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RNA-Binding Proteins of Coronavirus MHV: Detection of Monomeric and Multimeric N Protein with an RNA Overlay-Protein Blot Assay

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RNA-binding proteins of coronavirus MHV-A59 were identified using an RNA overlay-protein blot assay (ROPBA). The major viral RNA-binding protein in virions and infected cells was the phosphorylated nucleocapsid protein N (50K). A new 140K virus structural protein was identified as a minor RNA-binding protein both in virions and in infected cells. The 140K protein was antigenically related to N, and upon reduction, yielded only 50K N. Thus, the 140K protein is probably a trimer of N subunits linked by intermolecular disulfide bonds. Several cellular RNA-binding proteins were also detected. RNA-binding of N was not nucleotide sequence specific. Single-stranded RNA of MHV, VSV, or cellular origin, a DNA probe of the MHV leader sequence, and double-stranded bovine rotavirus RNA could all bind to N. Binding of MHV RNA was optimal between pH 7 and 8, and the RNA could be eluted in 0.1 M NaCl. The ROPBA is a useful method for the initial identification of RNA-binding proteins, such as N and the 140K protein of murine coronavirus.

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INTRODUCTION

The coronavirus mouse hepatitis virus (MHV, strain A59) consists of a helical nucleocapsid surrounded by an envelope containing two viral glycoproteins (Sturman and Holmes, 1977; Sturman *et al.*, 1980). The nucleocapsid is composed of a 5.5×10^6 Da positive-stranded genomic RNA and a phosphorylated 50K nucleocapsid protein, N (Stohlman and Lai, 1979). The nucleotide sequence of the mRNA encoding N has been determined for MHV-A59 and MHV-JHM (Armstrong *et al.*, 1983a, 1983b; Skinner and Siddell, 1983). During transcription and replication, the structural protein N and several of the five nonstructural viral proteins (Skinner *et al.*, 1985; Skinner and Siddell, 1985) probably bind to the coronavirus RNA.

A sensitive nucleic acid overlay-protein blotting method (Bowen *et al.*, 1980) has been useful for the initial identification of both DNA-binding proteins (Blair and Honess, 1983; Braun *et al.*, 1984; Ichihashi *et al.*, 1984; Roberts *et al.*, 1985; Petit and Pilot, 1985) and RNA-binding proteins of Rous sarcoma virus (Bowen *et al.*, 1980; Meric *et al.*, 1984) and ribosomes (Roziere and Mache, 1984). We have adapted the RNA overlay-protein blot assay (ROPBA) to detect the virus-specific RNA-binding proteins in coronavirus infected cells and virions. We found that in virions, N was the major RNA-binding protein, but a minor protein of mol wt 140K, not previously reported, also bound RNA. The 140K protein appears to be a multimer of N molecules linked by disulfide bonds.

MATERIALS AND METHODS

Virus and cells. The A59 and JHM strains of MHV (MHV-A59 and MHV-JHM) and the San Juan strain of vesicular stomatitis

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virus (VSV) were propagated and plaque assayed in the 17 Clone 1 (17 Cl 1) line of spontaneously transformed BALB/c 3T3 cells as described previously (Sturman *et al.*, 1980). Released MHV-A59 was purified by ultracentrifugation on discontinuous and continuous sucrose gradients (Sturman *et al.*, 1980). Vesicular stomatitis virus (VSV, San Juan strain) was propagated in the same cells. Bovine rotavirus (BRV, Lincoln strain), kindly provided by Dr. Albert Kapikian (NIH), was propagated in MA104 cells obtained from Monroe Vincent (USUHS), which were cultured in Medium 199 with Hank's balanced salts (Whittaker MA Bioproducts) with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Preparation of endogenously labeled RNA. Cytoplasmic RNA from MHV-infected cells was labeled endogenously with inorganic phosphate as described previously (Baric *et al.*, 1983), with modifications. Confluent 17 Cl 1 cell monolayers were inoculated with MHV-A59 at a m.o.i. of 5–10 PFU per cell. At 1.5 hr postinoculation (p.i.) the inoculum was replaced with PO₄-free minimum essential medium with Earle's salts (Eagle, 1959), 12% Dulbecco's medium (Grand Island Biological Co., GIBCO), 1% fetal bovine serum (GIBCO), and 5 µg/ml actinomycin D-mannitol (Sigma Chemicals). At 2.75 hr p.i., [³²P]orthophosphate (ICN Pharmaceuticals) was added to a final concentration of 130–180 µCi/ml. At 9 hr p.i., each flask was washed with ice-cold PBS, and cells were detached with disposable plastic scrapers and pelleted.

Cytoplasmic RNA was purified using SDS (1%), proteinase K (200 µg/ml) and diethylpyrocarbonate (0.05%, DEPC) as described by Maniatis *et al.* (1982), but omitting RNasin. The precipitated RNA was pelleted at 16,300 *g* for 15 min, air- and vacuum-dried, and resuspended in double-distilled water. RNA was stored in aliquots at –70°, and its concentration and purity were determined by spectrophotometric analysis at 260 and 280 nm. Specific activities ranged from about 4 × 10³ to 4 × 10⁴ cpm/µg. RNA samples were analyzed by electrophoresis for approximately 22 hr in 1% Seakem LE agarose

(FMC Corp.) horizontal slab gels (Zeevi *et al.*, 1981), using 6 *M* formaldehyde in both gel and sample buffers and 0.1% DEPC in the running buffer. Nick-translated, denatured [³²P]dCTP-labeled λ phage DNA digested with *Hind*III (Bethesda Research Laboratories) was used as a molecular weight standard. Intracellular virus-specific RNA was prepared similarly from VSV-infected cells harvested at 8.5 hr p.i. and BRV-infected cells at 8–9 hr p.i.

Preparation of radiolabeled DNA and RNA probes. A 22-nucleotide cDNA probe complementary to the MHV leader (5'-AATGTTTGGATTAGATTTAAAC-3') and HSV-1 DNA and calf thymus DNA were 5'-end labeled with [³²P]ATP with T4 polynucleotide kinase (Bethesda Research Laboratories; Maxam and Gilbert, 1980). Purified, double-stranded RNA genome segments from bovine rotavirus were 3'-end labeled with ³²P using T4 polynucleotide ligase as described previously (England and Uhlenbeck, 1978).

Preparation of cell extracts. Cytoplasmic extracts of 17 Cl 1 cells were prepared by washing mock-inoculated or MHV-A59-infected flasks with ice-cold PBS containing 0.01% phenylmethylsulfonyl fluoride (PMSF, Sigma), and then incubating for 10 min on wet ice with RIPA buffer (Okuno *et al.*, 1983), with 1 mM EDTA and 1% (v/v) NP-40 but without methionine and Triton, and containing the following protease inhibitors: 0.01% PMSF, 0.1 mg/ml soybean trypsin inhibitor (GIBCO), and 1% (v/v) aprotinin (Sigma). Floating cells were washed twice in PBS, pelleted, and added to the rest of the cell extract. After 15 min on ice, cell extracts were vortexed and insoluble material was removed by centrifugation. Cell extracts were stored at –70°. Protein concentrations were determined by the methods of Bradford (1976) or Lowry *et al.* (1951).

Gel electrophoresis and electroblotting. Cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8, 10, 12%, or 5 to 15% gradient slab gels (Laemmli, 1970). Gels were electrophoresed at 150 V for 2 to 3 hr. Proteins were electroblotted using a Transblot apparatus (Bio-Rad) from gels

onto nitrocellulose paper (0.45 μ m pore size; Schleicher and Schuell) at 30 V for 16 to 20 hr or at 55 V for 4 hr, with cooling (Towbin *et al.*, 1979; Bowen *et al.*, 1980). The transfer buffer consisted of 25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol. Blots were stained for protein using amido black (Towbin *et al.*, 1979). Blots which had been immunoblotted, dried, and stored at 25°, could be rehydrated in transfer buffer and used for ROPBA. Blots could be stored for as long as 3 weeks in transfer buffer at 4° prior to reaction with probes.

RNA overlay-protein blot assay. For reaction with labeled nucleic acid probes, blots were treated as described by Bowen *et al.* (1980) except that they were not soaked in urea, and reactions and washes were done on a rocker platform in small polystyrene trays (reservoir inserts; Dynatech Labs, Inc.). Briefly, blots were rinsed with standard binding buffer (SBB; 0.05 M NaCl, 1 mM disodium EDTA, 10 mM Tris-HCl, pH 7, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone (PVP-40)) for 30 min, incubated with 10^4 to 1.5×10^5 cpm/ml [32 P]RNA probe for 1 hr at room temperature, washed 3 times with SBB for 15 min each, then air-dried and autoradiographed against intensifying screens. Conditions were varied as described in the text.

Preparation of antisera. Rabbit antisera to purified MHV virions and to isolated E2 were prepared as previously described (Sturman *et al.*, 1980). Monospecific anti-N antibody was prepared similarly by immunizing rabbits with N protein extracted from an SDS gel of purified virus. Antiserum to the E1 glycoprotein was prepared by immunization of rabbits with a synthetic peptide to the carboxy terminus of E1 from MHV-A59 (prepared by Dr. Chris Richardson, NIH), coupled to keyhole limpet hemocyanin.

Mouse antibody to the N protein was raised in female BALB/c mice (Jackson Laboratories) as described previously (Russell *et al.*, 1970). Mice were inoculated with N protein eluted from a band of an SDS gel and were inoculated with complete Freund's adjuvant on Day 3. Ascites fluid was prepared by intraperitoneal inocula-

tion of fresh sarcoma 180 cells (kindly provided by Dr. Walter Brandt, Walter Reed Army Institute for Research, Washington, D. C.). Antibody specificities were determined by using immunoblot analysis on proteins of MHV virions and MHV infected or control cell extracts.

Immunoblot analysis. Viral proteins on nitrocellulose blots were identified by immunoblotting (Towbin *et al.*, 1979). Antisera and 125 I-staphylococcal protein A (SPA; Pharmacia) were diluted in 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, and 0.1% bovine serum albumin. Wash buffer was 150 mM NaCl with 0.05% Tween 20. Bound antibody was detected with 10^6 cpm/ml of 125 I-SPA. Radioiodinated SPA was prepared by the chloramine T method (Tsu and Herzenberg, 1980). Radiolabeled viral protein bands were detected by autoradiography with intensifying screens. Alternative development systems were horseradish peroxidase coupled anti-immunoglobulin (Kirkegaard and Perry Laboratories), followed by reaction with 3,3'-diaminobenzidine (DAB; Sigma), by the method of Partanen *et al.* (1983), except using 100 mM Tris-HCl, pH 7.4, or with 4-chloro-1-naphthol (Sigma) as described by Hawkes *et al.* (1982).

RESULTS

Electrophoresis of cytoplasmic MHV RNA. 32 P-labeled RNA from coronavirus-infected cells was used as a probe to detect RNA-binding proteins from purified virus. The probe was a mixture of genomic RNA and six subgenomic mRNAs which form a nested set with common 3' ends (Fig. 1; Robb and Bond, 1979; Spaan *et al.*, 1981; Wege *et al.*, 1981). By densitometric scan, 52% of the labeled RNA was found to be of genome size (band 1; 5.5×10^6). The molecular masses in megadaltons of the mRNAs were as follows: band 2, 3.41; band 3, 2.71; band 4, 1.16; band 5, 1.03; band 6, 0.79; band 7, 0.65. The band at the top of the gel was at the origin and probably consisted of RNA aggregates. The 32 P-labeled MHV-A59 RNA and cytoplasmic RNA from uninfected cells were degraded by RNase and were therefore single-stranded and free of protein (data not shown).

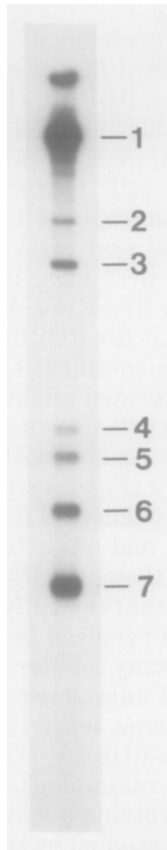


FIG. 1. Agarose gel electrophoresis of ^{32}P -labeled MHV cytoplasmic RNA. Cytoplasmic RNA was endogenously labeled with inorganic phosphate and purified from MHV-A59-infected 17 Cl 1 cells, as described in the text. The RNA sample loaded onto the gel was 7.25×10^8 cpm.

Identification of RNA-binding proteins in virions. RNA-binding proteins from purified virions were identified with the RNA overlay-protein blot assay using cytoplasmic ^{32}P -MHV RNA as the probe (Fig. 2). The major RNA-binding protein in MHV virions was the 50K nucleocapsid protein, N. A previously undetected 140K viral protein (Fig. 2) was also identified as an RNA-binding protein. It comprised approximately 3.4% of the total virion protein as estimated by densitometric scans of an immunoblot (lane A) and a Coomassie blue-stained gel. Preliminary studies suggest that the 140K protein bound RNA as effectively as did the N protein on a weight-by-weight basis. There was no detectable

binding of RNA by either form of the E2 glycoprotein, which forms the viral spikes. Glycosylated and unglycosylated forms of E1, the membrane glycoprotein, bound only trace amounts of RNA. ^{32}P -labeled RNA from VSV-infected cells also bound to the N and 140K proteins of MHV with high efficiency (Fig. 2, lane C), indicating that the observed RNA-binding was not nucleotide sequence specific.

Characterization of the 140K protein. The 140K protein was detected by immunoblotting purified virion proteins isolated from 17 Cl 1 cells, with monospecific antiserum against N protein, but not with monospecific antisera against the other viral proteins E1 and E2 (Fig. 3). Anti-E1 detected both monomeric and dimeric E1 (Fig. 3, lane D). A broad band with an average mol wt of 73K occasionally seen in heavily

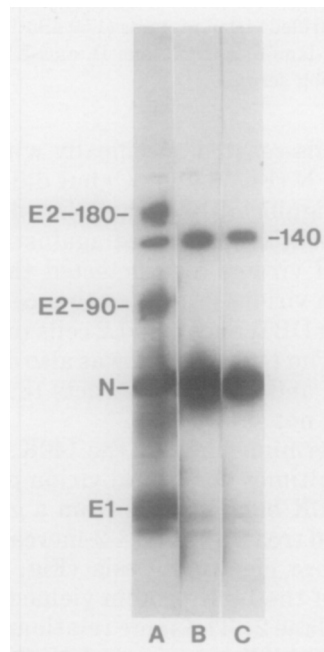


FIG. 2. RNA-binding proteins of purified MHV-A59 virions detected by the ROPBA. Gradient-purified virion proteins ($35 \mu\text{g}$) were resolved on a 5–15% SDS gel and transferred to nitrocellulose. The protein blot was cut into strips and individual strips were reacted with the following probes: lane A, virus-specific rabbit antiserum followed by ^{125}I -staphylococcal protein A; lane B, ^{32}P -labeled MHV-A59 cytoplasmic RNA (1.5×10^6 cpm/ml); lane C, ^{32}P -labeled VSV cytoplasmic RNA (1.3×10^6 cpm/ml).

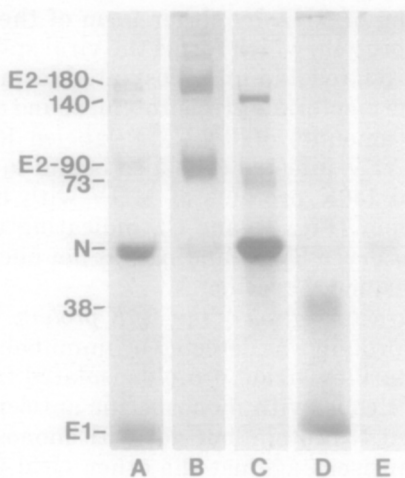


FIG. 3. The 140K protein shares common antigens with the N protein. Purified MHV-A59 (10 μ g) was electrophoresed on an 8% gel in each of the five lanes shown and electroblotted to nitrocellulose. Viral proteins stained with amido black are shown in lane A. Other lanes were probed with monospecific rabbit antisera to purified virion proteins (1:50 dilution). Lane B, anti-E2; lane C, anti-N; lane D, anti-E1; lane E, normal rabbit serum.

loaded gels reacted specifically with antiserum to N (Fig. 3, lane C) but did not associate significantly with RNA (data not shown). Antiserum raised against NP-40-disrupted virions also detected the 140K protein in virions grown in other cell types, including DBT, Sac-, and L2 cells (data not shown). The 140K protein was also detected in 17 Cl 1 and DBT cell extracts 18 to 24 hr p.i. (data not shown).

To determine whether the 140K protein was a multimer of N, total virion proteins of the 140K band excised from a gel were boiled and treated with 5% 2-mercaptoethanol before electrophoresis (Fig. 4). Reduction of the 140K protein yielded only N (Fig. 4B, lane 2). The same relationship between the 140K protein and N was demonstrated by reacting them with 32 P-labeled cytoplasmic RNA from MHV-infected cells (data not shown). Thus, the 140K protein appears to be a multimeric molecule composed of three N monomers held together by intermolecular sulfhydryl bonds.

Detection of RNA-binding proteins in mock-infected and MHV-infected cells. The

RNA-binding proteins of mock-infected and MHV-infected 17 Cl 1 cells were identified using the ROPBA (Fig. 5). N was readily detectable as an RNA-binding protein at 8 hr p.i. in MHV-A59- and MHV-JHM-infected cell extracts (data not shown). The electrophoretic mobility of N from MHV-A59 and MHV-JHM cell extracts differed by 3 kDa. In cell extracts prepared at 15 hr (MHV-A59) and 13 hr (MHV-JHM) p.i., the ROPBA detected two additional virus-specific RNA-binding proteins which migrated slightly ahead of N (Fig. 5B). These proteins, designated N' and N'' because they reacted with monospecific anti-N antibody (Fig. 5A), are believed to be cleavage products of N (Cheley and Anderson, 1981), and may be degradation products. That they have RNA-binding activity suggests that their RNA-binding domains were not removed by cleavage.

There were many cellular proteins in the protein blots of uninfected 17 Cl 1 cell extracts. Of these, at least 12 bound MHV-A59 RNA in the ROPBA (Fig. 5B). Cellular RNA-binding proteins, including ribosomal proteins and proteins involved in translation, have been studied by the ROPBA and

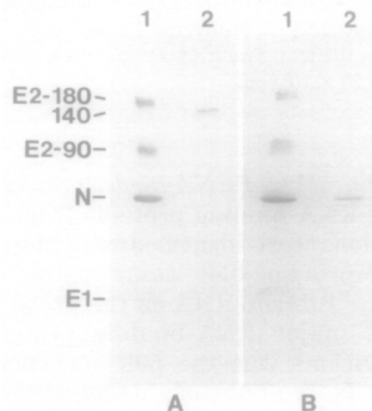


FIG. 4. The 140K protein is a multimer of N. Protein samples were run on a 5-15% SDS-polyacrylamide gradient gel after the usual 37° treatment for 30 min (A) or after boiling 5 min, followed by reduction with 5% 2-mercaptoethanol (B). After electroblotting, the blot was reacted with virion-specific antiserum (1:100 dilution), and developed with horseradish peroxidase and 4-chloro-1-naphthol. Lanes 1, purified MHV-A59 virus (2.8 μ g); lanes 2, gel-purified 140K protein.

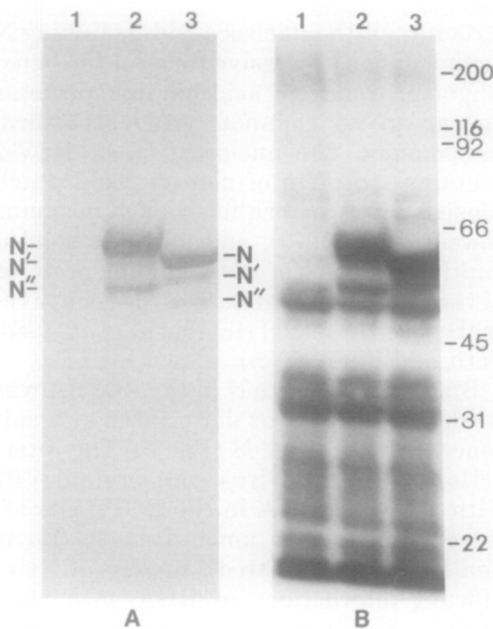


FIG. 5. N species are RNA binding proteins in MHV-A59- and MHV-JHM-infected cells. Protein samples (75 μ g) were electrophoresed on a 10% gel and electroblotted. (A) Immunoblot after probing with N-specific mouse ascites fluid and reacting with horseradish peroxidase and diaminobenzidine. (B) ROPBA using 32 P-labeled MHV-A59 RNA (2.5×10^4 cpm/ml). Lanes 1, mock-infected cell extract; lanes 2, 13-hr MHV-JHM-infected cell extract; lanes 3, 15-hr MHV-A59-infected cell extract. The molecular weights of marker proteins are shown on the right, and are in kilodalton units.

other methods (Rozier and Mache, 1984; Gourse *et al.*, 1981; Shatkin *et al.*, 1982).

Interaction of MHV RNA-binding proteins with nucleic acids. The affinity of viral RNA for proteins in infected cell extracts was studied by reacting separate strips of a protein blot with RNA in buffers containing different sodium chloride concentrations and washing in the corresponding buffer (Fig. 6). Increasing the salt concentration above 0.075 M reduced RNA binding and very little binding was detected above 0.1 M NaCl. Subsequent amido black staining of the same blot showed that proteins were not dissociated from the paper at this ionic strength. RNA binding was also pH dependent. It diminished above pH 8 and background increased below pH 7 (not shown).

To examine the nucleotide sequence specificity of the RNA-protein interaction, mock-infected and MHV-A59-infected 17 Cl 1 cell extracts were electroblotted from SDS gels and probed with several different 32 P-labeled nucleic acids (Fig. 7). Probes were equalized with respect to counts per minute per milliliter. In 15-hr MHV-A59 infected cell extracts, similar bands of RNA-binding proteins were detected using as probes MHV-A59 RNA, cellular RNA, MHV leader cDNA, and ds BRV RNA. N bound all of the probes tested. Longer exposure of Fig. 7C yielded the same pattern of viral and cellular RNA-binding proteins seen with the other probes. N also bound 32 P-5'-end labeled HSV-1 and calf thymus DNAs (not shown). These results showed that, in the ROPBA, N protein bound nucleic acids in a manner which was not specific for nucleotide sequence.

DISCUSSION

We have identified RNA-binding proteins of a coronavirus using a sensitive and direct method, the RNA overlay-protein blot assay (Bowen *et al.*, 1980). In MHV virions, the N protein was found to be the

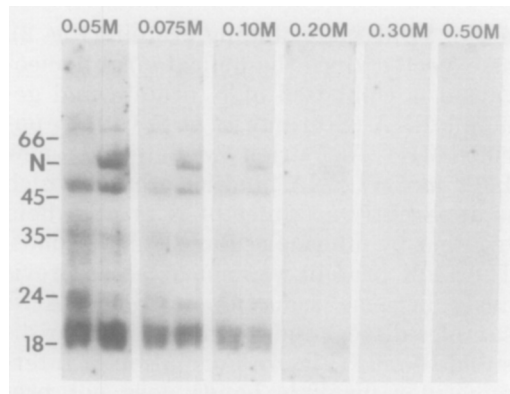


FIG. 6. Effect of salt concentration on RNA binding. Proteins from cell extracts were electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted. The blot was cut into strips and each strip was washed and reacted in an ROPBA with 32 P-labeled MHV RNA (4.4×10^8 cpm/ml) in buffers of the different NaCl concentrations indicated in the figure. Mock-infected cell extract (130 μ g) was run in the left lane of each panel; MHV-A59-infected cell extract (140 μ g), harvested at 16 hr p.i., was run in the right lane. Molecular weight markers are indicated at the left.

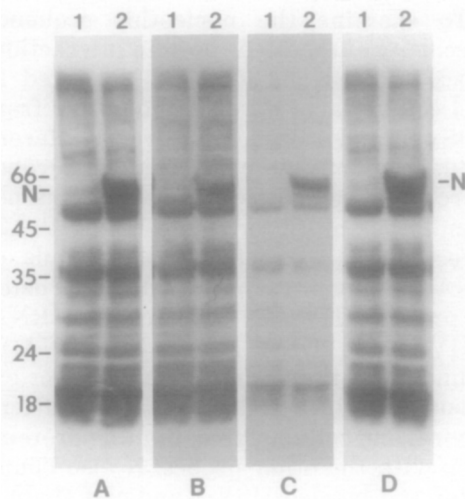


FIG. 7. Binding of intracellular proteins by different nucleic acid probes. Proteins from cell extracts (75 μ g) were electrophoresed on a 12% SDS-polyacrylamide gel and electroblotted. Blot strips were reacted in RNA or DNA overlay-protein blot assays with the following 32 P-labeled probes, all at 10^4 cpm/ml: (A) MHV-A59 cytoplasmic RNA; (B) 17 Cl 1 cellular RNA; (C) 22-nucleotide cDNA from the MHV leader, 5'-end labeled with 32 P; (D) double-stranded genome RNA from purified bovine rotavirus, 3'-end labeled with 32 P-pCp. Lanes 1, mock-infected cell extract; lanes 2, A59-infected cell extract, 15 hr p.i.

major viral RNA-binding protein (Fig. 2), as expected since the helical viral nucleocapsid is composed of N protein and genomic RNA (Sturman *et al.*, 1980). In purified MHV-A59 virions we detected a new 140K mol wt RNA-binding protein which is apparently a trimer of N protein held together by intermolecular disulfide bonds. The 140K protein was not detected previously because reduction of SDS-PAGE samples dissociated its intermolecular disulfide bonds (Fig. 4). Formation of intermolecular disulfide bonds does not prevent the N subunits of 140K from binding to RNA.

A trimeric assembly of N protein may be a general feature of coronaviruses. In bovine coronavirions a 160K disulfide-linked protein which reacts with antiserum to the nucleocapsid protein has recently been detected (Hogue *et al.*, 1984). Oligomerization of some molecules of N is possibly associated with nucleocapsid or virion assembly, perhaps stabilizing the helical

structure of the nucleocapsid by linking N monomers on successive turns of the helix. Oligomeric nucleic acid-binding proteins appear to be common virus structural components. The nucleocapsid of HSV-2 contains a protein of mol wt 350K which consists of two disulfide-linked proteins (Zweig *et al.*, 1979), and disulfide-bonded multimeric forms of DNA-binding proteins of vaccinia and hepatitis B viruses have recently been detected (Ichihashi *et al.*, 1984; Petit and Pillot, 1985).

Binding of RNA to N in the ROPBA was weak since RNA was dissociated at a salt concentration of 0.1 M (Fig. 6). The interaction of the rotavirus core protein VP2 with rotavirus RNA in the ROPBA is affected similarly by ionic strength (Boyle and Holmes, submitted). Biologically significant interactions of RNA with viral proteins can occur at low ionic strength. For example, *in vitro* assembly of tobacco mosaic virus (TMV) RNA and protein is routinely done at 0.1 M ionic strength, pH 7 (Butler, 1984).

The binding of RNA to N detected by the ROPBA was not nucleotide sequence specific, since several different types of nucleic acids bound to N. The biological significance of this weak, nonsequence specific RNA-binding is unclear. Partial denaturation of N during SDS-PAGE and electroblotting may have favored nonsequence specific binding. Alternatively, lack of nucleotide sequence specificity in N binding may reflect a property intrinsic to proteins which form helical nucleocapsids encasing the entire genome. During assembly of some viruses with helical nucleocapsids, such as VSV (Blumberg *et al.*, 1983) and TMV (Butler, 1984), a specific nucleation site on the viral RNA is required to bind the first protein molecules, but binding of subsequent protein monomers to the remainder of the genomic RNA is not nucleotide sequence specific. Sequence specific RNA-binding of N might be identified using short RNA probes for specific regions of the coronavirus genome in competitive binding studies using the ROPBA or filter binding assays (Riggs *et al.*, 1970).

The RNA-binding domains of N and the 140K trimer of N are probably in one or more of the five basic regions of the N pro-

tein (Armstrong *et al.*, 1983a, 1983b; Skinner and Siddell, 1983). In other nucleic acid-binding proteins, phosphorylation regulates nucleic acid-binding (Wilcox *et al.*, 1980; DeBenedetti and Baglioni, 1984; Leis and Johnson, 1984). In MHV N protein, a serine-rich region (ser 194-ser 220), which could be a site for phosphorylation, may be involved in RNA binding. Using a solid phase binding assay with synthetic peptides of N protein domains, it should now be possible to identify the RNA-binding domain(s) of the N protein.

The ROPBA has proved to be a useful approach for initial identification of the major coronavirus RNA-binding proteins. Additionally, this assay has been used to detect nonstructural RNA-binding proteins in extracts of rotavirus-infected cells (Boyle and Holmes, submitted). Coronavirus nonstructural proteins, which are present in low amounts in infected cells, were not detected with the ROPBA, possibly because they comigrate with cellular RNA-binding proteins. Two-dimensional electrophoresis may help to detect RNA-binding activity of coronavirus nonstructural proteins.

Note added in proof. While this manuscript was in preparation, the ROPBA was used to identify an RNA-binding protein of Berne virus, a member of the *Toviridae* (Horzinek *et al.*, 1985).

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