JOURNAL OF VIROLOGY, Nov. 1988, p. 4288–4295
0022-538X/88/114288-08$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 62, No. 11

Specific Interaction between Coronavirus Leader RNA and Nucleocapsid Protein

STEPHEN A. STOHLMAN,[1,2]* RALPH S. BARIC,[3] GARY N. NELSON,[2] LISA H. SOE,[2] LISA M. WELTER,[2] AND ROBERT J. DEANS[2]

Departments of Neurology[1] and Microbiology,[2] University of Southern California School of Medicine, Los Angeles, California 90033, and the Department of Parasitology and Laboratory Practice, University of North Carolina School of Public Health, Chapel Hill, North Carolina 27514[3]

Received 28 April 1988/Accepted 1 August 1988

Northwestern blot analysis in the presence of competitor RNA was used to examine the interaction between the mouse hepatitis virus (MHV) nucleocapsid protein (N) and virus-specific RNAs. Our accompanying article demonstrates that anti-N monoclonal antibodies immunoprecipitated all seven MHV-specific RNAs as well as the small leader-containing RNAs from infected cells. In this article we report that a Northwestern blotting protocol using radiolabeled viral RNAs in the presence of host cell competitor RNA can be used to demonstrate a high-affinity interaction between the MHV N protein and the virus-specific RNAs. Further, RNA probes prepared by in vitro transcription were used to define the sequences that participate in such high-affinity binding. A specific interaction occurs between the N protein and sequences contained with the leader RNA which is conserved at the 5′ end of all MHV RNAs. We have further defined the binding sites to the area of nucleotides 56 to 65 at the 3′ end of the leader RNA and suggest that this interaction may play an important role in the discontinuous nonprocessive RNA transcriptional process unique to coronaviruses.

The murine coronavirus mouse hepatitis virus (MHV) contains a single-stranded positive-polarity RNA of approximately 6×10^6 daltons (20). This RNA interacts with the 60,000-dalton nucleocapsid (N) protein to form a helical nucleocapsid structure (34). The MHV envelope contains two virus-specified envelope glycoproteins, designated E1 and E2, which are acquired during maturation at intracytoplasmic membranes (34). E2 is a 180,000-dalton N-linked glycoprotein which mediates cell-cell fusion and is the site of neutralizing-antibody attachment (13, 15, 35). E1 is an O-linked matrix glycoprotein of 23,000 daltons that is believed to interact with N in the formation of virions (34, 35).

Genomic-length mRNA and six subgenomic mRNAs are transcribed from a full-length negative-stranded template (19, 31). These intracellular mRNAs are both capped and polyadenylated and are arranged in the form of a nested-set array from the 3′ end of the genome, i.e., they have common 3′ termini and extend for various lengths in the 5′ direction (18, 31). In addition, each virus-specific mRNA contains an identical leader sequence approximately 72 nucleotides long which is derived from the 5′ end of genomic RNA (17, 18). After synthesis, the leader RNA disassociates from the negative-stranded template and rebinds at the initiation sites for each of the six subgenomic mRNAs, thereby serving as a primer for transcription (6, 8, 22). Transcription of the MHV mRNAs is discontinuous, and pausing occurs in AU-rich regions or regions of secondary structure (6). This leads to the generation of discrete small leader-containing RNAs which are present both as nascent plus-strands on the replicative intermediate (RI) RNA and as free molecules dissociated from the transcript complex. RNA species originate during synthesis of the individual genes, with 3′ termination corresponding to regions of the RNA with secondary structure or AU-rich regions (6). These discrete functional intermediate RNAs probably also function in *trans* as intermediates of RNA-RNA recombination (22).

In the accompanying article we have shown that MHV virion RNA as well as all seven intracellular RNAs are complexed with the N protein (5). Immunoprecipitation of the small leader-containing RNA species from infected cells demonstrated that the leader RNA sequences were sufficient to bind the N protein. Our inability to demonstrate N complexed to leader RNAs of less than 57 nucleotides along with its presence on RNAs of greater than 65 nucleotides suggested that either the N-RNA binding site was encoded within this 10-nucleotide stretch or there was a minimum-size constraint on the N-RNA interaction. In addition, the data suggested that N protein is associated with the RI RNA complex in infected cells. This may be due to the presence of N protein associated with the leader RNA sequences at the 5′ end of the nascent strands; however, we could not determine whether N bound the negative-stranded RNA directly via antisense leader RNA sequences. In this report, we describe the use of competitor RNA in a Northwestern blot analysis to identify high-affinity RNA-protein interactions and present additional evidence for the specificity of N binding to the leader segment of MHV RNA. Previous studies using this assay in the absence of competitor RNA were unable to detect sequence-specific binding of the N protein (26). This assay was used to distinguish between binding to the positive- and negative-stranded RNAs and provides additional evidence which suggests that N protein binds to leader RNA between nucleotides 56 and 65 from the 5′ end of all the MHV mRNAs.

MATERIALS AND METHODS

Virus and cells. The studies described used the A59 strain of MHV. Virus was propagated in the DBT murine astrocytoma cell line or L2 cells as previously described (20). Cells were grown in 150- or 100-mm-diameter petri plates (Becton Dickinson Labware, Oxnard, Calif.) and infected at a multiplicity of infection of 1 to 5. For experiments using $^{32}P_i$ to radiolabel MHV-specific mRNAs, infected cells were treated with 2 μg of actinomycin D per ml at 2.5 h postin-

* Corresponding author.

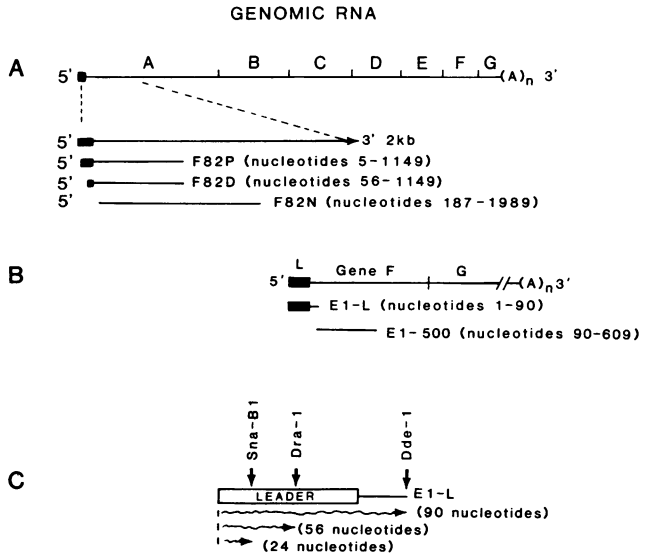


FIG. 1. Structure of the MHV cDNA probes used for Northwestern blot experiments. (A) Clone pF82P contains nucleotides 5 through 1149 derived from the 5′ end of the genome. Clone F82D is derived from F82P and contains nucleotides 56 through 1149 from the 5′ end of the genome. Clone F82N is derived from cDNA clone F82 and contains nucleotides 187 through 1989 from the 5′ end of the genome. (B) Clone E1-L contains the leader RNA (nucleotides 1 through 90) derived from the 5′ end of mRNA 6 (gene F). Clone E1-500 lacks the leader RNA (nucleotides 90 through 609) and is derived from the 5′ end of mRNA 6. (C) The derivation of the 24 and 56 nucleotide RNAs synthesized from E1-L after digestion with either *Sna*BI or *Dra*I.

fection (p.i.) and labeled with 500 μCi of $^{32}P_i$ (ICN Pharmaceuticals Inc., Irvine, Calif.) at 4.5 h p.i. RNA was harvested by phenol extraction of nucleus-free lysates of infected cells at 7.5 to 8.5 h p.i.

In vitro transcription of MHV strand-specific probes. The in vitro transcripts used in this study were derived from pT7 plasmids containing the bacterial T7 RNA polymerase initiation site. Inserts were obtained by cDNA cloning of either the A59 or JHM strains of MHV and are depicted in Fig. 1. The E1-L′ plasmids were obtained from the 5′ end of gene F of A59 virus by subcloning a *Bam*HI-*Dde*I fragment into the *Bam*HI site of pT7.1 or pT7.2. These constructs contain the 72-base leader plus 18 bases extending into the E1 coding region (2). The plasmid E1-L′(+) synthesizes RNA in the plus-stranded configuration, while the plasmid E1-L′(−) synthesizes the identical sequences in the negative-sense configuration. Plasmid E1-500 contains the coding region of gene F without the leader RNA sequences (nucleotides 90 to 609) and synthesizes plus-stranded RNA from the T7 promoter (5, 6).

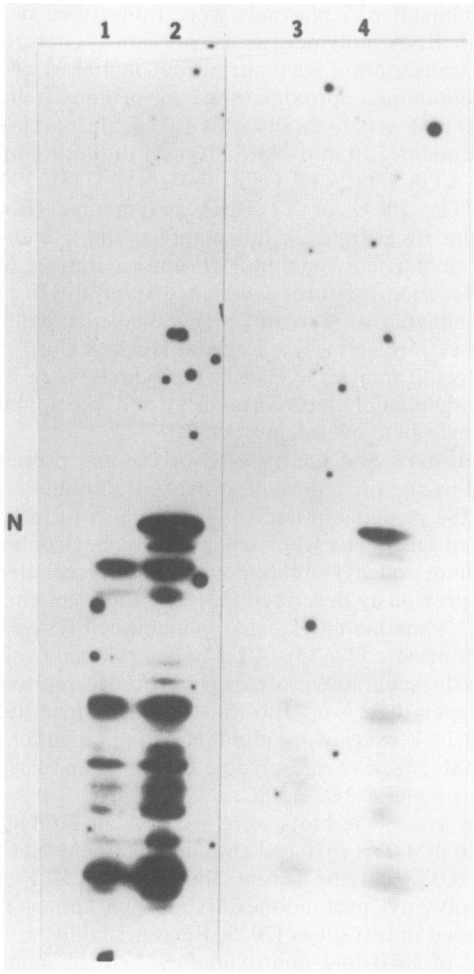
Details of the construction of pT7F82P (F82P), pT7F82D (F82D), and pT7F82N (F82N) have been previously described (28, 30). Briefly, plasmid F82P contains the initial 1.2 kilobases of nucleotides from the 5′ end of genomic RNA, including all but 5 nucleotides from the 5′ end of the leader sequence. Plasmid F82P(+) produces RNA in the plus-stranded configuration, while plasmid F82P(−) contains the identical insert in the opposite orientation and produces negative-stranded RNA. Plasmid F82D contains nucleotides 56 through 1149 from the 5′ end of genomic RNA. Plasmid F82N contains nucleotides 187 to 1989 from the 5′ end of genomic RNA. Both plasmids F82D and F82N produce plus-stranded RNA from the T7 promoter.

Recombinant pT7 plasmids were transcribed in vitro by using T7 RNA polymerase as previously described (5). Briefly, transcription was carried out in 100 μl of reaction buffer containing approximately 2 μg of linearized plasmid DNA; 40 mM Tris hydrochloride (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 1 mM each of ATP, CTP, and GTP; 0.01 mM UTP; 100 μCi of [α-^{32}P]UTP; 10 U of T7 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); and 40 U of RNasin ribonuclease inhibitor (Promega Biotec, Madison, Wis.). Reaction mixtures were incubated at 37°C for 1 h. DNA templates were removed by digestion with 2 U of RNase-free DNase I (New England Nuclear Corp., Boston, Mass.) for 30 min at 37°C. RNA products were extracted twice with phenol:chloroform (1:1) and precipitated with ethanol at −20°C overnight.

Northwestern blot analysis. RNA binding proteins were detected by the procedure described by Robbins et al. (26). Briefly, the proteins in nucleus-free lysates of infected and uninfected DBT cells were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (32, 33). Each gel contained a lane of [^{35}S]methionine-labeled infected-cell lysate to localize the N protein (32, 33). After polyacrylamide gel electrophoresis the separated proteins were transferred to nitrocellulose paper (0.22 μm, Bio-Rad Laboratories, Richmond, Calif.) at 14 V overnight without cooling in a buffer containing 25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol. Blots were stored −20°C.

Before assay, the blots were washed for 30 min in SBB buffer (10 mM Tris [pH 7.0] containing 50 mM NaCl, 1 mM EDTA, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinyl pyrrolidone). Strips (0.5 cm) of the blots were placed in test tubes (2025, Becton Dickinson) containing 4 ml of SBB and approximately 2×10^5 cpm of ^{32}P-labeled RNA, extracted from either virus-infected cells or in vitro transcription products. Competitor RNA (total cytoplasmic RNA from uninfected cells) was added at various concentrations to demonstrate the specificity of binding. Amounts of competitor RNA represented a 10^3 and 10^4 molar excess relative to the probes tested. After 1 h at room temperature with constant agitation, the strips were washed with three changes of SBB and dried, and the bound RNA was visualized by autoradiography.

Western blots (immunoblots). N protein was also localized on the nitrocellulose paper strips by Western blotting analysis. Briefly, 0.5-cm nitrocellulose strips were washed in buffer containing 10 mM Tris (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100 for 15 min at room temperature and then incubated overnight in the same buffer containing 3% bovine serum albumin at 4°C. The strips were placed in test tubes as described above with 4.0 ml of buffer containing 3% bovine serum albumin and monoclonal antibody A.1.10, specific for the N protein (15), and incubated overnight at 4°C with constant agitation. The antibody was removed, and the strips were washed at least three times before the addition of 1.0 μCi of ^{125}I-labeled *Staphylococcus* A protein (ICN). After overnight incubation at 4°C with constant agitation, the strips were washed at least four times with 10 mM Tris buffer (pH 8.0) containing 1.0 M NaCl, 1.0 mM EDTA, 0.5% Triton X-100, and 3% bovine serum albumin. The strips were dried, and the bound ^{125}I was visualized as described above.



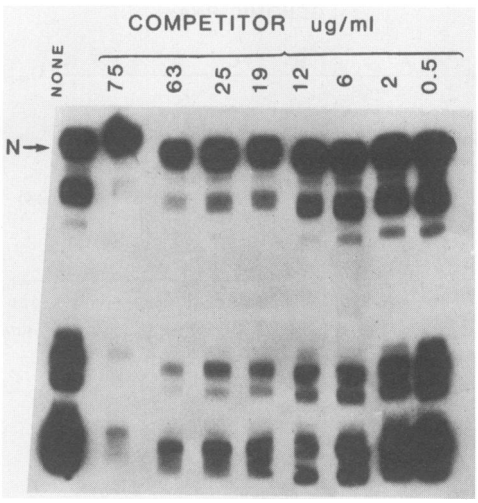


FIG. 2. Northwestern blot analysis of MHV-intracellular RNA in the presence of competitor RNA. (A) Lanes: 1 and 2, binding of MHV-RNA to the proteins in uninfected (lane 1) and infected (lane 2) cells in the absence of competitor RNA; 3 (uninfected cells) and 4 (infected cells), residual binding to N in the presence of 100 μg of competitor RNA per ml. (B) Titration of the competitor RNA levels, demonstrating the specificity of N-RNA binding. None, Absence of competitor RNA.

RESULTS

Specificity of the virus RNA-N protein interaction. The data presented in the accompanying article suggest that there is a specific interaction between the N protein and MHV RNA on the basis of the specific coimmunoprecipitation of virus RNA in complexes formed by the N protein and anti-N monoclonal antibody (5). The data further suggest that the N protein-RNA interaction is mediated, at least in part, by the sequences present in the MHV leader RNA sequences. To explore the specificity of the N protein-MHV RNA interaction and to develop an approach that would allow us to more clearly map the N binding site, RNA binding proteins in infected and uninfected cells were examined by Northwestern blot analysis (9, 26). Lysates of MHV-infected and uninfected cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The position of the N protein was verified by Western blot analysis of adjacent lanes in all experiments. In the initial experiments, ^{32}P-labeled MHV-specific RNA was extracted from infected actinomycin D-treated cells and used as a probe in the binding assays. We sought to determine whether the N protein-RNA binding previously described (26) exhibited the high-affinity binding characteristics of other sequence-specific nucleic acid-binding proteins by using competitor RNA to distinguish between high- and low-affinity binding (9, 14).

RNA extracted from MHV-infected cells, containing all seven MHV RNA species (18, 31), binds to the MHV N protein as well as to a variety of proteins derived from the cytoplasm of both uninfected and infected cells (Fig. 2A, lanes 1 and 2, respectively). To determine the relative affinity of binding of MHV RNA to the host cell-derived RNA-binding proteins, RNA prepared from uninfected DBT cells was used as a competitor. Competitor RNA (100 μg/ml) effectively inhibited the binding of MHV RNA to the host cell proteins but not to the N protein (Fig. 2A, lanes 3 and 4). Densitometric analysis indicated approximately 20% retention of binding of viral RNA to N at 100 μg of competitor RNA per ml, whereas there was less than 1% retention of binding to host cell RNA-binding proteins. Hence, this study demonstrates that viral RNA is bound specifically by the N protein and provides a useful approach for mapping binding signals within the MHV genome.

Similar results were obtained with individual MHV mRNAs 1, 6, and 7 isolated from infected cells and separated by agarose gel electrophoresis (data not shown). The use of specific MHV RNAs confirmed the presence of specific binding sites on all MHV RNAs, as suggested by the immunoprecipitation data presented in the accompanying article (5). In addition, we determined that mouse liver RNA, as well as L2 cell RNA, was a source of effective competitor RNA, while *Escherichia coli* rRNA and yeast tRNA were not effective competitors (data not shown). In separate assays, we demonstrated that double-stranded DNA also failed to compete with N-protein binding to viral leader sequences (L. M. Welter, unpublished results).

To more clearly demonstrate specificity of binding, competitor RNA was tested over a wide range (75 to 0.5 μg/ml) for its ability to quantitatively inhibit binding of viral RNA to the host cell RNA-binding proteins relative to N. The binding of MHV RNA to host cell proteins can be significantly reduced by the presence of 75 μg of competitor RNA per ml, and this binding changes in a dose-dependent manner (Fig. 2B). In contrast, the binding to N protein was relatively unaffected by the presence of competitor RNA, suggestive

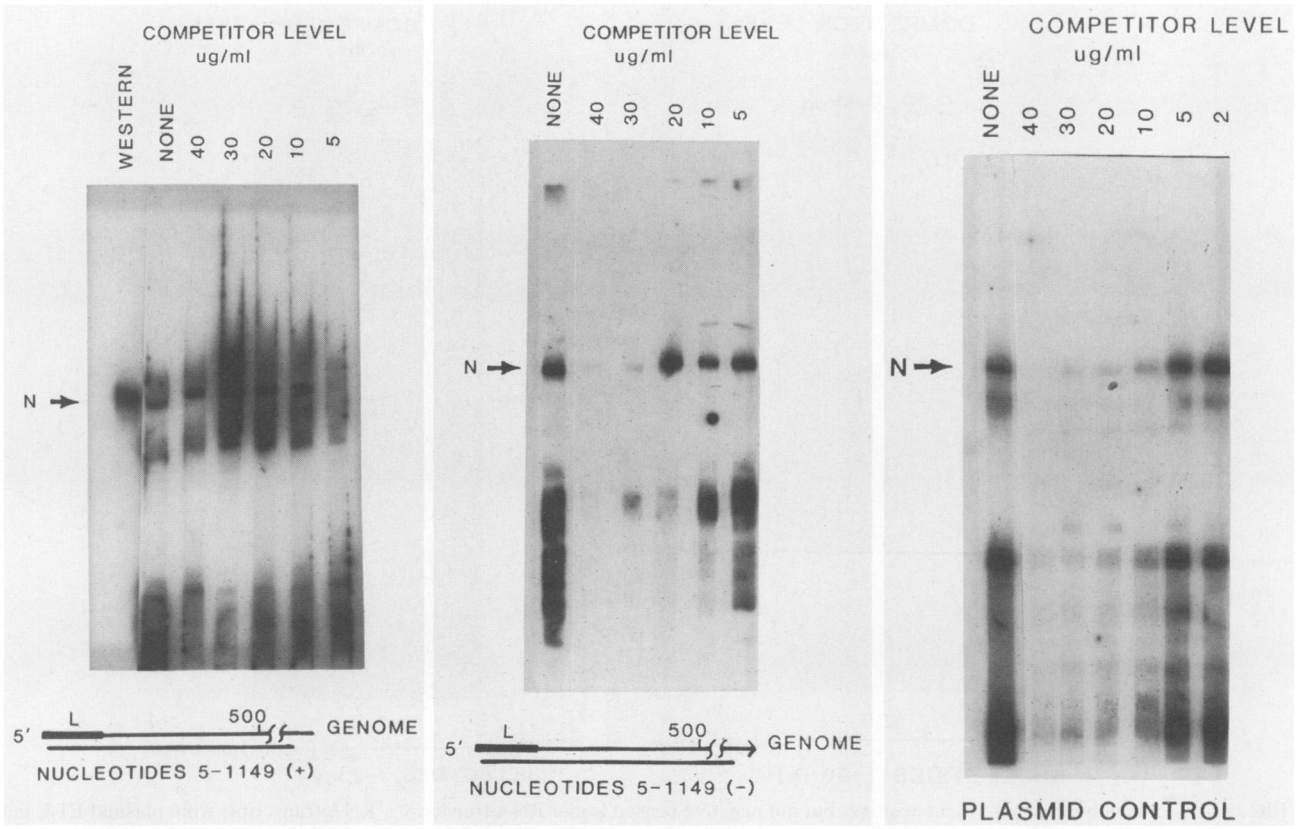


FIG. 3. Specific N-protein binding to in vitro RNA transcripts. (A) Specific binding of RNA transcripts from plasmid F82P in the presence of competitor RNA. (B) Absence of specific N binding by using a plasmid F82P RNA transcript in the negative sense. (C) Absence of specific N binding the RNA transcribed from plasmid F82P linearized at the *Xho* site 5′ of the MHV cDNA insert. None, Absence of competitor RNA.

of a specific and high-affinity binding. Therefore, these data confirm the specificity of the N protein-MHV RNA interaction demonstrated in the accompanying article (5).

Polarity-specific binding. The data in the accompanying article show that anti-N monoclonal antibody immunoprecipitates negative-stranded RNA (5). We suggested that this was due to the presence of N associated with the leader-containing nascent plus-strands on the RI RNA. However, we could not determine whether the negative-stranded RNA, as part of the RI complex, binds N via interaction with leader RNA sequences on the nascent plus-strands or whether N was associated with the transcription complex via protein-protein interactions. To determine whether an N binding site is present on plus- and negative-sense MHV RNA sequences, we first tested a 1.2-kilobase plus-sense leader-containing in vitro RNA transcript derived from the 5′ end of the MHV genome (F82+) (Fig. 1) for high-affinity binding to N. Figure 3A shows specific binding of this leader-containing transcript to the N protein. A second band of N, which migrates at a faster apparent molecular weight, was detected in some lysates (Fig. 2 and 3; see also Fig. 5). This band reacted with some, but not all, anti-N monoclonal antibodies (data not shown). Whether this species represents a degradation product or altered phosphorylated species is unclear. In contrast to the specific binding of the leader-containing transcript, the competitor RNA clearly inhibited binding of an identical transcript in the negative-sense configuration (Fig. 3B), as well as a transcript lacking MHV-specific RNA sequences (Fig. 3C). These data indicate that N binds to viral RNA via base sequence specific interactions contained within plus-sense sequences at the 5′ end of the genome and that in vitro transcripts can be used to define the N protein-RNA interaction. Conversely, negative-sensed RNAs derived from the 5′ end did not bind N. These data suggest that the N protein does not directly associate with negative-stranded RNA unless other binding sites are contained elsewhere on the genomic-length negative-stranded RNA species.

Binding by leader-containing RNAs. Small leader-containing RNA species immunoprecipitated with anti-N monoclonal antibody suggested that the N-RNA binding site was contained within the MHV leader RNA (5). To confirm the direct binding of N protein to the sequences contained within the leader RNA, we examined in vitro transcripts prepared from a pT7 plasmid containing an insert composed of the leader sequences plus 18 additional nucleotides derived from the 5′ end of MHV mRNA 6 (Fig. 1). This plus-stranded RNA bound to the N protein, and binding was not inhibited in the presence of competitor RNA (Fig. 4A). Confirming the requirements for polarity specificity, binding in the presence of competitor RNA was not detected by using the negative-sense 90-base transcript derived from plasmid E1-L′(−) (Fig. 4B). These data support the findings presented in the accompanying article and clearly indicate that an N-protein binding site is present within the leader RNA sequence.

Localization of the binding site. In the accompanying article (5) we demonstrate that N binds to the 65-nucleotide but not the 57-nucleotide leader RNA, suggesting that these leader RNA sequences are critical for N-RNA binding. However, it is not clear whether sequence 5′ to position 57

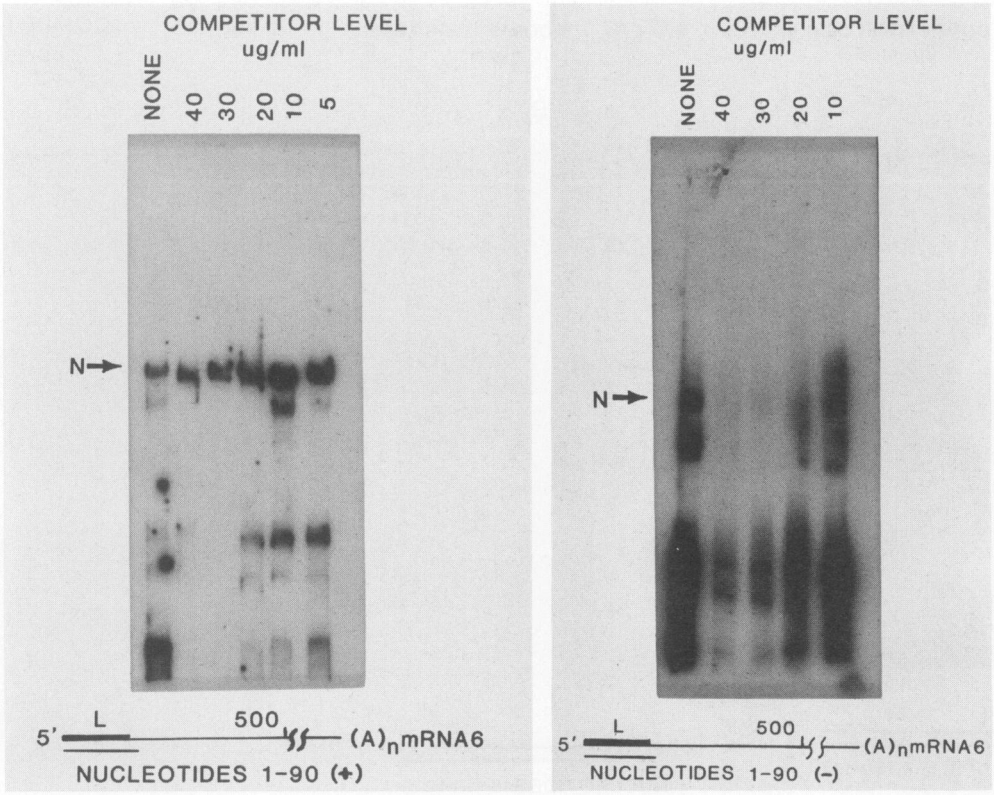


FIG. 4. Specific N binding activity in positive- but not negative-sensed leader RNA transcripts. RNA transcripts from plasmid E1-L in the positive (A) and negative (B) sense orientations were analyzed for their ability to bind N protein in the presence of various amounts of competitor RNA. None, Absence of competitor RNA.

also participates as part of the N binding site. To further localize the binding site, the plasmid E1-L'(+), containing the 90-base leader insert, was digested with either the *Dra*I or the *Sna*BI restriction endonucleases before in vitro transcription (Fig. 1). Digestion of this template with *Dra*I yields in vitro RNA transcripts containing the first 56 nucleotides at the 5' end of leader RNA, while digestion with the *Sna*BI endonuclease yields a 24-nucleotide 5' transcript. The sizes and integrity of these transcripts were confirmed by electrophoresis on denaturing polyacrylamide gels (data not shown). These truncated in vitro leader RNA transcripts do not exhibit specific binding in the presence of competitor RNA (Fig. 5B and C). This can be compared with the high-affinity binding detected with the full leader contained within the 90-base transcript (Fig. 5A). These data suggest that the sequences exhibiting specific N binding require nucleotides 56 through 90 in mRNA 6. To rule out specific interactions with the 18 nucleotides 3' to leader sequences derived from gene F which are contained in E1-L' (Fig. 1), we examined transcripts derived from plasmid F82D containing a cDNA insert representing nucleotides 56 through 1149 from the 5' end of genomic RNA (30). This cDNA clone diverges from the mRNA 6 sequence at position 67 (28). Specific high-affinity binding of this probe to the N protein in the presence of competitor RNA was found by using RNA transcribed from this plasmid (Fig. 5D). To confirm that no cryptic sites were contained in this relatively long transcript, we also tested a transcript derived from plasmid F82N containing a cDNA insert of nucleotides 187 to 1989 from the 5' end of genomic RNA (Fig. 1) and found no specific binding (Fig. 5E). It is unclear whether additional N binding sequences are present between nucleotides 67 and 187 in gene A, which potentially provides a packaging signal for genomic RNA.

RNA was also synthesized from a plasmid containing internal nucleotides 90 to 609 from the 5' end of mRNA 6 (E1-500) which lacks the first 90 nucleotides tested for Fig. 4A. These internal sequences do not exhibit specific binding to N (Fig. 5F). Therefore, these data suggest that specific and high-affinity binding of viral RNA by the N protein is necessary and sufficiently supplied by nucleotides in the viral leader spanning positions 56 to 67 of genomic RNA.

DISCUSSION

In the accompanying article we have demonstrated that the MHV N protein binds not only to the positive-stranded genomic-length RNA but also to all six subgenomic mRNAs (5). This binding appears to be mediated by sequences located at the 5' end of each mRNA. After infection, the MHV genomic RNA is transcribed by an early polymerase activity into a full-length negative-stranded RNA (10, 11, 19). Transcription of plus-stranded genomic-length RNA and the six subgenomic-length RNAs that make up the nested set of MHV mRNAs is via a unique leader-primed discontinuous nonprocessive mechanism (8, 22). Evidence for this mechanism includes the finding of free leader dissociated from the negative-stranded template and a temperature-sensitive mutant which synthesizes leader RNAs but not mRNAs at the nonpermissive temperature (6, 8). The leader RNA is also found at the 5' end of all positive-stranded MHV RNAs, including the 5' ends of nascent RNA on the RI

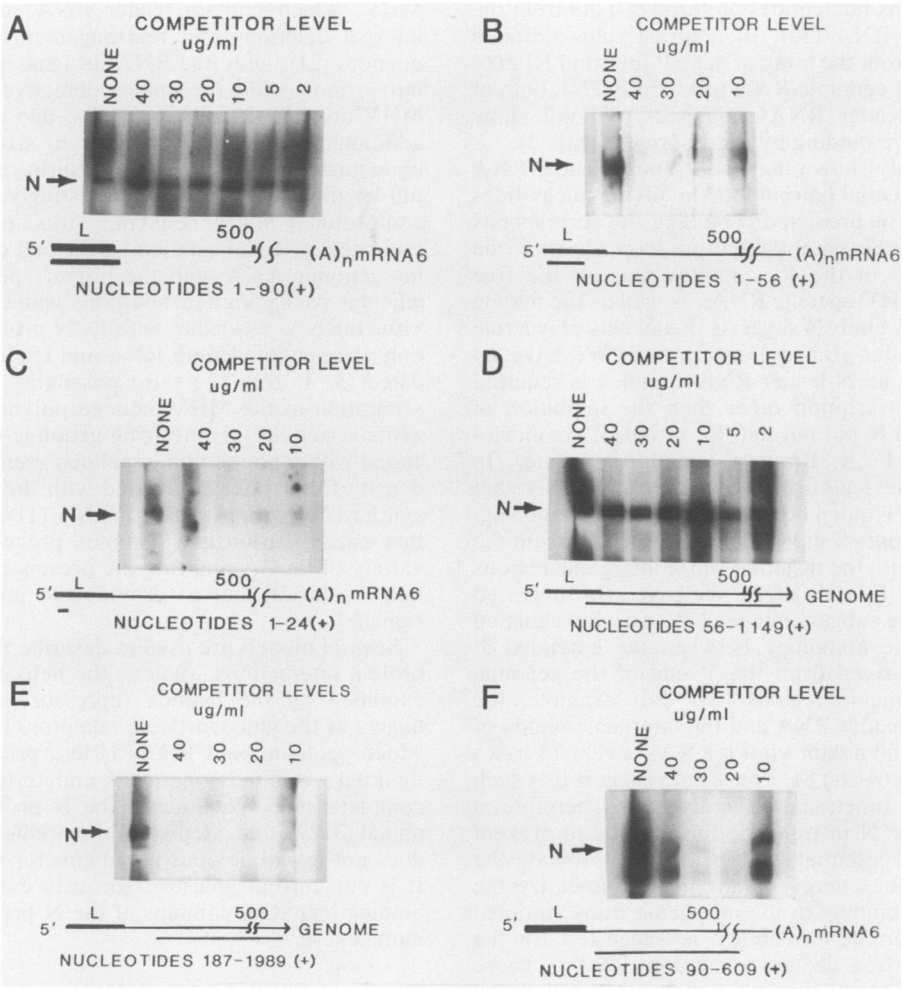


FIG. 5. Localization of the RNA binding site within the leader RNA sequence. (A) High-affinity binding by the RNA transcribed from E1-L(+). (B) Absence of specific binding by RNA containing nucleotides 1 through 56. (C) Absence of specific binding by RNA containing nucleotides 1 through 24. (D) Specific binding by RNA transcripts from plasmid F82D (nucleotides 56 through 1149). (E) Absence of specific binding by RNA transcripts from plasmid F82N (nucleotides 187 through 1989). (F) Absence of specific binding by RNA transcripts from plasmid E1-500 (nucleotides 90 through 609). None, Absence of competitor RNA.

complex (7, 17, 18). Further, the leader RNA sequences reassort at high frequency during mixed infection, indicating that these RNAs act in *trans* (21). The data presented in this and the accompanying article suggest that an N binding site is present within the MHV leader RNA sequence.

Negative-stranded as well as positive-stranded RNA is coimmunoprecipitated with N protein (5), suggesting either that N binds to negative-sensed RNA or that the leader-containing nascent chains of the RI complex (7) were associated with the N protein. The experiments described in this article examined the possibility of N binding domains on the negative-stranded RNA. Neither the 1.2-kilobase RNA transcript derived from the 5′ end sequences of genomic RNA nor the 90-base leader-containing RNA, both transcribed in the negative-sense configuration, was bound to N with specificity. In contrast, the complementary plus-sense RNA derived from the 5′ ends of both gene A and gene F sequences showed specific high-affinity binding. These data indicate that an N protein binding site is present on the leader RNA sequences contained on the nascent plus-strands of the RI complex (7) and further suggest a role for N in the transcription complex, as previously suggested by the inhibition of transcription by anti-N monoclonal antibody (12). These data also suggest that if the N protein binds to negative-stranded RNA, the interaction is mediated by sequences other than those at the 5′ end.

Analysis of the small MHV-specific leader-containing RNAs in infected cells by immunoprecipitation with anti-N monoclonal antibody suggests that N was present on RNA species greater than 65 nucleotides long (5). However, it could not be determined whether N bound directly to leader RNA sequences or whether it bound indirectly via association with other viral RNA-binding proteins. We have used RNAs produced by in vitro transcription to localize the site of N protein binding to the leader RNA. Northwestern blot analysis of high-affinity N-protein RNA binding in the presence of competitor RNA showed that N binds directly to the 90-base leader-containing RNA sequences derived from gene F. Analysis of RNAs deleted for the bases 1 through 56 confirmed that the sequences 1 through 56 alone do not exhibit high-affinity N binding. However, these data do not rule out the possibility of a minor contribution from the nucleotides 5′ of nucleotide 56. In addition, specific binding was demonstrated by using transcripts from the plasmid

F82D, which contains nucleotides 56 through 1149 from the 5′ end of genomic RNA (30). In contrast, plus-stranded sequences derived from the body of gene F (plasmid E1-500) or from the 5′ end of genomic RNA (plasmid F82N), both of which lacked the leader RNA sequences, did not show demonstrable specific binding by the N protein (Fig. 5).

We have previously shown that the 3′ end of leader RNA contains a weak potential hairpin loop involving nucleotides 52 through 76 (28). The predicted N binding site corresponds to sequences within this potential hairpin loop. Further, the demonstration of N in the RI complex and on the free leader-containing MHV-specific RNAs as well as the finding that free leader RNA binds N suggests that N may play a role in regulation of leader-primed transcription. We have no direct evidence that an N-leader RNA complex is required for subgenomic transcription other than the inhibition of transcription by anti-N but not anti-E1 or anti-E2 monoclonal antibodies (11; P. R. Brayton, unpublished data). In addition, the precise length of the free leader RNA that functions as primer is not known. However, we have suggested that it may contain more than 72 nucleotides, on the basis of homology with the negative-sense intergenic regions preceeding genes E, F, and G (28). We have also suggested that expression of the subgenomic mRNAs may be regulated by the extent of the homology between the extended 3′ leader sequences derived from the 5′ end of the genomic RNA and the intergenic regions (28). For example, the homology between leader RNA and the intergenic region of mRNA 7, the most abundant viral mRNA, covers 18 bases between nucleotides 63 and 81. These data suggest that such an RNA will have a functional N binding site. Therefore, a possible function for N in transcription may be to prevent base pairing at this potential hairpin loop and obviate the need to overcome the energy requirement to linearize the leader RNA before binding to the intergenic transcriptional start sites. An alternate possibility is suggested by the cytoplasmic small free leader-containing RNAs. These RNAs are believed to represent functional transcriptional intermediates (6) that may participate in the high-frequency RNA-RNA recombinational events described during coronavirus replication (22). N binding to leader transcripts of sufficient length to contain the specific binding site, i.e., longer than 72 nucleotides, may serve to target these RNA-protein complexes to the transcription complex through protein-protein interactions. This is supported by our finding that anti-N monoclonal antibody immunoprecipitates negative-stranded RNA from the membrane fraction (5), the site of RNA polymerase activity in infected cells (10, 11). Finally, our data cannot rule out the possibility that the binding of N and its subsequent self-assembly on the nascent strands may serve to prevent RNA-RNA base pairing after transcription.

We have tentatively localized the N-RNA binding site between nucleotides 56 and 65 in the leader RNA; therefore, it is not surprising that the subgenomic viral mRNAs are bound in ribonuclear protein complexes. Similar complexes involving mRNAs that are not packaged into virions have been identified during both vesicular stomatitis virus and La Crosse virus replication (4, 25, 27). These data suggest that additional *cis*-acting sequences must be present in the MHV genome to ensure efficient encapsidation of the genomic RNA. Alternatively, either a size constraint permitting only encapsidation of full-length genomic RNA or subcellular compartmentalization of genomic RNA may be required for proper packaging. The first two possibilities are supported by studies characterizing the defective interfering RNAs of MHV, which contain leader RNA sequences as well as internal deletions and rearrangements in the internal sequences (21). Only the RNA designated DIssA is packaged into virions, while the smaller defective interfering RNAs of MHV are not (21). This may be due either to the lack of additional packaging signals or to size constraints placed upon nucleocapsid formation requiring a full-length or nearly full-length genomic RNA. Interestingly, Rous sarcoma virus and Moloney murine leukemia virus appear to have at least two sites for encapsidation (23) which coordinate in providing genomic RNA with the proper specificity required for efficient packaging. In addition, while vesicular stomatitis virus mRNAs associate with its N protein in infected cells, only the genome-length RNA and leader RNA are encapsidated (3, 4, 16). The third possibility is suggested by the separation of the MHV-induced polymerases (11). The enzymatic activity synthesizing genomic-length RNA is associated with a population of cellular membranes that differ in density from those associated with the polymerase activity synthesizing virus-specific mRNA (11). These data suggest that encapsidation is a complex process which involves a variety of factors and that the presence of bound N protein may be insufficient to provide the appropriate packaging signal(s).

Several models are used to describe the structure of DNA protein interactions, such as the helix-turn-helix model exemplified by the lambda repressor and the zinc-binding fingers of the glucocorticoid receptor (1, 24). The manner in which proteins bind RNAs is less predictable, because of their less ordered secondary and tertiary structures. The complete gene sequence of the N protein has been determined (3, 29), and a search of the predicted protein sequence does not reveal obvious paradigms for nucleic acid binding. It is our current goal to rigorously define the nucleic acid binding including domains of the N protein by site-directed mutagenesis.

ACKNOWLEDGMENTS

We thank Michael M. C. Lai for helpful discussions and criticism and Carol Flores for preparation of the manuscript.

This work was supported by National Science Foundation grant DMB-87-17148, American Heart Association grant 40073, and U.S. Public Health Service grants AI 31386, AI 19244, NS 18146, NS 00796, and AI 23946 from the National Institutes of Health.

LITERATURE CITED

1. **Anderson, J. E., M. Ptashne, and S. Harrison.** 1985. A phage repressor-operator complex at 7A resolution. Nature (London) **316**:596–601.
2. **Armstrong, J., S. Smeekens, and P. Rottier.** 1983. Sequence of the nucleocapsid gene from murine coronavirus MHV-A59. Nucleic Acids Res. **11**:883–891.
3. **Arnheiter, H., N. L. Davis, G. Wertz, M. Schubert, and R. A. Lazzarini.** 1985. Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. Cell **41**:259–267.
4. **Banerjee, A.** 1987. Transcription and replication of rhabdoviruses. Microbiol. Rev. **51**:66–87.
5. **Baric, R. S., G. W. Nelson, J. O. Fleming, R. J. Deans, J. G. Keck, N. Casteel, and S. A. Stohlman.** 1988. Interactions between coronavirus nucleocapsid protein and viral RNAs: implications for viral transcription. J. Virol. **62**:4280–4287.
6. **Baric, R. S., C.-K. Sheih, S. A. Stohlman, and M. M. C. Lai.** 1987. Analysis of intracellular small RNAs of mouse hepatitis virus: evidence for discontinuous transcription. Virology **156**: 342–354.
7. **Baric, R. S., S. A. Stohlman, and M. M. C. Lai.** 1983. Characterization of replicative intermediate RNAs of mouse hepatitis virus: presence of leader RNA sequences on nascent chains. J.

Virol. **48**:633–640.
8. **Baric, R. S., S. A. Stohlman, M. K. Razavi, and M. M. C. Lai.** 1985. Characterization of the leader-related small RNAs in coronavirus-infected cells: further evidence for leader-primed mechanism of transcription. Virus Res. **3**:19–33.
9. **Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub.** 1980. The detection of DNA-binding proteins by protein blotting. Nucleic Acid Res. **8**:1–20.
10. **Brayton, P. R., M. M. C. Lai, C. D. Patton, and S. A. Stohlman.** 1982. Characterization of two RNA polymerase activities induced by mouse hepatitis virus. J. Virol. **42**:847–853.
11. **Brayton, P. R., S. A. Stohlman, and M. M. C. Lai.** 1984. Further characterization of mouse hepatitis virus RNA-dependent RNA polymerases. Virology **133**:197–201.
12. **Compton, J. R., D. B. Rogers, K. V. Holmes, D. Fertsch, J. Remenick, and J. J. McGowan.** 1987. In vitro replication of mouse hepatitis virus strain A59. J. Virol. **61**:1814–1820.
13. **Fleming, J. O., S. A. Stohlman, R. C. Harmon, M. M. C. Lai, and J. A. Frelinger.** 1983. Antigenic relationship of murine coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus. Virology **131**:296–307.
14. **Fried, M. G., and D. M. Crothers.** 1984. Equilibrium studies of the cyclic AMP receptor protein-DNA interaction. J. Mol. Biol. **172**:241–262.
15. **Gilmore, W., J. O. Fleming, S. A. Stohlman, and L. P. Weiner.** 1987. Characterization of the structural proteins of the murine coronavirus strain A59 using monoclonal antibodies. Proc. Soc. Exp. Biol. Med. **185**:177–186.
16. **Kurilla, M. G., and J. D. Keene.** 1983. The leader RNA of vesicular stomatitis virus is bound by a cellular protein reactive with anti-La lupus antibodies. Cell **34**:837–845.
17. **Lai, M. M. C., R. S. Baric, P. R. Brayton, and S. A. Stohlman.** 1984. Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic RNA virus. Proc. Natl. Acad. Sci. USA **81**:3626–3630.
18. **Lai, M. M. C., C. D. Patton, and S. A. Stohlman.** 1982. Further characterization of mRNAs of mouse hepatitis virus: presence of common 5′-end nucleotides. J. Virol. **41**:557–565.
19. **Lai, M. M. C., C. D. Patton, and S. A. Stohlman.** 1982. Replication of mouse hepatitis virus: negative-stranded RNA and replicative form RNA are of genomic length. J. Virol. **44**: 487–492.
20. **Lai, M. M. C., and S. A. Stohlman.** 1978. RNA of mouse hepatitis virus. J. Virol. **26**:236–242.
21. **Makino, S., N. Fujioka, and K. Fujiwara.** 1985. Structure of the intracellular defective viral RNAs of defective interfering particles of mouse hepatitis virus. J. Virol. **54**:329–339.
22. **Makino, S., S. A. Stohlman, and M. M. C. Lai.** 1986. Leader sequences of murine coronavirus mRNAs can be freely reassorted: evidence for the role of free leader in RNA transcription. Proc. Natl. Acad. Sci. USA **83**:4204–4208.
23. **Mann, R., and D. Baltimore.** 1985. Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. J. Virol. **54**:401–407.
24. **Miller, T., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domains in the protein transcription factor III A from Xenopus oocytes. EMBO J. **4**:1609–1614.
25. **Raju, R., and D. Kolakofsky.** 1987. Unusual transcripts in La Crosse virus-infected cells and the site of nucleocapsid assembly. J. Virol. **61**:667–672.
26. **Robbins, S. G., M. F. Frana, J. J. McGowan, J. F. Boyle, and K. V. Holmes.** 1986. RNA-binding proteins of MHV: detection of monomeric and multimeric N protein with an RNA overlay-protein blot assay. Virology **150**:402–410.
27. **Rosen, C. A., H. L. Ennis, and P. S. Cohen.** 1982. Translational control of vesicular stomatitis virus protein synthesis: isolation of an mRNA-sequestering particle. J. Virol. **44**:932–938.
28. **Shieh, C.-K., L. H. Soe, S. Makino, M.-F. Chang, S. A. Stohlman, and M. M. C. Lai.** 1987. The 5′-end sequences of murine coronavirus genome: implications for multiple fusion sites in leader-primed transcription. Virology **156**:321–330.
29. **Skinner, M. A., and S. G. Siddell.** 1983. Coronavirus JHM: nucleotide sequence of the mRNA that encodes nucleocapsid protein. Nucleic Acids Res. **11**:5045–5054.
30. **Soe, L. H., C.-K. Shieh, S. C. Baker, M.-F. Chang, and M. M. C. Lai.** 1987. Sequence and translation of the murine coronavirus 5′-end genomic RNA reveals the N-terminal structure of the putative RNA polymerase. J. Virol. **61**:3968–3976.
31. **Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeijst.** 1981. Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). Virology **108**:424–434.
32. **Stohlman, S. A., J. O. Fleming, C. D. Patton, and M. M. C. Lai.** 1983. Synthesis and subcellular localization of the murine coronavirus nucleocapsid protein. Virology **130**:527–532.
33. **Stohlman, S. A., and M. M. C. Lai.** 1979. Phosphoproteins of murine hepatitis viruses. J. Virol. **32**:672–675.
34. **Sturman, L. S., and K. V. Holmes.** 1983. The molecular biology of coronaviruses. Adv. Virus Res. **28**:35–112.
35. **Sturman, L. S., K. V. Holmes, and J. Behnke.** 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. J. Virol. **33**:449–462.