The amino and carboxyl domains of the infectious bronchitis virus nucleocapsid protein interact with 3′ genomic RNA

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Abstract

Previous studies indicated that the nucleocapsid (N) protein of infectious bronchitis virus (IBV) interacted with specific sequences in the 3′ non-coding region of IBV RNA. In order to identify domains in the N protein that bind to RNA, the whole protein (409 amino acids) and six overlapping fragments were expressed as fusion polypeptides with six histidine-tags. Using gel shift assays, the intact N protein and amino polypeptides, from residues 1 to 171 and residues 1 to 274, and carboxyl polypeptides, extending from residues 203 to 409 and residues 268 to 407, were found to interact with positive-stranded IBV RNA representing the 3′ end of the genome. The two ^{32}P-labeled probes that interacted with N and the amino and carboxyl fragments of N were RNA consisting of the IBV N gene and adjacent 3′ non-coding terminus, and RNA consisting of the 155-nucleotide sequences at the 3′ end of the 504-nt 3′ untranslated region. In contrast, the polypeptide fragment from the middle region, residues 101–283, did not interact with these 3′ IBV RNAs. The binding site in the amino region of N was either not present or only partially present in the first 91 residues because no interaction with RNA was observed with the polypeptide incorporating these residues. Cache Valley virus N expressed with a histidine tag, bovine serum albumin, and the basic lysozyme protein did not shift the IBV RNA. The lower molarities of the carboxyl fragment compared with residue 1–274 fragment needed for equivalent shifts suggested that the binding avidity for RNA at the carboxyl domain was greater than the amino domain. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nucleocapsid (N) protein; Infectious bronchitis virus (IBV); Amino polypeptides; Carboxyl polypeptides

1. Introduction

Infectious bronchitis virus (IBV) is a member of the Coronaviridae. The virion contains four structural proteins: membrane, spike, nucleocapsid (N) and the small membrane proteins. The IBV N protein, a basic, phosphorylated structural protein

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of 409 residues, is highly conserved among IBV strains, especially within the middle region (Williams et al., 1992).

The putative functions of the murine hepatitis virus (MHV) N protein depend on its interaction with viral RNA (Baric et al., 1988). Encapsidation of MHV RNA requires that the N protein recognize the packaging signal, a 69-nt stem-loop structure positioned at the end of ORF 1b, whereas the packaging signal in the porcine coronavirus, transmissible gastroenteritis virus apparently lies in the ORF 1a (Van der Most et al., 1991; Fosmire et al., 1992; Izeta et al., 1999). Interactions between the MHV N protein and the packaging signal have been reported (Molenkamp and Spaan, 1997). A 1.4-kb sequence, corresponding to IBV sequence at the 5′ end of the 1b gene, may be involved in the packaging of IBV RNAs or form part of a cis-acting replication element (Penzes et al., 1996). However, subgenomic RNAs, lacking the described packaging signals, have been detected in the packaged IBV particles (Zhao et al., 1993). MHV N protein has also been shown to interact specifically with the 5′ leader-containing sequences (Baric et al., 1988; Stohlman et al., 1988). Anti-N protein monoclonal antibodies were reported to precipitate both full-length and subgenomic RNAs, as well as replicative intermediate RNA (Stohlman et al., 1988). Compton et al. (1987) found that RNA synthesis was greater than 90% inhibited by antibodies to MHV N protein in an in vitro synthesis system prepared from the infected cells. All these data suggest that the coronavirus N protein is likely to be involved in viral RNA replication, transcription and packaging.

The 3′ end RNA sequences of MHV were found to bind to cellular proteins (Yu and Leibowitz, 1995). Zhang et al. (1999) have shown that heterogeneous nuclear ribonucleoprotein A1 interacts with intergenic sequences and 3′ end sequences of the negative-strand MHV RNA forming a ribonucleoprotein complex. A 55-nt sequence in the 3′ terminus of genomic RNA and the poly (A) tail of the MHV RNA were found to be required for minus-strand RNA synthesis (Lin et al., 1994). In addition, Lin et al. (1996) also found that the 3′ non-coding region of MHV RNA is required for the positive-sense subgenomic RNA transcription from a DI RNA. Hsue and Masters (1997) identified a stem-loop structure in the MHV 3′ untranslated region which is essential for RNA replication. It is evident that the 3′ end untranslated region plays very important roles in viral RNA replication and transcription.

Our previous studies demonstrated that IBV N protein interacts specifically with RNA sequences within the 3′ end non-coding region of genomic RNA (Zhou et al., 1996). The interactions between the IBV N protein and the 3′ non-coding region sequences may be correlated with in vivo functions of the N protein, although what role the IBV N protein plays in the viral RNA replication and transcription is still unknown. In this study, the intact IBV N protein and six overlapping polypeptides were expressed in a bacterial expression system and their interactions with the 3′ end of genomic RNA were examined using gel shift assays (Zhou et al., 1996). Two RNA binding domains for the 3′ non-coding terminal RNA were found in the IBV N protein, one located within the amino region and one at the carboxyl region.

2. Materials and methods

2.1. Preparation of protein and polypeptides

The N protein was analyzed with ISOELECTRIC and MOTIF programs. Isoelectric points of the amino, carboxyl and middle regions and the whole N protein were predicted using ISOELECTRIC (University of Wisconsin, GCG, Version 9.1). Possible motifs were searched against the database using the MOTIF program. The whole N gene and six overlapping cDNA sequences were amplified from the G2 plasmid by PCR. A *Bam*HI site was included at the 5′ end and a *Hin*dIII site at the 3′ end. The PCR amplified cDNAs were cloned into TA cloning vectors (Invitrogen, Carlsbad, CA). Plasmids containing the inserts were digested with *Bam*HI and *Hin*dIII, and the inserts were purified in 1% agarose gels and then ligated into pQE8 vectors, which were

also fully digested with *Bam*HI and *Hin*dIII (Sambrook et al., 1989). The fusion protein and polypeptides expressed from pQE8 plasmid contained six histidine tags at the amino termini. M15/pREP cells were transformed with the ligated constructs and incubated on LB plates supplemented with kanamycin and ampicillin. Single colonies were screened with mini-preparation and *Hin*dIII digestion. cDNAs from positive colonies with inserts were sequenced to confirm the orientation and fidelity with ABI-PRISM (Model 377, Version 2.1.1, DNA Technology Facilities, Department of Veterinary Pathobiology, Texas A&M University).

Clones with inserts in correct orientation were examined for protein expression as described, with modification (Zhou et al., 1996). Briefly, the bacterial cells were induced with IPTG and proteins were purified with Ni^{2+}-NTA resin (Qiagen, Valencia, CA, USA). The eluted samples were then analyzed with SDS-15% polyacrylamide gel electrophoresis.

SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl in 1 l of water with 20 mM glucose) was used for mass production of the whole N protein and the polypeptides (Sambrook et al., 1989). Recombinant polypeptides, with six histidine tags at the amino termini, were enriched and purified with Ni^{2+}-NTA (nitrilo-tri-acetic acid) resin (Zhou et al., 1996). Polypeptides equal to or greater than 274 amino acids were further purified with gel filtration through Sephadex G200 while peptides equal to or smaller than 207 amino acids were purified with Bio-gel P60 (Ausubel et al., 1987). A total of 8 ml of each

sample was loaded onto the column before washing with PBS (0.05 M phosphate, 0.3 M NaCl, pH 7.0). Fractions were monitored with the Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA), and the protein-containing fractions were analyzed by 15% polyacrylamide gel electrophoresis (Sambrook et al., 1989; Coligan et al., 1994). The fractions containing recombinant protein were pooled and concentrated with Ni^{2+}-NTA resin and eluted with 0.20 M imidazole (0.20 M imidazole, 0.04 M phosphate, 0.3 M NaCl, pH 7.0). A Sephadex G50 column was used to remove the imidazole (Ausubel et al., 1987).

The purified polypeptides were quantitated with the Bio-Rad Protein Microtitre Assay (Bio-Rad, Richmond, CA) and analyzed by SDS–PAGE using standard protocols (Sambrook et al., 1989). Western blot assays were used to determine the viral specificity of the recombinant polypeptides (Ausubel et al., 1987). Briefly, 200 ng of polypeptides were separated on 15% polyacrylamide gel and blotted onto the nitrocellulose membranes. The membranes were then reacted sequentially with chicken anti-IBV Gray serum and alkaline phosphatase-labeled goat anti-chicken IgG.

2.2. Preparation of RNA

The 1832-nt G RNA, corresponding to the IBV N gene plus the genomic 3′ non-coding terminus, and CD RNA, corresponding to the 155 nucleotides at the 3′ terminus of genomic RNA, were prepared as previously described (Zhou et al., 1996) (Fig. 1). RNAs were transcribed in vitro from linearized plasmids with T7 RNA polymerase (Promega, Madison, WI). Radioactive probes were synthesized by incorporating the α-^{32}P-CTP into the transcribed RNAs. RNAs were quantitated after gel electrophoresis by comparing the band intensity with that of control RNA observed with UV light (Sambrook et al., 1989).

2.3. Gel shift assay

Gel shift assays were performed on 0.5 and 1% agarose gel for G and CD RNAs, respectively (Zhou et al., 1996). Protein or polypeptides were diluted by twofold, and 5 µl of diluted samples
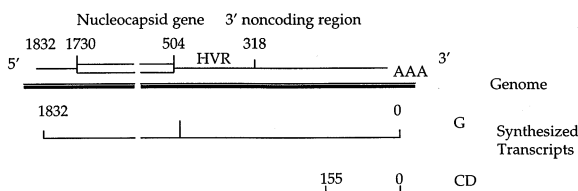

Fig. 1. Diagrams of G and CD RNA probes. G RNA contains an 1832-nucleotide genomic sequence, including the N gene, 102 nucleotides upstream of the N gene and 504 non-coding nucleotides at the 3′ end of the genome. CD corresponds to 155 nucleotides at the 3′ end of the positive-stranded genome.

Nucleocapsid protein (N)

pI

Amino terminus (A)	Middle region (M)	Carboxyl terminus (C)		

1 ————————————————————— 409   10.47

A91
1 □ 91   11.63

A171
1 □——————□ 171   10.97

A274
1 □——————————□ 274   10.97

M174
101 □————————□ 283   10.53

C207
203 □————————————□ 409   8.83

C140
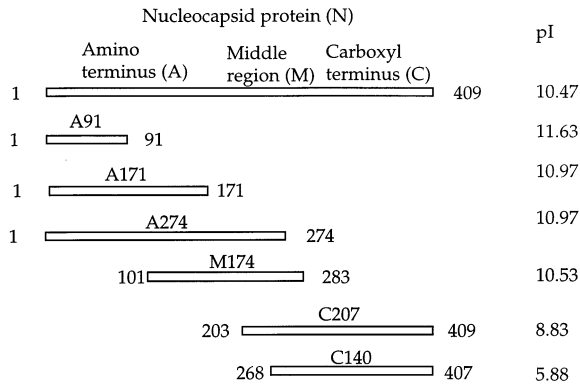268 □————————□ 407   5.88

Fig. 2. The IBV N polypeptides expressed in the Qiagen expression system and their putative isoelectric points. Peptides corresponding to the amino region and carboxyl region are designated as A91, A171, A274, C207 and C140. The peptide corresponding to the middle region is labeled as M174. The numbers following A, C or M are the length in residues of the polypeptides.

were added to individual wells on a 72-well Terasaki plate. The 500-μl probe mixture was prepared by mixing 200 μl $5 \times$ gel shift assay buffer (125 mM Hepes, 125 mM EDTA, 750 mM NaCl, 25 mM DTT, 50% glycerol, pH 7.4), 250 ng labeled RNA probe, 50 μg yeast tRNA and 1000 U RNasin (Promega, Madison, WI). A total of 5 μl of probe mixture were added to each well and incubated with protein sample. After 20-min incubation at room temperature, 1 μl of RNA loading buffer was added to each well and the samples were loaded onto an agarose gel and electrophoresed. The electrophoresis was performed in Tris-borate and EDTA buffer (Sambrook et al., 1989) at 120 V (Zhou et al., 1996). The gels were fixed in a solution of 45 methanol:10 acetic acid:45 water and dried. The presence of probes in the dried gel was detected by autoradiography.

3. Results

3.1. Protein preparation

Intact N protein and six overlapping polypeptides were expressed in the Qiagen expression system (Fig. 2). Since the recombinant polypep-

tides all contain a six histidine-tag at the amino terminus, they were first purified with Ni^{2+}-NTA resin (Zhou et al., 1996). However, SDS–PAGE indicated that the samples eluted from the Ni^{2+}-NTA column often contained more than one polypeptide band (data not shown). Therefore, A274, N and C207 were further purified with Sephadex G200, and C140 and A171 with a Bio-gel P60 column.

The purified N protein and polypeptides were analyzed by SDS–PAGE (Fig. 3A). Each sample contained the polypeptide of the expected molecular weight. Western blot analysis was performed to examine the viral specificity of the polypeptides (Fig. 3B). Chicken anti-IBV Gray antibody recognized all the polypeptides, although the reaction with A91 in lane 1 was weak. Western blot analysis also detected some polypeptides smaller than expected (lanes 3 and 4, Fig. 3B), which small fragments are likely to be the products of degradation or truncation. No reaction was detected between anti-IBV serum and BSA (lane 8, Fig. 3B).

3.2. Gel shift of the 1832-nt IBV G RNA

Interactions of protein with the labeled RNA result in the formation of complexes which migrate more slowly than the unbound RNA, causing a shift in the agarose gel or polyacrylamide gel. A total of 5 μM of N protein and polypeptides were reacted with 1 ng of ^{32}P-labeled G RNA (Fig. 4). N protein, and A171, A274, C207 and C140 polypeptides shifted the G RNA probe (Fig. 4) while the 173 residue fragment in the middle of N protein did not (lane 5, Fig. 4). C140 and C207 shifted the RNA probes further than amino terminal polypeptides and the intact N protein did. The C140 and C207 polypeptides, which had a tendency to aggregate during column chromatography purification in the absence of RNA, also formed complexes with radioactive RNA probe complexes that did not migrated into the gel (data not shown). Therefore, the radioactivity in the bands of lower molecular weight were reduced in intensity. To rule out that G RNA interacts with N protein and polypeptides simply because they are basic, lysozyme, a basic protein,

was used as control protein in this assay. No interaction between lysozyme and G RNA was observed (lane 9, Fig. 4). Cache Valley Virus N protein, which is also expressed in pQE and contains a histidine tag at amino terminus, did not shift G RNA (lane 8, Fig. 4). This result indicated that the histidine tag does not interact with IBV G RNA under these conditions.

The interactions were further examined in the gel shift assays using varying amounts of N protein and polypeptides, and fixed concentrations of G RNA (Fig. 5). Twofold dilutions beginning with equivalent 3 µg of N protein or polypeptides were interacted with G RNA. The greatest molar concentrations for the intact N protein were 6 µM and ≈ 27 µM for the smallest peptide, A91. The greatest molar concentrations of the other polypeptides range from ≈ 9 to 18 µM. The amino terminus polypeptide, A91, did not shift the G RNA even at a concentration of ≈ 27 µM (Fig. 5, lane 2) while as little as 1.25 µM of N protein did shift the G RNA. The most diluted concentrations that still shifted the G RNA ranged from 1.5 to 4.5 µM for A171, A274

and C207, C140, respectively (Fig. 5). The polypeptides from the middle region did not shift G RNA at any concentration used (Fig. 5).

3.3. Gel shift of the CD RNA

The smaller CD RNA, representing the 155 nucleotides at 3′ non-coding region of the IBV genome, had been shown to interact with the whole N protein by the gel shift assay (Zhou et al., 1996). The interactions of CD RNA with varying µg of recombinant polypeptides were examined in this experiment. Polypeptides from both carboxyl and amino regions were found to interact with CD RNA and cause shifts in the probe (Fig. 6). The minimum concentration at which IBV N protein shifted the CD was 0.14 µM (lane 7). Minimum shifts in IBV RNA were observed with 0.375 µM of C207 (lane 7) and 0.56 µM of C140. The polypeptide at the amino terminus appeared to shift the CD RNA less efficiently than C207 and C140 because shifts were observed only when more than 1.68 µM of A171 were used. Again, the polypeptide representing the middle
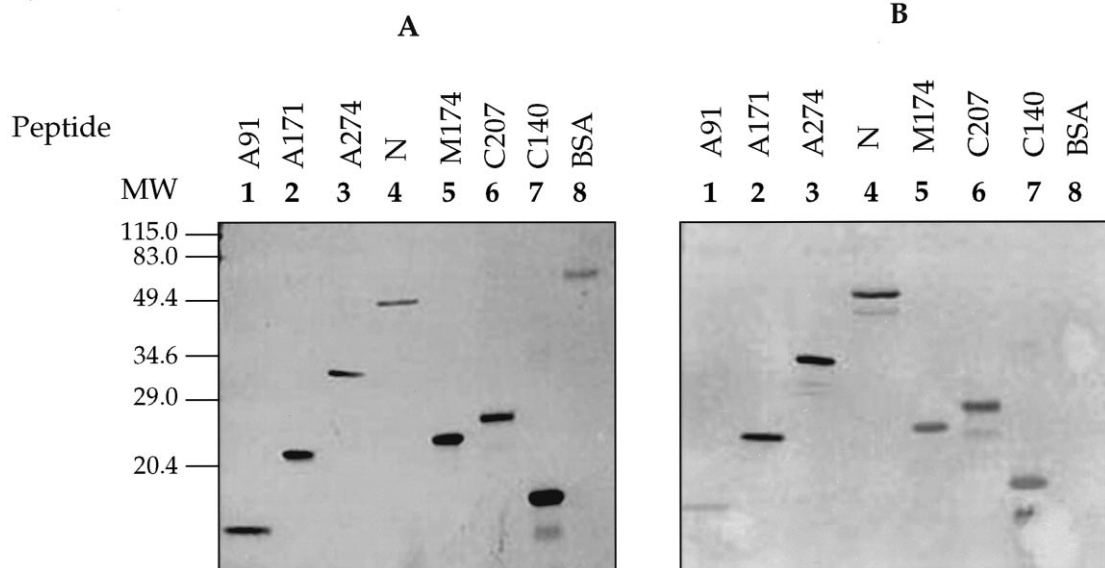

Fig. 3. SDS–PAGE and Western blot analyses of IBV N protein and polypeptides. (A) The polypeptides were stained with Coomassie brilliant blue R250. (B) Western blot analyses of expressed polypeptides using chicken anti-IBV Gray antibody and alkaline phosphatase-labeled goat anti-chicken IgG. Lane 1 contains A91; 2, A171; 3, A274; 4, N; 5, M174; 6, C207; 7, C140; and 8, BSA.
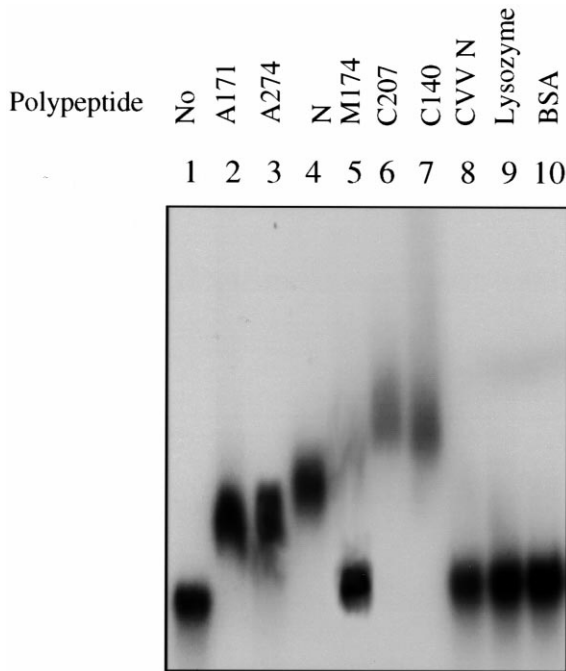
Fig. 4. The gel shift of labeled G RNA is specific for N and N polypeptides. A total of 1 ng of labeled G RNA was reacted with 5 μM of N protein or polypeptides and control proteins. Lane 1 contains no protein. A171, A274, N, M174, C207, C140, CVV N, lysozyme and BSA were added into lanes 2–10, respectively.

region, M173, as well as BSA, did not interact with CD RNA at any dilution used in this study.

4. Discussion

Although the IBV N protein has been shown to interact with the 3' non-coding region of its genomic RNA (Zhou et al., 1996), computer analyses of N protein could not identify any known motif which is specific for RNA binding. Based on the sequence conservation, hydrophobicity and isoelectric point, IBV N protein can be divided into three regions, which are very similar to the MHV N protein (Parker and Masters, 1990). Computer analyses indicated that N protein is highly conserved among IBV strains, especially in the middle region. The amino terminus is highly basic while the carboxyl terminus is relatively acidic. The amino and carboxyl regions and the conserved middle region may contain distinct functional domains.

Regions which interacted with RNA from the 3' non-coding region of IBV genomic RNA have been located at the amino and carboxyl regions using the gel shift assay. The two RNA binding domains of the IBV N protein may differ in binding affinity. The gel shift assays suggested that interactions with the carboxyl domain result in greater shifts at lower concentrations than interactions with the amino domain. The carboxyl region polypeptides shifted the G RNA probe more than the equivalent molar concentration of the amino region polypeptide (A171). The A91 fragment does not contain the complete binding site because this polypeptide alone could not shift the G RNA and CD RNA. Residues between 91 and 171 may be important for direct interactions with the RNAs or maintaining a certain structure of the peptide.

The middle region of the N IBV protein did not interact with RNAs from the 3' non-coding region in either the ROPBA (data not shown) or gel shift assay although the corresponding middle region of MHV has been shown to interact with 5' leader sequences (Masters, 1992). A highly homologous region of ten amino acids was identified between 91 and 110 in the IBV N protein and between 128 and 137 in the MHV N protein. Out of the ten residues, nine were identical. The N proteins of IBV and MHV may bind to distinct RNA sequences or structures, reflecting the differences in the 5' and 3' end sequences of coronavirus RNA and associated N protein functions. Our preliminary data have suggested that the middle region of N protein can interact with the 5' end sequences of IBV RNA.

The affinity differences between the binding of a protein to specific and non-specific RNAs defines the specificity of the RNA-protein interaction. The gel shift assays were performed in the presence of 200-fold excess of non-specific RNA. Therefore, interactions with the G RNA and CD RNA indicate the strong binding affinities of the N polypeptides with viral RNAs. Moreover, none of the control proteins, BSA, CVV N protein and lysozyme, interacted with G and CD RNA. Confi-

rming a preference for specific RNA, interactions of N with yeast tRNA and ribosomal RNA, minus-strand RNA from the 3′ genomic end (data not shown), and fragment EF from the 3′ genomic end are consistently negligible (Zhou et al., 1996).

Interactions between the N and IBV RNA may be the consequence of multiple binding sites in the N, RNA or both. The shift of IBV RNA always increases with increasing amount of protein. Multiple bands can even be observed with some inter-
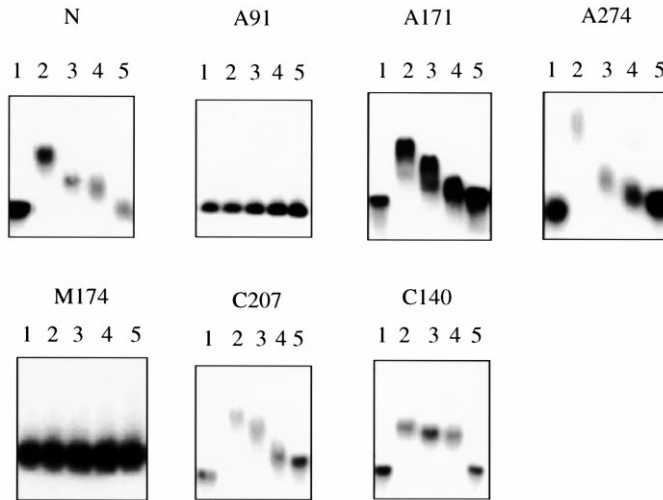

Fig. 5. Gel shift assays of G RNA with varying concentrations of IBV N polypeptides. IBV N polypeptides, C207, C140, A91, N, A274 and A171, as indicated on top of each panel. Lane 1 in each panel represents the migration free RNA probe. Twofold dilutions of N protein and polypeptides were added from lane 2 to lane 5, starting with 3 µg in lane 2. The starting molar concentrations were 6, 27, 14, 9, 14, 12 and 18 µM for N, A91, A171, A274, M174, C207 and C140, respectively.
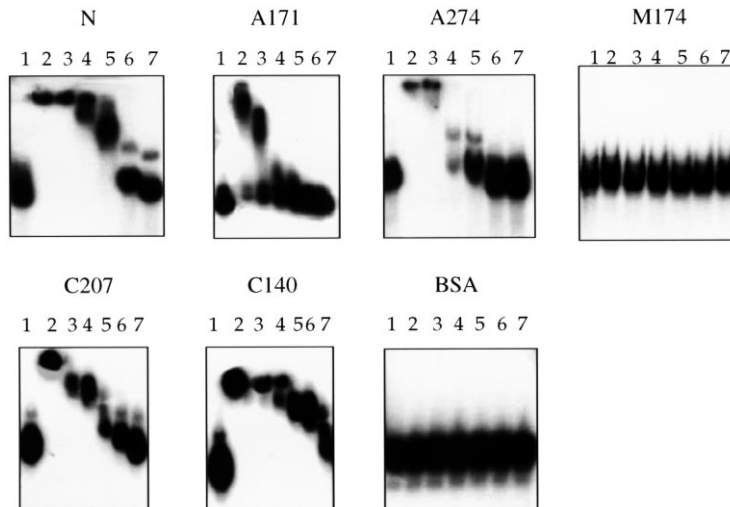

Fig. 6. Gel shift assays of CD RNA with varying concentrations of IBV N polypeptides. IBV polypeptides used were M173, C207, A171 and C140, as indicated on top of each panel. Lane 1 in each panel represents the migration of free RNA probe. Twofold diluted polypeptides were added from lane 2 to lane 7, starting with 3 µg in lane 2. The starting molar concentrations were 6, 14, 9, 14, 12 and 18 µM for N, A171, A274, M174, C207 and C140, respectively.

actions. Furthermore, the carboxyl polypeptides, C140 and C207, shifted the RNA probe further than amino region polypeptides. Protein-protein interactions may also contribute to the increased complexity of interactions with RNA. This phenomenon may be explained by the presence of multiple binding sites in the RNA and/or protein. The carboxyl region polypeptides, even the smaller C140 fragment, were found to form large complexes in the absence of RNA during filtration purification through Bio-Gel P60 (data not shown). Formation of N multimers was also observed by Robbins et al. (1986) where multiple bands were detected by labeled RNA with RNA protein blot analysis.

The binding of the N protein to the 3′ non-coding region may be speculated to regulate transcription or replication. Studies on MHV also indicated that both N protein and sequences in the 3′ non-coding region are involved in regulating the viral RNA replication and transcription (Compton et al., 1987; Lin et al., 1996). The putative roles that the IBV N protein plays in replication and transcription could be speculated. Critical amounts of N protein may act as an inhibitor competing with transcriptional factors for the binding sites. Alternatively, the N protein could promote transcriptional factors to the sites. The 3′ non-coding region of MHV has been shown to interact with cellular proteins (Yu and Leibowitz, 1995); the actual involvement of those proteins in viral RNA transcription is not clear.

The interactions of the MHV and BCV N proteins with packaging signals, which are located on the end of the polymerase gene, have been observed with in vitro assays (Van der Most et al., 1991; Cologna and Hogue, 1997). However, subgenomic RNAs, lacking the described packaging signals, have been detected in the packaged IBV particles (Zhao et al., 1993). Virion incorporation of these RNAs could have resulted from interactions between the N protein and the 3′ non-coding region found in all subgenomic RNA. Packaging may occur through interactions of sequences in the 3′ end.

The RNA structures or sequences within the 3′ end of the IBV RNA required for the interaction are being studied. Further studies are also needed to determine the unique motifs on N protein that are required for the RNA protein interactions.

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