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The Infectious Bronchitis Virus Nucleocapsid Protein Binds RNA Sequences in the 3′ Terminus of the Genome

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The infectious bronchitis virus (IBV) nucleocapsid protein was expressed as a bacterial fusion protein which differed from the native protein only in the addition of six amino terminus histidine residues. Using RNA overlay protein blot assays, the recombinant protein was shown to bind to RNA fragments specific for the positive sense 3′ noncoding end of the IBV genome. At greater concentrations of sodium chloride, the native and fusion nucleocapsid proteins similarly bound to G RNA, representing the terminal 1805 3′ nt of the genome, whereas bovine serum albumin and allantoic fluid protein did not bind to labeled G RNA. Competitive gel shift assays with labeled G RNA indicated that the protein interacted with several unlabeled RNA representing sequences at the 3′ noncoding end of the IBV genome. Cache Valley virus (a bunyavirus) mRNA transcribed from the small segment cDNA also inhibited the interaction with IBV G RNA to approximately the same extent as homologous unlabeled G RNA, whereas reactions with bovine liver RNA and yeast tRNA were considerably weaker. Whereas yeast tRNA did not inhibit the interaction with the labeled large G RNA, interactions of the fusion protein with EF, a region from 78 to 217 nt from the 3′ terminus of the IBV genome, were also apparently weaker than interactions with fragment CD which consisted of the 3′ terminal 155 nt. On a molar basis, the latter interacted in an identical nature to a RNA consisting of CD and an additional 1053 nt of plasmid sequences. Compared to bovine liver RNA, unlabeled G specifically inhibited binding to the two smaller labeled IBV fragments in gel shift assays. The binding of IBV nucleocapsid protein with RNA probably requires specific sequences and/or structures that are present on the genome, and may represent a common mechanism used by similar viral nucleoproteins whose functions depend on binding to RNA.

INTRODUCTION

The nucleocapsid protein of the avian coronavirus, infectious bronchitis virus (IBV), is a highly basic, phosphorylated structural protein of 409 residues or about 50 kDa (Boursnell *et al.,* 1985; Williams *et al.,* 1992). Coronavirus nucleocapsid sequences vary among major antigenic groups but are highly conserved within these groups (Williams *et al.,* 1992). The amino acid sequences of various IBV strains are greater than 94% conserved. In addition, the proteins are more conserved in the central region than at the ends. A highly basic region in the IBV protein between residues 238 and 293 is identical among the strains that have been examined (Williams *et al.,* 1992). The corresponding region of mouse hepatitis virus (MHV) has been shown to bind to RNA (Masters, 1992; Nelson and Stohlman, 1993).

In addition to being closely associated with the RNA genome, the coronavirus nucleocapsid protein has been shown to be involved in transcription and possibly in translation (Robbins *et al.,* 1986; Compton *et al.,* 1987; Stohlman *et al.,* 1988; Baric *et al.,* 1988; Masters, 1992). The MHV nucleocapsid protein specifically binds to small leader-containing RNAs, and within the cytosol of MHV-infected cells it can interact with membrane-bound small leader RNA in transcription complexes (Stohlman *et al.,* 1988). Anti-nucleocapsid monoclonal antibodies reportedly precipitate both full-length and subgenomic mRNA, as well as replicative intermediate RNA (Baric *et al.,* 1988). The amount of protein found associated with the genome and the putative functions of this protein suggested that it could readily associate with additional regions of coronavirus RNA.

Because transcription of the negative template must initiate at the 3′ end of genomic RNA and because nucleocapsid protein may be involved, it is likely that the 3′ noncoding region will also readily associate with this protein. A relatively A-U-rich hypervariable region (HVR) with an as yet undefined function can be found in the 3′ noncoding region of nearly all IBV strains (Williams *et al.,* 1993). This study examined the association of a recombinant IBV nucleocapsid protein with the 3′ end of the IBV genome, representing the terminal 1805 nt, and overlapping 151- to 256-nt fragments representing the noncoding region. Enriched nucleocapsid recombinant protein, obtained with an *Escherichia coli* expression vector were used in competitive binding gel shift assays

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that indicated the nucleocapsid protein specifically interacts with several regions of the 3′ noncoding terminus of the IBV genome.

MATERIALS AND METHODS

Cloning into the pQE8 expression vector

The nucleocapsid gene of the IBV Gray strain was excised from a pCR1000 recombinant plasmid (Williams *et al.,* 1992) using *Bam*HI and ligated into the pQE8 expression vector with T4 DNA ligase (Promega, Madison, WI). M15/pRep4 cells were then transfected with the plasmid construct by electroporation at 12.5 kV/cm for 5 msec (Sambrook *et al.,* 1989; BTX, San Diego, CA; Qiagen, Chatsworth, CA). The IBV cDNA inserts were then sequenced using Sequenase kit (USB, Cleveland, OH) to determine the orientation and to confirm the fidelity of the nucleocapsid sequence.

Expression and enrichment of the recombinant nucleocapsid gene

Single colonies containing the pQE8 with nucleocapsid cDNA insert were grown in LB media with ampicillin and kanamycin according to the Qiagen manual and protein expression was induced with IPTG (Qiagen, Chatsworth, CA). The fusion protein was expressed in cells collected from 1 ml of the culture fluid, and analyzed by SDS–PAGE following Coomassie blue staining, while IBV specificity of the fusion protein was determined by Western blot analysis using primary chicken anti-IBV and secondary goat anti-chicken antibodies (Sneed *et al.,* 1989; Parr and Collisson, 1993).

For cytosolic localization, the fusion protein was induced in 100-ml cell cultures as described in the Qiagen manual (Qiagen, Chatsworth, CA). Immediately prior to and following induction of protein, 1-ml samples were taken from the cultures and stored in lysing buffer before separating the proteins by SDS–PAGE. The cells from induced cultures were harvested by centrifugation at 4000 $\times$ *g* for 10 min and the pellet was resuspended in 5 ml sonication buffer (50 m*M* NaH_2PO_4, pH 8, 300 m*M* NaCl), supplemented with 1 mg/ml lysozyme, and kept on ice for 5 min before processing for cytosolic localization.

Cells from a 1-liter culture were induced and pelleted as described above and resuspended in 6 ml of sonication buffer supplemented with 1 mg/ml lysozyme (Qiagen manual, Qiagen, Chatsworth, CA). The sample was incubated for 5 min on ice before adding 0.66 ml 3 *M* NaCl and incubating on ice for another 5 min. The cells were sonicated for 5 min on ice and centrifuged at 10,000 *g* for 30 min. The supernatant was collected and loaded onto an 8-ml Ni^{2+}-NTA column (Qiagen, Chatsworth, CA) preequilibrated with sonication buffer. The column was washed with 10 vol of sonication buffer supplemented with 20 m*M* imidazole to reduce the background of cellular proteins. The recombinant nucleocapsid protein was eluted with sonication buffer, pH 7, supplemented with 250 m*M* imidazole. Two-milliliter fractions were collected from the column, and fractions containing the recombinant protein were concentrated using an Amicon centriprep 10 concentrator (Amicon, Beverly, MA). Western blot assays were used to compare native nucleocapsid protein prepared from purified Gray strain IBV grown in embryonating chicken eggs (Sneed *et al.,* 1989) with the fusion protein.

Source of RNA

The *in vitro* transcripts were produced from the pCR1000 and the pGEM3Z vectors with the T7 promoter (Promega, Madison, WI). Inserts specific for selected fragments of the 3′ end of the Gray genome were obtained by cDNA cloning of PCR products (produced using Taq polymerase from Promega, Madison, WI) that corresponded to the sequences of interest. In addition, *Bam*HI or *Hin*dIII sites were inserted downstream of the transcript with the amplifying primers. The resulting PCR products were cloned into the pCR1000 vector through the A overhang resulting from the polymerase reaction or into the pGEM3Z vector following restriction enzyme digestion (Sambrook *et al.,* 1989). Electroporation (described above) was used to transform JM109 cells (Stratagene, La Jolla, CA) with the constructed plasmids.

Template plasmids containing specific IBV sequences were linearized by digesting with *Bam*HI before extracting with phenol/chloroform and precipitating with ethanol (Sambrook *et al.,* 1989). Transcription was carried out as described in the Promega Protocol and Applications Guide (Promega) with T7 RNA polymerase and the transcripts, terminated at the 3′-incorporated *Bam*HI or *Hin*dIII sites, were labeled by the addition of [α^{32}P]CTP (NEN, Boston, MA). Cache Valley virus (CVV) RNA, used as competitor, was produced by transcribing *Bam*HI-digested S segment cDNA that had been cloned into pGEM3Z (Chung, 1992). Transcripts with 1052 nt of plasmid RNA were also prepared following *Pst*I digestion of pCR1000 plasmids (Sambrook *et al.,* 1989). The RNA products were quantitated by comparing the ethidium bromide-stained samples with known standards of yeast tRNA (Promega). Bovine liver RNA (ribosomal RNA) used as a control was obtained from Sigma Chemical Co. (St. Louis, MO).

RNA overlay protein blot analysis (ROPBA)

RNA sequences binding to the nucleocapsid proteins (whole virus or synthetic preparations) and control proteins (bovine serum albumin or egg allantoic proteins) were detected by ROPBA (Robbins *et al.,* 1986; Sambrook *et al.,* 1989). Proteins (4.2 μg/well) were separated by SDS–PAGE on a 10% acrylamide gel and transferred to nitrocellulose. The nitrocellulose membrane blots were

washed for 30 min at room temperature in SBB buffer (0.05 to 0.3 *M* of NaCl, 10 m*M* Tris, pH 7, 1 m*M* EDTA, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinyl pyrrolidone). Strips of the blots were placed in petri dishes containing 4 ml SBB and 2×10^5 cpm of [α^{32}P]CTP-radiolabeled RNA (approximately 10 ng RNA). Bovine liver RNA was added to a final concentration of 20 μg/ml to remove nonspecific binding. After 1 hr at room temperature with constant agitation, strips were thoroughly washed with SBB and dried, and the bound RNA was then visualized by autoradiography. The integrated optical densities of the bands were quantitated with a BioImage Visage Image Analysis System with Sun Sparc 10 Workstation (Ann Arbor, MI).

Gel-shift analysis

Protein–RNA interactions were analyzed by a modified gel-shift assay (Chodosh, 1992). One ng of RNA and varying concentrations of nucleocapsid protein were coincubated for 20 min at room temperature in 10 μl of gel-shift buffer (25 m*M* Hepes, 25 m*M* EDTA, 150 m*M* NaCl, 5 m*M* DTT, 10% glycerol, and 20 units rRNasin, Boehringer Mannheim Corp., Indianapolis, IN). Following the addition of 1 μl 10× sample buffer, the reaction mixtures were loaded onto a 0.5% (larger RNA fragment) or 1% agarose gel (smaller fragments) and electrophoresed at 60 V in 1× Tris–borate–EDTA (Sambrook *et al.,* 1989). Gels were then dryed and autoradiographed. Competition assays were performed using a constant 3.0 μg of nucleocapsid protein and a mixture of 1 ng of [α^{32}P]CTP-radiolabeled RNA as indicated and varying concentrations of unlabeled homologous or heterologous competitor RNA.

RESULTS

Recombinant nucleocapsid protein

Nucleocapsid protein from the Gray strain of IBV was expressed in a pQE8 bacterial expression vector in order to obtain quantities of relatively enriched protein used for studying interactions with RNA. A protein band corresponding to the expected 50-kDa position was seen in the IPTG-induced cultures by 3 hr and peaked at 5 hr (data not shown). Induced and uninduced samples from clone 68 obtained 5 hr following exposure to IPTG are shown in Fig. 1. The soluble cytoplasmic fraction was used as the source for the IBV nucleocapsid fusion protein.

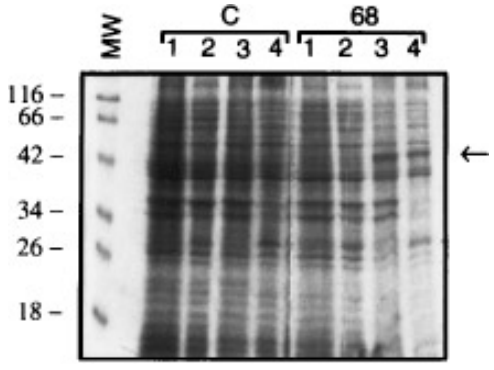

FIG. 1. Crude protein preparations of uninduced (lanes 1 and 2) and induced (lanes 3 and 4) cultures of clone 68 and a bacteria cell control without plasmid (C). The recombinant protein band is indicated by an arrow.

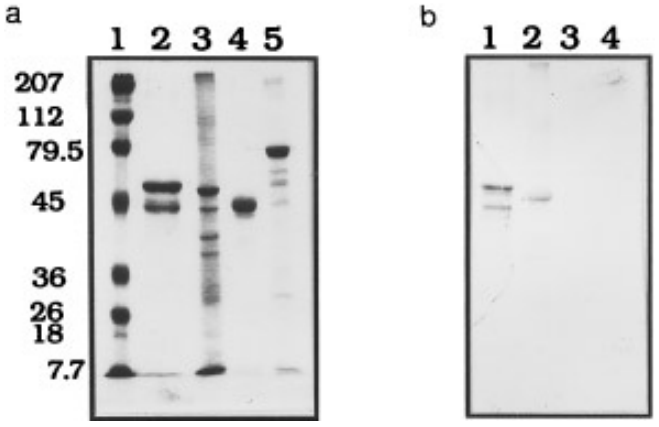

FIG. 2. Comparison of the purified, concentrated recombinant protein with the native protein from gradient purified virus by (a) SDS–PAGE and (b) Western blot. In a, lane 1 represents the marker protein standards; 2, recombinant protein; 3, native protein; 4, egg allantoic protein; and 5, bovine serum albumin. In b, lane 1 represents recombinant protein; 2, native protein; 3, egg allantoic protein; and 4, bovine serum albumin.

The fusion protein with tandem histidine residues at the amino terminus was enriched from the soluble cytoplasmic fraction by Ni^{2+}-NTA affinity chromatography. Figure 2 shows that in an SDS–polyacrylamide gel, the migration of recombinant nucleocapsid protein was similar to that of native nucleocapsid protein derived from IBV. Moreover, both recombinant and native proteins reacted with anti-IBV serum in a Western blot assay (Fig. 2). Anti-IBV antibody did not react with bacterial proteins. In addition to the whole nucleocapsid protein, smaller bands could sometimes be seen in the Western blot assay in the recombinant protein lane. The reaction of smaller polypeptides with the anti-IBV antisera suggested that they were produced by premature termination of translation. As the histidine tag is at the amino terminus of the protein, any such premature termination products would be absorbed onto the column and thus copurified with the full-length nucleocapsid protein (Qiagen Manual). Alternatively, they could have originated from proteolysis.

RNA binding to immobilized protein

The ROPBA was used to initially determine potential binding of the synthetic nucleocapsid protein to RNA corresponding to regions in the 1805 base 3′ terminus

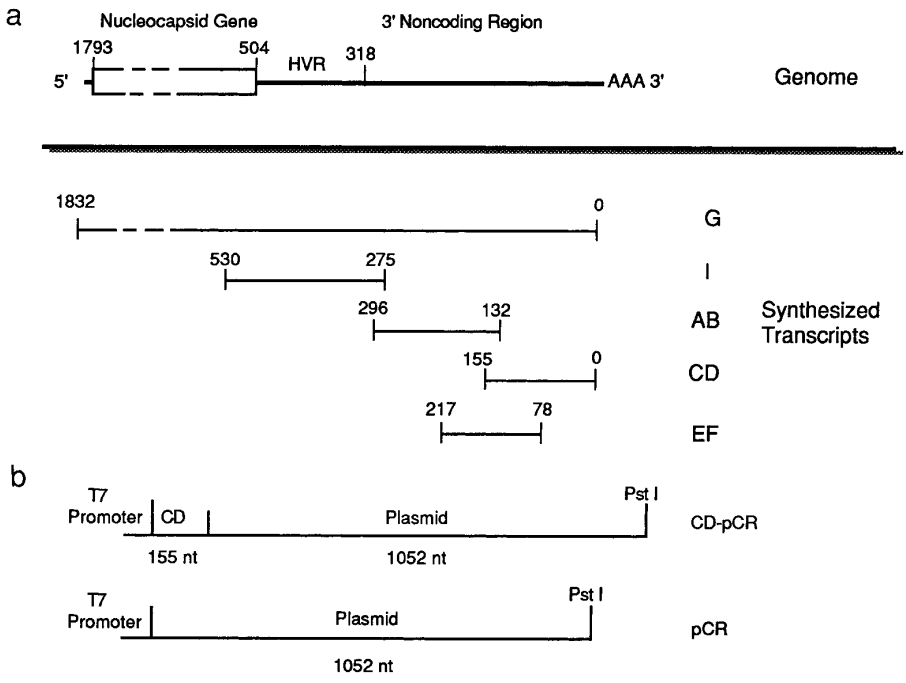

FIG. 3. (a) Diagram indicating the position of each RNA transcript (right of figure) on the Gray IBV genome. The numbers indicate the nt included in each transcript as numbered from the 3′ end of the genome. (b) DNA template for plasmid control RNA. pCR represents RNA transcribed from pCR1000 and CD-pCR, RNA transcribed from the pCR1000 with CD cloned into the *Hin*dIII site.

of the Gray IBV genome. Positive sense RNAs specific for the 3′ noncoding end of the genome were synthesized as targets for the synthetic nucleocapsid protein binding studies (Fig. 3). RNA G (1805 nt) was transcribed from the whole nucleocapsid gene, and RNA I represents the 3′ HVR (256 nt). The RNA fragments AB (165 nt), CD (155 nt), and EF (140 nt which partially overlapped with both AB and CD) represent conserved regions extending from the HVR to the 3′ terminus of the genome. Although G and CD included the 3′ terminus of the genome, neither included a poly(A) tail. A *Bam*HI site was used to terminate the cDNA template for all transcripts except CD in which an *Eco*RI site was used to linearize the plasmid.

Each IBV-specific RNA fragment was reacted with 4.2 μg of fusion nucleocapsid protein (Fig. 4). Using a 0.05

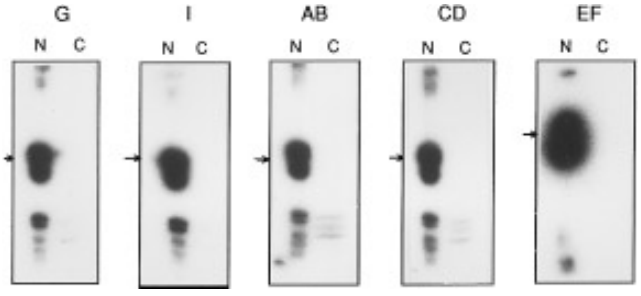

FIG. 4. RNA overlay protein blot analysis (ROPBA) with recombinant nucleocapsid protein binding to positive-sense RNA from the 3′ end of the IBV genome. Each panel shows the reaction with the indicated labeled transcript explained in Fig. 3. Lane N in each panel contains the synthetic nucleocapsid protein and lane C, the bacterial cell control.

M concentration of sodium chloride, all the radiolabeled IBV-specific RNA transcripts, including the large G transcript, and the smaller I, AB, CD, and EF, bound to the nucleocapsid protein. The intensity of the radioactive RNA signal was greatest at the position on the blot corresponding to full-length recombinant nucleocapsid protein.

ROPBA were repeated with the large G transcripts to examine the effects of sodium ion concentration on interactions of IBV RNA with recombinant and native nucleocapsid protein. Potential interactions with unrelated bovine serum albumin and egg allantoic proteins were included as controls. Autoradiographs of the blots and a graph of the integrated optical intensities of each band are shown in Fig. 5. Labeled G interacted similarly with both recombinant and native nucleocapsid protein at all concentrations used, whereas no interaction was detected with either bovine serum albumin or egg allantoic proteins used as protein controls. Because the decrease in binding of both recombinant and native proteins was more dose dependent between 0.05 *M* and 0.1 *M* and the recombinant protein bound with somewhat less efficiency at 0.3 *M*, 0.15 *M* sodium chloride concentration was used in additional experiments evaluating protein–RNA interactions.

Gel shift analyses

Gel shift assays were used to further examine protein–RNA interactions to determine relative shifts in mobility of the various species of RNA, including transcripts corre-

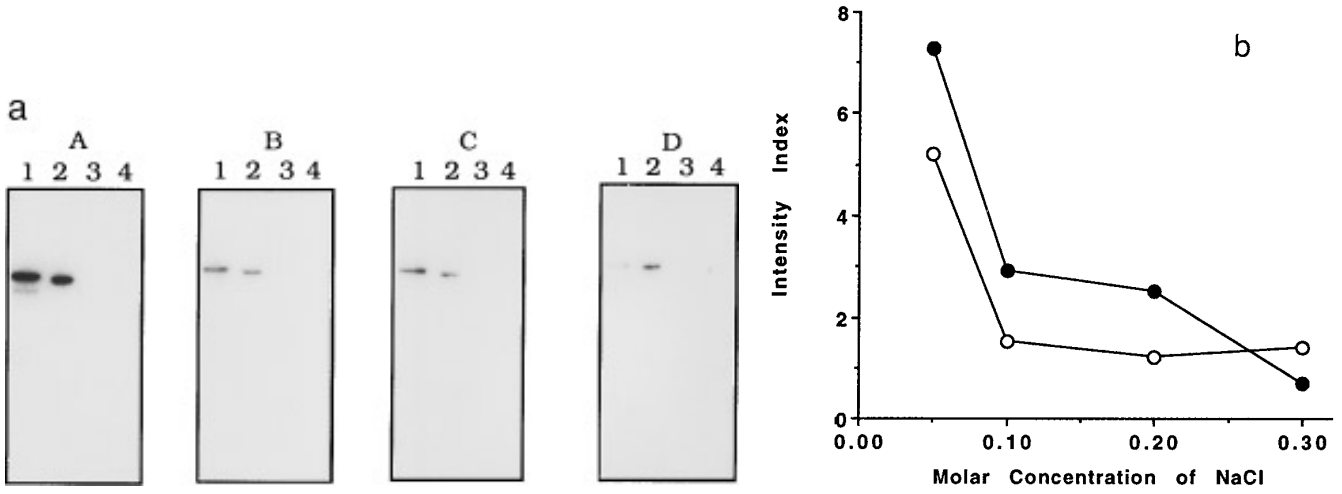

FIG. 5. The effects of sodium chloride concentrations on radiolabeled G RNA interactions with IBV nucleocapsid proteins. (a) The autoradiograph of the ROPBA in which lane 1 represents the recombinant protein; lane 2, the native proteins; lanes 3 and 4, egg allantoic proteins and bovine serum albumin, respectively. Panel A represents 0.05 *M* of sodium chloride; B, 0.1 *M*; C, 0.2 *M*; and D, 0.3 *M*. (b) Integrated optical intensities from the autoradiographs.

sponding to the 3′ terminus of the IBV genome. Interactions between G and the fusion protein could be identified by the decrease in mobility of the radiolabeled RNA probe (Fig. 6a). No shift in mobility could be observed when labeled RNA probe was mixed with bovine serum albumin. The effects of protein concentration on formation of G RNA–protein complexes on gel shift assays were first determined in the absence of competing RNA (Fig. 6b). The mobility decreased, that is, the size of the complexes increased, with increasing concentrations of nucleocapsid protein. In the presence of IBV RNA, the greatest concentrations of protein resulted in formation of additional large, megacomplexes that did not migrate into the gel. No shift in mobility of the labeled RNA was observed when reacted with an equivalent concentration of either egg allantoic proteins (Fig. 6a) or bovine serum albumin (data not shown).

Competitive gel shift analyses

Competition gel shift assays were done using 3 μg, a nonsaturating concentration, of the fusion protein. In addition to unlabeled RNA representing the various regions of the 3′ terminus of the genome, CVV RNA, yeast tRNA, and bovine liver RNA were used to compete with the interaction of G RNA and the recombinant nucleocapsid protein (Fig. 7). Radiolabeled G was mixed with 100 ng of each competing RNA species before reacting with the nucleocapsid protein. Differences could be seen in the efficiency of inhibition of G interaction with the nucleocapsid protein by each RNA species. Yeast tRNA appeared to have no effect on the mobility of radiolabeled G, whereas the autologous unlabeled G RNA was most efficient, almost eliminating the protein interaction with labeled G. Surprisingly, CVV RNA, corresponding to the positive sense RNA of the small genome segment, ap-

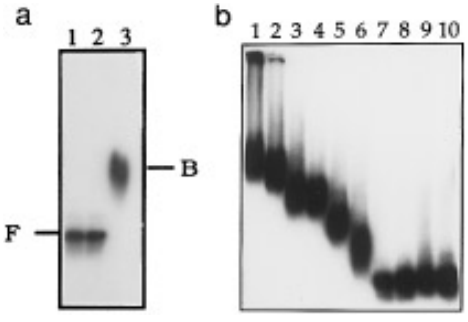

FIG. 6. Gel-shift assay demonstrating the binding of radiolabeled G RNA to the synthetic nucleocapsid protein. (a) Free α-^{32}P-labeled G in the absence of protein is shown in lane 1, radiolabeled G with 3 μg bovine serum albumin is shown in lane 2, and the binding of 1 ng radiolabeled G with 3 μg of nucleocapsid protein is shown in lane 3. (b) Lanes 1 to 9 show the effects of twofold dilutions of protein beginning in lane 1 with 20 μg of protein. The free labeled G is shown in lane 10.

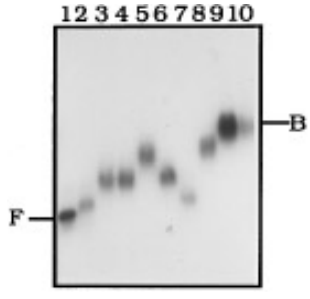

FIG. 7. Gel-shift assay in which the interaction of α-^{32}P-labeled G RNA and recombinant nucleocapsid protein is competed with 100 ng of unlabeled RNA. Lane 1 represents free labeled G, lane 10 represents labeled G and protein in the absence of competitor RNA, and lane 2, labeled G with the protein and unlabeled autologous G. Lanes 3 to 9 represent labeled G with protein and unlabeled competitor AB, CD, EF, I, CVV, bovine liver, and yeast RNA, respectively.

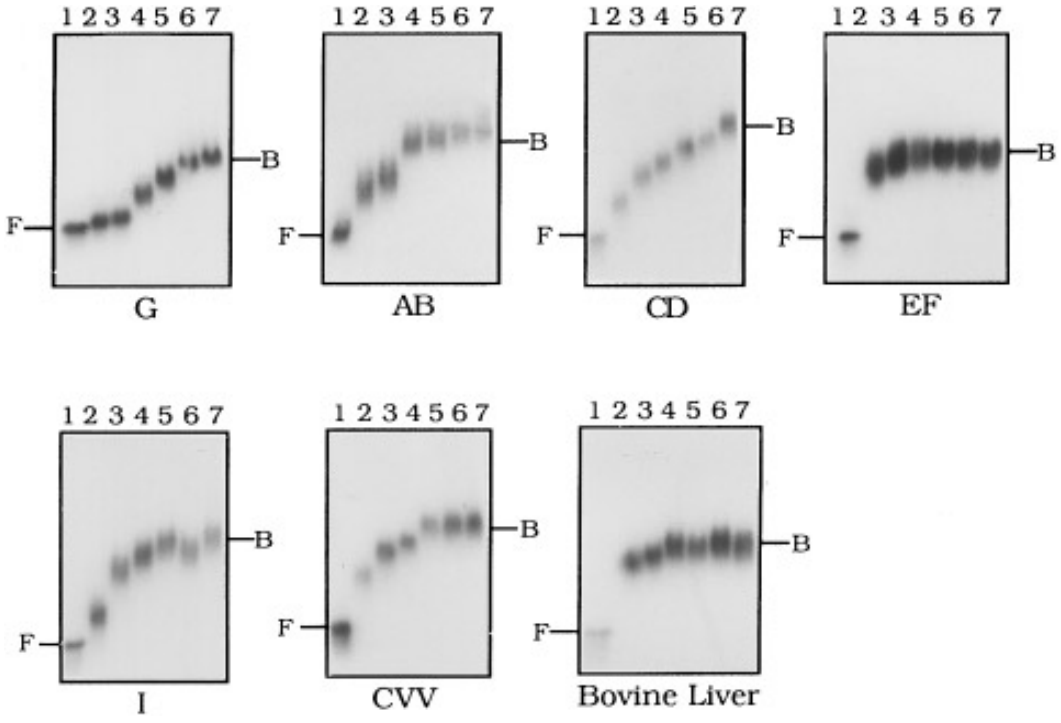

FIG. 8. Competitive gel-shift assay in which the interactions of the recombinant nucleocapsid protein with radiolabeled G RNA are inhibited with varying concentrations of the unlabeled RNA. The corresponding competitor RNA used is indicated below each autoradiograph. Lanes 1 represent free labeled G RNA in the absence of protein and competitor, lanes 2 to 6, labeled G with protein and twofold dilutions of competitor beginning with 100 ng in lane 2, and lane 7 represents labeled G in the presence of protein and no competitor.

peared to bind at least as well, if not better than I, AB, and CD. In contrast, the EF region was a very inefficient inhibitor, comparable in this assay to bovine liver RNA, which was of ribosomal origin.

The shifts in G mobility resulting from the interaction of protein were further characterized using varying concentrations of unlabeled competing RNA of each species mixed with labeled RNA before the addition of recombinant nucleocapsid protein. The shift in the mobility in the presence of the competitor was compared with the maximum shift in the absence of the competitor. Unlabeled G, AB, CD, I, and CVV RNA again appeared to interact relatively efficiently with the nucleocapsid protein, whereas the protein interactions with EF and bovine liver RNA were relatively inefficient (Fig. 8). All IBV fragments from the 3′ end appeared to react with the recombinant nucleocapsid protein with relatively high efficiency except the EF fragment which corresponded to nt 78 to 217 from the 3′ end of the genome. The efficiency of interaction with protein appeared to be at least as poor as that of bovine liver RNA. CVV RNA interactions with the nucleocapsid protein did suggest a similar efficiency in inhibition in mobility to that of the homologous G RNA.

Transcribed RNA of a similar length as G, a 1052-nt fragment designated pCR, prepared from *Pst*I-digested pCR1000, and RNA transcripts from pCR1000 with the CD immediately downstream of the promoter, a 1207-nt fragment designated CD-pCR (Fig. 3b), were also used as inhibitors (Fig. 9). No competition of G binding to nucleocapsid protein was observed with pCR RNA, which did not contain IBV sequences (Fig. 9a). Competition with CD and CD-pCR, an equivalent amount of CD-pCR RNA was reproducibly (in three separate experiments) less efficient at interacting with nucleocapsid protein (Figs. 9b and 9d). Because the molar ratio of CD to CDpCR was about 8:1, competitive inhibition was also examined with a molar equivalent of CD and CD-pCR RNA (Figs. 9b and 9c). As might be predicted if CD contains binding sites for the nucleocapsid protein and therefore specifically interacts with nucleocapsid competed, the observed inhibition of the two preparations with G was nearly identical. The IBV sequences within the CD fragment specifically interacted with the recombinant protein. Considering the excess of non-IBV sequences in CD-pCR, the inhibition of this fragment was greater than might be expected, possibly indicating a degree of nonspecific cooperative binding in the presence of specific binding.

In order to further confirm that IBV nucleocapsid protein interactions with IBV RNA were not the result of nonspecific binding to RNA, competitive gel shift assays were done with [α^{32}P]CTP-radiolabeled CD or EF RNA and unlabeled G or yeast tRNA. A shift assay using varying concentrations of recombinant protein reacted with 1 ng of labeled CD or EF indicated that 2 μg represented a concentration of nonsaturated protein (data not shown).

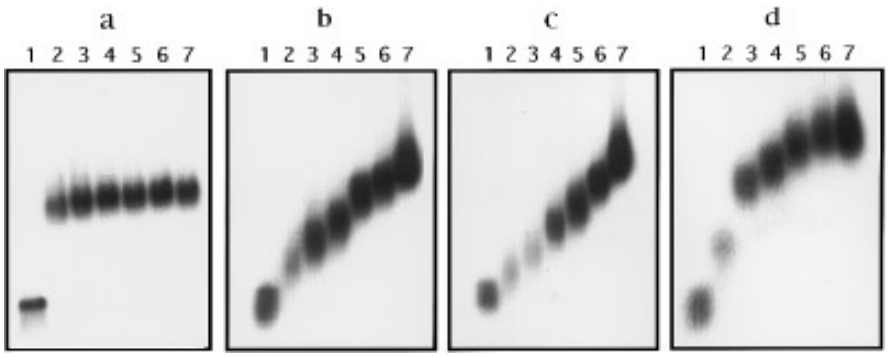

FIG. 9. Competitive gel-shift assay using pCR RNA (a), CD RNA (b), and CD-pCR RNA (c and d) as competitors. Lane 1. represents 1 ng labeled G RNA probe, lanes 2 to 6, labeled G with protein and twofold dilutions of competitor beginning with 100 ng (a, b, and d) or 800 ng (c) in lane 2, and lane 7 represents labeled G in the presence of protein and no competitor.

One nanogram of labeled CD and varying concentrations of unlabeled G were mixed with 2 μg of the recombinant protein. A shift in mobility of labeled CD or EF was observed in the presence of protein (Fig. 10). A small, but reproducible difference in the inhibition of EF and CD with unlabeled G could be observed, suggesting that EF interacts somewhat less efficiently than CD. The decrease in mobility of EF probes could be inhibited even with 6.25 ng of cold G. The inhibition of CD mobility was less affected by the same concentration of G or in parallel experiments not affected by the addition of unlabeled G (data not shown). In contrast, the presence of unlabeled yeast tRNA had no effect on the nucleocapsid protein associated shift of EF. Although the presence of unlabeled yeast tRNA had a slight effect on the interaction with CD, it was not comparable to the inhibition observed with G. Therefore, the recombinant protein generated in these experiments specifically interact with the 3′ end of the IBV genome.

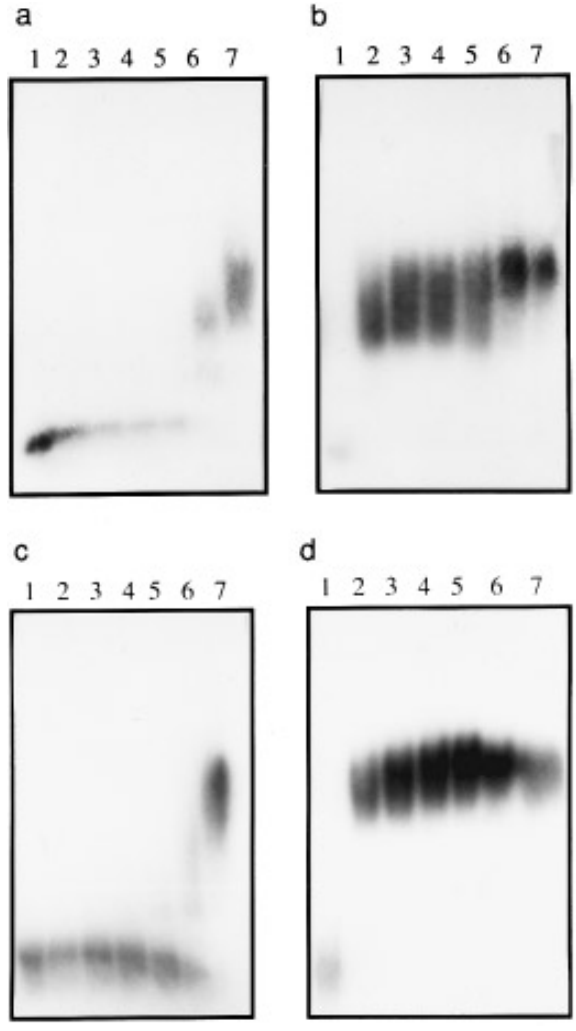

FIG. 10. Competitive gel-shift assay in which the interactions of 2 μg of recombinant nucleocapsid protein with 1 ng of α-^{32}P-labeled CD (a and b) or EF (c and d) were inhibited with unlabeled G (a and c) or yeast tRNA (b and d). Lanes 1 represent 2 ng of free labeled RNA in the absence of protein and competitor RNA, lanes 2 to 6, 1 ng of labeled CD or EF with protein and twofold decreasing concentrations of unlabeled G beginning with 100 ng in lanes 2, and lanes 7 represent labeled CD or EF in the presence of protein and no competitor.

DISCUSSION

The nucleocapsid protein of MHV has been shown to bind specifically to RNA corresponding to its leader sequence (Stohlman *et al.,* 1988; Nelson and Stohlman, 1993). In this paper, we have shown that the nucleocapsid protein of IBV is able to interact with relatively high affinity to RNA representing specific regions at the 3′ end of the IBV genome. Competitive binding analyses indicated that the nucleocapsid protein bound with similar specificity to transcripts which spanned the length of the entire 3′ noncoding region, but the interaction between the recombinant fusion protein and an overlapping fragment, from 217 to 78 nts from the 3′ terminus, was comparatively weaker. The interactions with unlabeled RNA indicated that the protein interaction with RNA fragment EF was consistently less efficient than that with autologous labeled G. Therefore, within the positive sense 3′ noncoding region, fragments I, AB, CD, and G, as well as the CVV RNA, contained sequences that

promoted nucleocapsid protein binding under conditions used in these studies, whereas sequences or structures required for optimal interactions with nucleocapsid protein were not present or were sufficiently altered in EF and bovine liver RNA. The interactions of labeled CD and EF with protein were both inhibited by unlabeled G. The reproducible difference observed in the inhibition of CD and EF with G might also suggest that EF interacts less efficiently than CD. Inhibition with the relatively large G may produce a less sensitive assay for comparing interactions with the nucleocapsid protein than inhibitions with the unlabeled smaller fragments. In addition, unlike the competitive studies with labeled G, partial inhibition of the smaller fragments could be observed, that is, the labeled RNA migrated both at the position of free RNA and of protein-complexed RNA.

The relative interactions of each RNA fragment might be expected to depend on the number of binding sites, as well as binding affinity at each site. However, a molar equivalent of CD and CD-pCR (with a non-IBV 1052-nt sequence) inhibited G binding in an identical manner. CD appears to encode sequences that can specifically bind to the nucleocapsid protein although we can not rule out the possibility that G encodes additional binding sites. Correlation of reactivity strictly with fragment size was not evident, because CD-pCR RNA did not compete as efficiently as an equivalent amount of G nor on the basis of amount of nucleotides available, as well as CD. The G fragment of 1807 nt in length, CVV of approximately 800 nt and CD of 155 nt were most efficient at equivalent total RNA concentrations. Those RNA transcripts that bound less efficiently included the small yeast tRNA, 1052-nt fragment of pCR1000, 2300- or 4200-nt ribosomal RNA of the bovine liver preparation, and sequences in the 140-nt EF transcripts. The sequences of the latter were included within either AB or CD. Therefore, the RNA sites required for binding to the recombinant nucleocapsid protein are not ubiquitous in the genome or in nature but require a degree of sequence and/or structure specificity.

Since the fusion protein used for these studies had tandem histidine residues at the amino terminus and was produced in a bacterial system, this product would not be expected to be identical to the viral protein synthesized in the eukaryotic host. However, the effects of sodium ions on G RNA and nucleocapsid interactions suggest that the fusion protein functions at least in some respects as the native protein at salt concentrations below 0.3 *M*. The difference observed in reactions in the 0.3 *M* sodium chloride buffer may reflect alterations in the binding site(s) of the synthetic protein or the presence of additional functional site(s) on the native counterpart. Differences may also exist in the phosphorylation status of the native and synthetic proteins. Any phosphorylation of the fusion protein would depend on the bacterial cell or less likely on autophosphorylation. Although it might be expected that altered phosphorylation in the eukaryotic host cell could result in variations of the RNA reactions of the native protein, it was obvious that this recombinant protein was at least functional with respect to its basic interaction with RNA and its antigenicity. Therefore, it should provide a valuable source for further characterization of the functions of the nucleocapsid in IBV replication.

The nucleocapsid protein used in these experiments appeared to interact preferentially to specific sequences and/or structures found on the IBV genome, as well as the RNA of at least one other virus. These interactions may be indicative of a common strategy for certain viral protein interactions with viral RNA. The RNA of CVV may have structures or sequences that not only interact with IBV nucleocapsid protein but also may react in a similar fashion with its own nucleocapsid protein. Computer-generated comparisons between the G and CVV RNA did identify a short region of 18 nt of 88% similarity (personal observations). Putative amino acid sequences of the IBV and CVV nucleocapsid protein indicate that both are lacking zinc fingers (Williams *et al.,* 1992; Chung, 1992) and therefore must bind to RNA through a mechanism independent of such structures. Although computer generated hairpin turns can be found in all the fragments used, requirements for secondary structure or base pairing still need to be evaluated.

The nature of the binding between nucleocapsid protein and RNA is not known. Presumably cooperative binding among multivalent RNA and protein molecules was responsible for the continuously increasing size of the complexes resulting from increasing concentrations of protein. Large complexes that could not enter the gel were observed at the greater concentrations of protein. Similar concentrations of the recombinant protein alone as shown by Western blot analyses did not form these megacomplexes (data not shown) which could also be simulated at the lower concentrations of recombinant nucleocapsid protein when spermidine was added to the reaction mixture (Williams, 1993). Because these large complexes were not seen in the absence of RNA, they must be related to interactions involving both RNA and protein. The increased complexity of the shift in mobility of RNA–protein observed in the presence of the larger G fragment compared with that in the presence of EF or CD suggests that G may have more sites of interaction and be capable of greater cooperative binding with the nucleocapsid protein. The increased binding of CD on a molar basis with the addition of pCR RNA (which alone does not bind) may be explained by the presence of cooperative binding following specific interactions with CD.

Following the presumed binding of viral protein at a packaging signal sequence on genomic RNA, packaging of the entire genome may dictate the necessity of nucleocapsid protein binding in a cooperative manner to re-

peated sites along the molecule. These interactions would not appear to be promiscuous, but, in fact, require sequences or structures that are not uniform even on the IBV genome. Although the specificity might be dictated by primary sequences, the 3′ HVR with its distinctly enriched U-A composition bound better than EF which has an overall similar U-A composition as AB and CD. Putative hairpin loop structures present in these fragments (unpublished observations), as suggested by computer analyses, contributed to the binding specificity. We are now examining the effects of antisense CD RNA which will have a similar structure but differ in sequence on RNA-nucleocapsid binding. Ongoing experiments are examining the reaction with smaller RNA fragments in order to identify the critical length, composition, and/or structure of RNA needed for IBV nucleocapsid interactions.

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