction. The mixtures were then separated by electrophoresis on a 5% polyacrylamide gel (mono:bis 37.5:1) in 0.5× TBE (45 mM Tris – Cl (pH 7.5)), 45 mM boric acid, 1 mM EDTA) for 6 h at 5 mA (fixed). Subsequently, the gel was dried and exposed to X-ray film with an intensifying screen at −80°C.

UV cross-linking

RNA binding reactions were performed as described above. Subsequently the samples were irradiated at a 2-cm distance with 1.8 J/cm² of 254-nm UV light in a Stratalinker 1800 (Stratagene). The complexes were then incubated with a mixture of 500 ng RNase A (Pharmacia) and 2.5 units RNase T1 (Gibco BRL) for 20 min at 37°C. The complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% gels. Alternatively, cross-linked and RNase treated samples were immunoprecipitated prior to electrophoresis as described earlier (Bos et al., 1996) with monoclonal antibody 5B188.2 (Talbot and Buchmeier, 1985) or rabbit polyclonal MHV-A59 antiserum k134.

RESULTS

Ps290 RNA binds specifically to proteins from MHV-infected and mock-infected cells.

In the studies described here we have analyzed the interaction between the MHV-A59 N protein and RNAs containing the 69-nt Ps signal. In order to identify this
interaction we have first used gel mobility shift assays using protein lysates from both MHV-infected and mock-infected cells and an in vitro transcript containing the Ps (Fig. 1). From pilot experiments using 10 ng of labeled RNA we determined that approximately 4 μg of protein lysate was required to observe a distinct retarded band (data not shown). We therefore used these amounts as standard in all binding experiments.

An interaction between Ps290 RNA and proteins from infected cells (I-lysate), as well as from mock-infected cells (MI-lysate) was readily observed (Fig. 2, lanes 2 and 9). However, the complex formed between Ps290 RNA and proteins from the MI-lysate migrated slightly slower in the gel and appeared to be more diffuse, indicating that there are some differences in binding activity between I- and MI-lysates.

In order to determine the specificity of the interaction, competition experiments were performed. Competition of the interaction between Ps290 RNA and proteins from the I-lysate with a 10-fold molar excess of nonlabeled Ps290 RNA resulted already in a decrease of intensity of the retarded band (Fig. 2, lane 5). At a level of 30-fold molar excess of nonlabeled Ps290 RNA the retarded band was no longer visible and all of the labeled RNA migrated at the position of the unbound RNA probe (Fig. 2, lane 7). Competition with a 100-fold molar excess of yeast tRNA did not affect the intensity or the mobility of the retarded band, indicating that the observed interaction between Ps290 RNA and proteins from the I-lysate is specific for the Ps290 RNA (Fig. 2, lane 8). Competition of the interaction between Ps290 RNA and proteins from the MI-lysate with non-labeled Ps290 RNA or yeast tRNA showed a similar result. A 30-fold molar excess of unlabelled Ps290 RNA competed entirely for protein binding (Fig. 2, lane 14), whereas a 100-fold molar excess of yeast tRNA had no effect on the interaction (Fig. 2, lane 15). These observations indicate that there is a specific interaction between Ps290 RNA and proteins from MHV-infected cells as well as with proteins from mock-infected cells.

The MHV-A59 nucleocapsid protein interacts specifically with Ps290 RNA

To investigate whether the N protein is part of the protein-Ps290 RNA complex, a supershift assay using a N-specific antibody was performed. If the N protein is indeed part of the complex, binding to N of a N-specific antibody should result in the formation of a large complex composed of Ps290 RNA, N protein, and N-specific antibody. This complex is expected to migrate slower in the gel as compared to the protein–Ps290 RNA complex and should be visible as a so-called supershift. The monoclonal N-specific antibody 5B188.2 (Talbot and Buchmeier, 1985) was used to analyze the protein–Ps290 RNA complex and the monoclonal β-galactosidase-specific antibody was used as a control. When N-specific antibody 5B188.2 was added to the complex formed between Ps290 RNA and proteins from the I-lysate, a second complex, migrating slightly slower than the protein–Ps290 RNA complex was readily observed (Fig. 3, lane 5). In contrast, addition of 5B188.2 to the complex formed between Ps290 RNA and proteins from the MI-lysate did not result in a supershift (Fig. 3, lane 13). This demonstrates the presence of the MHV-A59 N protein in the complex formed between Ps290 RNA and proteins from the I-lysate.

The β-galactosidase-specific antibody was not able to shift the complex formed between Ps290 RNA and proteins from the I-lysate or from the MI-lysate (Fig. 3, lanes 11 and 19). Furthermore, incubation of Ps290 RNA and antibody 5B188.2 or β-galactosidase-specific antibody without protein lysate, did not result in the formation of...
Cross-linked and RNase treated samples were immunoprecipitated with the monoclonal N-specific antibody 5B188.2 or the rabbit polyclonal MHV-A59 antiserum k134 as indicated in the figure legends.

An interaction between the N protein and Ps180 RNA was readily observed (Fig. 4, lane 1), but no interaction was observed between the N-protein and PsΔHP RNA (Fig. 4, lane 2). Complexes formed between proteins from the MI-lysate and Ps180 RNA or PsΔHP RNA could not be immunoprecipitated with the N-specific antibody (Fig. 4, lanes 3 and 4). When the Ps180 and PsΔHP RNAs were used in a competitive gel retardation experiment with radiolabeled Ps290 and the I-lysate, complete competition was observed with the Ps180 RNA, whereas no competition was observed with the PsΔHP RNA (data not shown). These results show that the interaction between the N-protein and Ps180 RNA can also be demonstrated by using an UV cross-linking assay followed by immunoprecipitation and that this interaction is dependent on the presence of the 69-nt Ps hairpin.

To determine the specificity of the nucleocapsid protein–Ps180 RNA interaction in UV cross-linking assays, competition experiments were performed. Addition of a 100-fold molar excess of nonlabeled Ps290 competitor RNA resulted in the complete inhibition of N protein binding (Fig. 5, lane 2), whereas, a 100-fold molar excess of tRNA had no effect on the interaction between the N protein and Ps180 RNA (Fig. 5, lane 3). This clearly demonstrates the specificity of the interaction between the N protein and Ps180 RNA.

UV cross-linking demonstrates specific nucleocapsid protein Ps180-RNA interaction

Another assay for studying the interaction between the N protein and Ps containing RNAs is UV cross-linking. To exclude the possibility that the non-MHV-specific sequences present in Ps290 RNA are involved in the interaction between the nucleocapsid-protein and Ps290 RNA, an RNA probe (Ps180) containing only MHV-specific sequences was constructed. Furthermore, in order to investigate whether the 69-nt hairpin of Ps180 (and Ps290) RNA is responsible for specific nucleocapsid protein binding, PsΔHP RNA was constructed. This RNA is identical to Ps180 RNA except that the largest part of the 69-nt Ps hairpin has been removed. The U contents of Ps180 and PsΔHP are similar (approx. 25%).

Pilot experiments were performed to estimate the optimal UV dose. From these experiments it was determined that an UV dose of 1.8 J/cm² gave the most distinct bands with the least background (data not shown).

In all experiments performed with Ps180 RNA or PsΔHP RNA, 5 μg of total cytoplasmic RNA extracted from uninfected cells was added as nonspecific competitor RNA. Cross-linked and RNase treated samples were immunoprecipitated with the monoclonal N-specific antibody 5B188.2 or the rabbit polyclonal MHV-A59 antiserum k134 as indicated in the figure legends.

FIG. 3. Gel supershift assay performed with Ps290 probe and protein lysates from infected (I) or mock-infected (MI) cells. The complex was supershifted with either N-specific antibody (5B188.2) or nonspecific β-galactosidase antibody (β). Competition was performed using a 100-fold molar excess of yeast tRNA. The positions of the unbound RNA (probe), complexed RNA (complex), and supershift are indicated.

RNA–protein complexes (Fig. 3, lanes 8–10 and 12), clearly indicating that the observed supershift was not the result of an interaction between the RNA probe and the N-specific antibody.

Addition of a 100-fold molar excess of nonlabeled Ps290 competitor RNA resulted in the complete inhibition of protein binding to the RNA probe (Fig. 3, lane 6). In contrast, a 100-fold molar excess of tRNA did not affect the formation of RNA–protein and RNA–protein–antibody complexes (Fig. 3, lane 7), which demonstrates the specificity of the interaction between the N protein and Ps290 RNA.

Nucleocapsid protein from purified viruses and recombinant expressed nucleocapsid protein interact specifically with Ps180 RNA

During RNA encapsidation or particle formation, the N protein might undergo structural changes or other modifications. It would therefore be interesting to see if the N protein incorporated in virus particles is still able to inter-
INTERACTION BETWEEN NUCLEOCAPSID PROTEIN AND PACKAGING SIGNAL

observed using the vTF7-lysate (Fig. 6C, lane 2). These experiments clearly demonstrate that no virus specific modifications of the N protein or any other viral proteins are required for the specific interaction with Ps180.

DISCUSSION

Specific interaction of the MHV nucleocapsid protein with Ps containing RNAs

Initiation of encapsidation by a specific interaction between the (nucleo-) capsid protein and a RNA packaging signal is a common mechanism used by positive stranded RNA viruses. Such an interaction has already been identified and studied in vitro in alphaviruses (Owen and Kuhn, 1996; Weis et al., 1989), retroviruses (Zhang and Barklis, 1995; Berkowitz et al., 1995; Dupraz and Spahr, 1992; Aldovini and Young, 1990) but also in E. coli bacteriophages like R17 and MS2 (Witherell et al., 1991).

In this report we describe the specific in vitro interaction of the mouse hepatitis virus A59 N protein with transcripts containing the RNA packaging signal of MHV. This interaction was studied by gel retardation and UV cross-linking assays using an RNA probe containing the 69-nt Ps and the N protein from MHV-A59 infected cell lysates. A similar interaction was identified between the N protein from purified virus and recombinant N-protein expressed by the vTF7 expression system.

In addition to the N-Ps RNA complex we also observed that cellular proteins bind to Ps containing RNAs. The nature of these cellular proteins and the significance of this interaction, however, remains unclear.
The nature of the nucleocapsid protein–Ps RNA interaction

The N-protein of MHV does not possess any well known RNA binding domains, such as the ARM (Lazinski et al., 1989; Talbot and Buchmeier, 1985) or zinc fingers (Draper, 1995). It interacts, although, specifically with leader-RNA (Stohlman et al., 1988; Baric et al., 1988) and an RNA binding domain has been identified (Nelson and Stohlman, 1993). This domain comprises the two major hydrophobic basic regions in the middle of the protein. Since this central part of the N protein contains a high degree of basic amino acids, it is possible that the Ps recognition domain might also be positioned somewhere within this part of the N protein.

In infected cells the N protein of MHV is very rapidly phosphorylated on serine residues (Stohlman and Lai, 1979) and part of it concomitantly becomes associated with a cell membrane fraction (Stohlman et al., 1983; Anderson and Wong, 1993). It is still unknown if phosphorylation is carried out by a host cell or viral encoded protein kinase and its (biological) role remains unclear. It has been suggested that it might govern the tightness of the interaction between N and RNA (Laude and Masters, 1995). It will be interesting to compare the phosphorylation of the N protein from infected cells and the recombinant N protein and to investigate the possible role of phosphorylation in RNA binding. Phosphorylation and likewise dephosphorylation could also induce a major conformational change of the N protein. It has been suggested that dephosphorylation of the nucleocapsid after virus entry is involved in uncoating of the viral RNA (Mahondas and Dales, 1991).

In general the recognition of an RNA signal by a protein involves secondary structure elements in addition to the primary sequence of the RNA signal (Draper, 1995). There is accumulating evidence for an induced-fit mechanism in RNA–protein interactions affecting both the RNA and the protein (Beck and Nassal, 1997; Allain et al., 1996). Sufficient flexibility in the RNA molecule could be a major determinant in protein binding. From that perspective, the recognition of the Ps by the N protein could be highly dependent on the secondary structure of the Ps domain. When a short RNA probe consisting of only the 69-nt Ps was used in our studies, no gel retardation or UV cross-linking to the N protein was observed (unpublished observations). Computer prediction of the secondary structure of this small 69-nt RNA molecule showed that the secondary structure was entirely different from that of the Ps in the ORF1b context. This suggests that the flanking ORF1b sequences are necessary to force the Ps in its specific structure or that the small RNA probe lacks the flexibility to form the specific structure. Recently, it has been shown that the 69-nt Ps, flanked by non-MHV sequences could confer specific encapsidation to a heterologous RNA in MHV infected cells (Woo et al., 1997). Although it is not known whether this encapsidation was efficient, it might be possible that the heterologous flanking sequences have increased the flexibility of the RNA molecule, allowing it to adapt the proper structure for encapsidation.

Significance of the nucleocapsid protein–Ps RNA interaction

Since MHV virions contain only MHV genomic RNA, it is evident that RNA encapsidation is a highly specific process. The location of the encapsidation signal in ORF1b ensures the specific encapsidation of only genomic RNA. It is unclear if the MHV Ps is by itself sufficient for efficient encapsidation. It is known that Rous sarcoma virus has multiple encapsidation sites, which are required to interact for efficient packaging of the genome (Sorge et al., 1983; Pugatsch and Stacey, 1983). Recently, it was shown that a subgenomic RNA of MHV containing the Ps can be encapsidated specifically but the efficiency was much lower as compared to the encapsidation efficiency of a defective interfering RNA (Boo et al., 1997).

Reassembled nucleocapsids have never been observed in coronavirus infected cells, but an electron-dense structure, which may correspond to the nucleocapsid, can be found at the cytoplasmic face of the budding site (Dubois-Dalcq et al., 1982; Holmes, 1991). Nucleocapsid incorporation in budding virions is expected to be mediated by M–N protein interactions. Association of the M protein to the nucleocapsid in NP-40-disrupted virions has been reported (Sturman et al., 1980); however, the same study demonstrated that the M protein was able to bind RNA in the absence of N. It is still unclear if the interaction between M and N is a prerequisite for RNA encapsidation in vivo. This interaction could position the N protein in a favorable way to interact with the genomic RNA. This must then be followed by interaction with additional N proteins, forming the helical nucleocapsid. Involvement of other viral proteins in Ps RNA binding, however, was not observed in our in vitro studies. Recombinant nucleocapsid protein, expressed in the vTF7 expression system, interacts also specifically with the Ps RNA (Fig. 6C), although the efficiency of this interaction was not determined. A striking feature of coronaviruses is that the nucleocapsid has a helical symmetry (MacNaughton et al., 1978; Holmes and Behnke, 1981), in contrast to the nucleocapsids of all other positive stranded RNA viruses, which are icosahedral or spherical (Murphy et al., 1995). However, electron microscopy studies on the transmissible gastroenteritis coronavirus and MHV (Risco et al., 1996) have revealed recently a spherical core structure inside the virion (internal core) and this structure reacted with M- and N-specific antibodies. A structural model for coronaviruses was proposed, in which a spherical core, composed of a combination of N and M proteins, was present in addition to
a helical nucleocapsid. It will be of interest to study the possible relationship between the morphology of the nucleocapsid or internal core and the mechanism of encapsidation initiation.

It has been shown that, as opposed to alphavirus assembly, nucleocapsid formation and RNA encapsidation are not strictly required for virion formation (Vennema et al., 1996; Bos et al., 1996; Strauss and Strauss, 1994). Coexpression of M and E protein was sufficient for particle formation. However, incorporation of nucleocapsids during the budding process could greatly increase the efficiency of virion formation.

The study of the encapsidation of coronaviruses is greatly hampered by the absence of an infectious clone. The in vitro binding assay, described in this report, can greatly enhance our understanding of the encapsidation mechanism. We have shown that recombinant nucleocapsid protein expressed in the VTF7 expression system is able to interact with the Ps in a similar fashion as the nucleocapsid protein produced in infected cells. Mutational analysis of both the nucleocapsid protein and the Ps should provide us with information that allows us to understand the encapsidation mechanism in more detail.

ACKNOWLEDGMENTS

We thank Willem Luytjes, Evelyne Bos, Guido van Marle, Jessika Dobbe, and Heleen Gerritsma for stimulating discussions. R.M. was supported by Grant 700-31-020 from the Dutch Foundation for Chemical Research (SON).

REFERENCES

Aldovini, A., and Young, R. A. (1990). Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging in production of noninfectious virus. J. Virol. 64, 1920–1926.

Allain, F. H.-T., Gubser, C. C., Howe, P. W. A., Nagai, K., Neuhaus, D., and Varani, G. (1996). Specificity of ribonucleoprotein interaction determined by RNA folding during complex formation. Nature 380, 646–650.

Anderson, R., and Wong, F. (1993). Membrane and phospholipid binding by murine coronavirus nucleocapsid N protein. Virology 194, 224–232.

Armstrong, J., Smeekens, S., and Rottier, P. J. M. (1983). Sequence of the Nucleocapsid gene from murine coronavirus MHV-A59. Nucleic Acids Res. 11, 883–891.

Baric, R. S., Nelson, G. W., Fleming, J. O., Deans, R. J., Keck, J. G., Casteel, N., and Stohlman, S. A. (1988). Interactions between coronavirus nucleocapsid protein and viral RNAs: Implications for viral transcription. J. Virol. 62, 4280–4287.

Beck, J., and Nassal, M. (1997). Sequence- and structure-specific determinants in the interaction between the RNA encapsidation signal and reverse transcriptase of avian hepatitis B viruses. J. Virol. 71, 4971–4980.

Berkowitz, R. D., Ohagen, A., Hodglin, S., and Goff, S. P. (1995). Retroviral nucleocapsid domains mediate the specific recognition of genomic viral RNAs by chimeric gag polyproteins during RNA packaging in vivo. J. Virol. 69, 6445–6456.

Bos, E. C. W., Luytjes, W., Meulen van der, H., Koerten, H. K., and Spaan, W. J. M. (1996). The production of recombinant infectious DI-particles of a murine coronavirus in the absence of helper virus. Virology 218, 52–60.

Bos, E. C. W., Dobbe, J., Luytjes, W., and Spaan, W. J. M. (1997). A subgenomic mRNA transcript of the coronavirus mouse hepatitis virus strain A59 defective interfering (DI) RNA is packaged when it contains the DI packaging signal. J. Virol. 71, 5684–5687.

Bredenbeek, P. J. (1990). "Nucleic Acid Domains and Proteins Involved in the Replication of Coronaviruses." Ph.D. Thesis, University of Utrecht, Utrecht, The Netherlands.

Burk, C. G., and Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. Science 265, 615–621.

Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983). Acurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11, 1475–1489.

Draper, D. E. (1995). Protein-RNA recognition. Annu. Rev. Biochem. 64, 593–620.

Dubois-Dalcq, M. E., Doller, E. W., Haspel, M., and Holmes, K. V. (1982). Cell tropism and expression of mouse hepatitis viruses (MHV) in mouse spinal cord cultures. Virology 119, 317–331.

Dupraz, P., and Spahr, P. F. (1992). Specificity of Rous sarcoma virus nucleocapsid protein in genomic RNA packaging. J. Virol. 66, 4662–4670.

Fisette, F., Peng, D., Hingley, S. T., Weiss, S. R., and Masters, P. S. (1997). The internal open reading frame within the nucleocapsid gene of mouse hepatitis virus encodes a structural protein that is not essential for viral replication. J. Virol. 71, 996–1003.

Fosmire, J. A., Hwang, K., and Makino, S. (1992). Identification and characterization of a coronavirus packaging signal. J. Virol. 66, 3522–3530.

Holmes, K. V. (1991). In "Fundamental Virology" (B. N. Fields and D. M. Knipe, Eds.), pp. 471–486. Raven Press, New York.

Holmes, K. V., and Behnke, J. N. (1981). Evolution of a coronavirus during persistent infection in vitro. Adv. Exp. Med. Biol. 142, 287–299.

Lai, M. M. C., Baric, R. S., Brayton, P. R., and Stohlman, S. A. (1984). Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic virus RNA. Proc. Natl. Acad. Sci. USA 81, 3626–3630.

Laude, H., and Masters, P. S. (1995). In "The Coronaviridae" (S. G. Siddell, Ed.), pp. 141–163. Plenum Press, New York.

Lazinski, D., Gradzielska, E., and Das, A. (1989). Sequence specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif. Cell 59, 207–215.

Luytjes, W. (1995). In "The Coronaviridae" (S. G. Siddell, Ed.), pp. 33–44. Plenum, New York.

Luytjes, W., Gerritsma, H., Bos, E. C. W., and Spaan, W. J. M. (1997). Characterisation of two temperature-sensitive mutants of coronavirus mouse hepatitis virus strain A59 with maturation defects in the nucleocapsid protein and viral RNAs: Implications for viral transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Virology 11, 646–650.

MacNaughton, M. R., Davies, H. A., and Nermut, M. V. (1978). Ribonucleoprotein-like structures from coronavirus particles. J. Gen. Virol. 39, 545–549.

Mahondas, D. V., and Dales, S. (1991). Endosomal association of a protein phosphatase with high dephosphorylation activity against a coronavirus nucleocapsid protein. FEBS Lett. 282, 419–424.

Makino, S., Shieh, C., Soe, L. H., Baker, S. C., and Lai, M. M. C. (1988). Primary structure and translation of a defective interfering RNA of murine coronavirus. Virology 166, 1–11.

Masters, P. S. (1992). Localization of an RNA-binding domain in the nucleocapsid protein of the coronavirus mouse hepatitis virus. Arch. Virol. 125, 141–160.

Most, v. d., R. G., Bredenbeek, P. J., and Spaan, W. J. M. (1991). A domain in the 3' end of the polymerase gene is essential for encapsidation of coronavirus defective interfering RNAs. J. Virol. 65, 3219–3226.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martinelli, G. P., Mayo, M. A., and Summers, M. D. (1995). "Virus
Taxonomy, Classification and Nomenclature of Viruses.* Springer-Verlag, New York.

Nelson, G. W., and Stohlman, S. A. (1993). Localization of the RNA-binding domain of mouse hepatitis virus nucleocapsid protein. J. Gen. Virol. 74, 1975–1979.

Owen, K. E., and Kuhn, R. J. (1996). Identification of a region in the sindbis virus nucleocapsid protein that is involved in specificity of RNA encapsidation. J. Virol. 70, 2757–2763.

Parker, M. M., and Masters, P. S. (1990). Sequence comparison of the N genes of five strains of the coronavirus mouse hepatitis virus suggests a three domain structure for the nucleocapsid protein. Virology 179, 463–468.

Pugatsch, T., and Stacey, D. W. (1983). Identification of a sequence likely to be required for avian retroviral packaging. Virology 128, 505–511.

Risco, C., Anton, I. M., Enjuanes, L., and Carrascosa, J. L. (1996). The transmissible gastroenteritis coronavirus contains a spherical core shell consisting of M and N proteins. J. Virol. 70, 4773–4777.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schlesinger, S., Makino, S., and Linial, M. L. (1994). Cis-acting genomic elements and trans-acting proteins involved in the assembly of RNA viruses. Semin. Virol. 5, 39–49.

Snijder, E. J., Wassenaar, A. L. M., Dinten, v., L. C., Spaan, W. J. M., and Gorbalenya, A. E. (1996). The arterivirus Nsp4 protease is the prototype of a novel group of chymotrypsin-like enzymes, the 3C-like serine proteases. J. Biol. Chem. 271, 4864–4871.

Sorge, J., Ricci, W., and Hughes, S. H. (1983). Cis-Acting RNA packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. J. Virol. 48, 667–675.

Spaan, W. J. M., Rottier, P. J. M., Horzinek, M. C., and Van der Zeijst, B. A. M. (1981). Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). Virology 108, 424–434.

Spaan, W. J. M., Delius, H., Skinner, M. A., Armstrong, J., Rottier, P. J. M., Smeekens, S., Van der Zeijst, B. A. M., and Siddell, S. G. (1983). Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. EMBO. J. 2, 1839–1844.

Spaan, W. J. M., Cavanagh, D., and Horzinek, M. C. (1988). Coronaviruses: structure and genome expression. J. Gen. Virol. 69, 2939–2952.

Stohlman, S. A., Fleming, J. O., Patton, C. D., and Lai, M. M. C. (1983). Synthesis and subcellular localization of the murine coronavirus nucleocapsid protein. Virology 130, 527–532.

Stohlman, S. A., Baric, R. S., Nelson, G. W., Soe, L. H., Welte, L. M., and Deans, R. J. (1988). Specific interaction between coronavirus leader RNA and nucleocapsid protein. J. Virol. 62, 4288–4295.

Stohlman, S. A., and Lai, M. M. C. (1979). Phosphoproteins of murine hepatitis viruses. J. Virol. 32, 672–675.

Sturman, L. S., Holmes, K. V., and Behnke, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. Virology 115, 463–468.

Talbot, P. J., and Buchmeier, M. J. (1985). Antigenic variation among murine coronaviruses: Evidence for polymorphism on the peplomer glycoprotein, E2. Virus Res. 2, 317–328.

Vennema, H., Goddeke, G.-J., Rosset, J. W. A., Voorhout, W. F., Horzinek, M. C., Opstelten, D.-J. E., and Rottier, P. J. M. (1996). Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. EMBO J. 15, 2020–2028.

Weis, B., Nitschko, H., Ghattas, I., and Schlesinger, S. (1989). Evidence for specificity in the encapsidation of Sindbis virus RNAs. J. Virol. 63, 5310–5318.

Witherell, G. W., Gott, J. M., and Uhlenbeck, O. C. (1991). Specific interaction between RNA phage coat proteins and RNA. Proc. Natl. Acad. Sci. U. S. A. 88, 2015–2020.

Zhang, Y., and Barklis, E. (1995). Nucleocapsid protein effects on the specificity of retrovirus RNA encapsidation. J. Virol. 69, 5204–5209.

Zhou, M., Williams, A. K., Chung, S., Wang, L., and Collier, E. W. (1997). The infectious bronchitis virus nucleocapsid protein binds RNA sequences in the 3' terminus of the genome. Virology 217, 191–199.