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Research Note—

In Vitro Analysis of a Hammerhead Ribozyme Targeted to Infectious Bronchitis Virus Nucleocapsid mRNA

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SUMMARY. Hammerhead ribozymes are catalytic RNA molecules that specifically cleave a target RNA molecule. Herein, we report the design, synthesis, and *in vitro* analysis of a hammerhead ribozyme targeted to the infectious bronchitis virus (IBV) nucleocapsid mRNA. At a concentration of 0.5 or 10 μM, the ribozyme, designated IBV-N-Rz, effectively cleaved target RNAs *in trans* (37 C, 10 mM $MgCl_2$, 50 mM Tris). Cleavage products were visualized by agarose gel analysis. The time course of the ribozyme reaction was monitored by agarose gel analysis and relative quantitative reverse transcription–polymerase chain reaction. The amount of target RNA continually declined over a 5-hr period, indicating that the ribozyme was truly catalytic. Although stability and delivery problems must be overcome, a hammerhead ribozyme targeted to the IBV nucleocapsid mRNA most likely has antiviral activity and may be an effective therapeutic/prophylactic reagent in the future.

RESUMEN. *Nota de Investigación*—Análisis *in vitro* de la ribozima tipo cabeza de martillo dirigida contra el ARN mensajero del nucleocápsido del virus de bronquitis infecciosa.

Las ribozimas tipo cabeza de martillo son moléculas catalíticas de ARN que cortan una molécula blanco de ARN en una región específica. Se reporta el diseño, síntesis, y análisis *in vitro* de una ribozima tipo cabeza de martillo dirigida contra el ARN mensajero del nucleocápsido del virus de bronquitis infecciosa. A una concentración de 0.5 o 10 μM, la ribozima, designada como IBV-N-Rz, cortó efectivamente las moléculas blanco de ARN *in trans* (37 C, 10 mM $MgCl_2$, 50 mM Tris). Se visualizaron y analizaron los productos cortados en un gel de agarosa. Se evaluó el tiempo requerido para la acción de la ribozima mediante el análisis en gel de agarosa y una prueba de cuantificación relativa de la transcriptasa reversa-reacción en cadena por la polimerasa. Se observó una disminución continua en la concentración de la molécula de ARN del nucleocápsido sobre un periodo de 5 horas, indicando la naturaleza catalítica de la ribozima. Aunque se deben resolver los problemas relacionados con la estabilidad y los métodos de aplicación, la ribozima tipo cabeza de martillo dirigida contra el ARN mensajero del nucleocápsido posee muy probablemente actividad antiviral y puede ser un reactivo profiláctico y terapéutico efectivo en el futuro.

Key words: infectious bronchitis virus, nucleocapsid mRNA, hammerhead ribozyme, antiviral, MFOLD

Abbreviations: ct = cycle threshold; EDTA = ethylenediaminetetraacetic acid; HIV = human immunodeficiency virus; IB = infectious bronchitis; IBV = infectious bronchitis virus; LCMV = lymphocytic choriomeningitis virus; Mass 41 = Massachusetts 41; MHV = mouse hepatitis virus; RT-PCR = reverse transcriptase–polymerase chain reaction; UTR = untranslated region

Ribozymes are catalytic RNA molecules that specifically cleave another target RNA molecule *in cis or trans.* Ribozymes occur naturally in pathogenic plant viroids (15), viruses (5), bacteria (3), protozoans (8), newts (13), and schistosomes (14). Many different types of ribozymes exist, including hammerhead, group I intron, hairpin, and others (12).

The focus of this study was the hammerhead ribozyme. Hammerhead ribozymes are made up of two components, a catalytic core and flanking sequences. The catalytic core is responsible for cleavage of the target RNA, while the flanking sequences determine the ribozyme specificity (15). Hammerhead ribozymes have been constructed as antiviral molecules against viruses from families including, but not limited to, the *Arenaviridae* (25,26), *Retroviridae* (16), *Birnaviridae* (1), *Paramyxoviridae* (2), *Picornaviridae* (20), and *Coronaviridae* (22).

Infectious bronchitis (IB) is an acute, highly contagious, upper respiratory tract disease in chickens. Clinical signs include tracheal rales, nasal exudate, coughing, and sneezing. The etiologic agent of IB is infectious bronchitis virus (IBV), a member of the *Coronaviridae* family. The virion is enveloped with club-shaped surface projections. The viral genome is a single-stranded, positive-sense, 27.5-kilobase RNA molecule. The genome codes for four major structural proteins: a spike glycoprotein, the integral membrane glycoprotein, the envelope protein, and the nucleocapsid protein that associates with the RNA genome (18).

As a result of the proposed multiple roles of *Coronavirus* nucleocapsid protein in replication (4,9,10), the interaction of IBV nucleocapsid proteins with the IBV viral genome (27), and the 3′ untranslated region (UTR) sequences necessary for IBV replication (11), we chose to target the mRNA encoding the nucleocapsid protein of IBV *in trans* with a hammerhead ribozyme. If functional *in vitro*, this molecule could hinder the production of nucleocapsid protein *in vivo* and possibly block IBV replication.

MATERIALS AND METHODS

Virus. The IBV strain used in this study was Massachusetts 41 (Mass 41) (17). The virus was propagated by allantoic sac inoculation of 9-to-11-day-old embryonating chicken eggs. After 48 hr of incubation, the allantoic fluid was harvested and stored at −70 C (24).

Primer and ribozyme sequences. The primers and ribozyme sequences used in this study are listed in Table 1.

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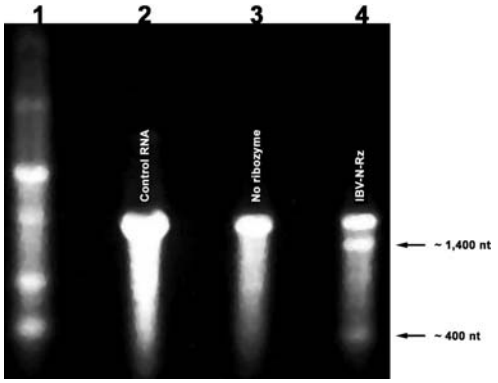

Fig. 1. Cleavage of nucleocapsid gene target RNA by IBV-N-Rz. Lane 1 = RNA ladder with bands of 9000, 7000, 5000, 3000, 2000, 1000, and 500 nt (New England Biolabs, Inc.); Lane 2 = Control RNA, kept on ice with no other chemicals added; Lane 3 = Target RNA incubated at 37 C for 1 hr with no ribozyme added; Lane 4 = Target RNA incubated at 37 C for 1 hr with IBV-N-Rz added.

Nucleocapsid and membrane gene reverse transcriptase–polymerase chain reaction (RT-PCR), cloning, sequencing, and target RNA production. Viral RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics Corp., Indianapolis, IN) and was used as template for RT-PCR (Titan One-Tube RT-PCR System, Roche Diagnostics Corp.), with a primer set (BeauU30, BeauL27585) that flanks the entire subgenomic nucleocapsid and membrane mRNA. The RT-PCR products were purified as previously described (7) and cloned (TOPO© XL PCR Cloning Kit, Invitrogen Corp., Carlsbad, CA). Clones containing the complete nucleocapsid and membrane gene, including the 3′ UTR of the IBV genome, were found by sequencing. Two clones, designated N-9 (nucleocapsid gene) and M-4 (membrane gene), were selected and used in all subsequent steps. The complete insert of the N-9 clone was sequenced (ResGen, Invitrogen Corp., Huntsville, AL) and assembled using MacDNASIS Pro V3.5 computer software (Hitachi Software Engineering Corp., San Bruno, CA). The N-9 clone had an insert that mapped to nucleotide positions 1 to 1651 relative to GenBank accession number M28566 (N-9 had a 49-bp insert at the 3′ end not present in the previously reported Mass 41 nucleocapsid gene sequence). The complete insert of the M-4 clone was not fully sequenced, but the ends were sequenced and the cloned insert mapped to nucleotide positions 24,418 to 27,608 relative to GenBank accession number M95169.

Target RNA was synthesized with the RiboMax Large Scale RNA Production System–T7 (Promega Corp., Madison, WI) using *Hin*dIII (New England Biolabs, Inc., Beverly, MA) linearized N-9 and M-4 clone DNA as the template. Production of correctly sized RNA was analyzed by agarose gel electrophoresis. Briefly, 200 ng of RNA was mixed with 5.0 µl of Tris-borate/ethylenediaminetetraacetic acid (EDTA) urea sample buffer (Bio-Rad, Hercules, CA), heated to 95 C for 4 min and then placed on ice. The mixture was then loaded onto a 1% agarose gel without formaldehyde (19) and electrophoresed for approximately 1 hr at 80 volts in 1× Tris-acetate/EDTA running buffer.

Ribozyme design and synthesis. A search for possible hammerhead ribozyme target sequences was performed using MacDNASIS Pro V3.5 computer software (Hitachi Software Engineering Corp.). Secondary structure predictions were performed using the MFOLD algorithm available over the Internet (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) (23,28). Regions with minimal secondary structure were defined using the single-stranded plot option. Then, specific hammerhead target sites were analyzed within those regions. An average value for the 10 nucleotides before and after the NUH (where N = any nucleotide and H = A, C, T, or U) target site was calculated. The target site and surrounding nucleotides having the highest average single strandedness was chosen as the hammerhead ribozyme target site. A hammerhead ribozyme complementary to this target site was chemically synthesized (Integrated DNA Technologies, Inc., Coralville, IA).

Table 1. Sequences and location of the primers and ribozyme used in this study.

Name	Sequence (5′→3′)	Position
BeauU30	TAC ACT AGC CTT GCG CTA GA	30–49[A]
BeauL27585	TGC TCT AAC TCT ATA CTA GCC TAT	27608–27585[A]
M41NF1	C**G**A GCG GTA AGG CAA CTG GAA AGA[B]	106–129[C]
M41NR1	ACC CTT ACC AGC AAC CCA CAC TAT	461–438[C]
M41NF2	ACG CCC AGA CTT CAA CCA GAT	963–983[C]
M41NR2	CGT TCG TTT CCA GGC TAC TAA	1441–1421[C]
IBV-N-Rz	CUU G**GU** UUU A*CU GAU GAG GCC GAA AGG CCG AA*A UUU UCA UUA U[BD]	295–274[C]

[A]Numbers represent the position of the primers relative to GenBank accession number NC_001451.

[B]Bold nucleotides represent differences in sequence for the Massachusetts 41 strain used in this study, as compared to the Massachusetts 41 nucleocapsid gene sequence reported in GenBank (M28566).

[C]Numbers represent the position of the primers and ribozyme relative to GenBank accession number M28566.

[D]The IBV-N-Rz target site sequence is 5′-AUAAUGAAAAU-**C**↓UAAAACCAAG-3′. The bold **C** does not hybridize with any of the IBV-N-Rz nucleotides, and cleavage occurs at the down arrow. Underlined nucleotides represent ribozyme flanking sequences that are complementary to the RNA target site. Italic nucleotides represent the ribozyme catalytic core sequence (not complementary to target site).

Ribozyme activity and time course assays. For determination of ribozyme activity, several different target RNAs were mixed with IBV-N-Rz, and its ability to mediate cleavage was assessed. The reaction components, incubation temperatures, and times are listed in Table 2. After the appropriate incubation time, 5.25 µl of 0.5 M EDTA was added, the tube was placed on ice, and the RNA was extracted as described above. Cleavage of the target RNA was analyzed by agarose gel electrophoresis (nucleocapsid and membrane RNA runoffs) or by relative quantitative RT-PCR (full-length genomic RNA) (LightCycler™ RT-PCR Kit, Roche Diagnostics Corp.).

To study IBV-N-Rz–mediated cleavage of target RNA over time, two time course assays were performed. For both time course assays, the reaction components, incubation temperatures, and times are listed in Table 2. The cleavage products were analyzed on a 1% agarose gel and by relative quantitative RT-PCR (LightCycler™ RT-PCR Kit, Roche Diagnostics Corp.). For RT-PCR, one primer set (M41NF1,R1) flanked the target site of IBV-N-Rz, while the other set did not (M41NF2,R2). The primer sets were used in a RT-PCR with the RNA extracted from each time point as the template.

RESULTS

Target RNA was produced from the cloned nucleocapsid and membrane gene of the Mass 41 strain of IBV using T7 RNA polymerase. By agarose gel analysis, cleavage of the target RNAs into appropriately sized products of 1400 nucleotides and 400 nucleotides for the nucleocapsid RNA (Fig. 1) and 1800 nucleotides and 1400 nucleotides for the membrane RNA (Fig. 2) were observed in the reactions containing the IBV-N-Rz. Full-length genomic RNA extracted from virus in allantoic fluid was also susceptible to cleavage in reactions containing the IBV-N-Rz, as measured by

Table 2. Reaction components and incubation times for ribozyme activity and time course assays.

Target RNA	Amount of target RNA (μg)	Final [IBV-N-Rz] (μM)	Incubation time (hr)	Assay
N gene RNA runoffs[A]	1.0	0.5	1	Activity
M gene RNA runoffs[A]	1.0	10.0	1	Activity
Full-length genomic RNA[A]	?[B]	0.5	1	Activity
N gene RNA runoffs[A]	1.0	0.5	0, 1, 2, 3	Time course 1
N gene RNA runoffs[A]	0.2	10.0	0, 1, 2, 3, 4, 5	Time course 2

[A]Each reaction contained target RNA and IBV-N-Rz as shown in the table, along with $MgCl_2$, 10 mM; Tris, 50 mM; and diethylpyrocarbonate-treated water added to a final volume of 100 μl. All the reactions were incubated at 37 C.
[B]An unknown amount of RNA was used as target RNA for this assay.

relative quantitative RT-PCR (data not shown). No cleavage was observed by agarose gel analysis or relative quantitative RT-PCR in any reactions that excluded the ribozyme.

To provide further evidence that IBV-N-Rz was actually catalytic, a time course experiment was performed using the nucleocapsid RNA runoffs as target RNA. For the initial time course assay, a final ribozyme concentration of 0.5 μM was used. Both agarose gel analysis (data not shown) and relative quantitative RT-PCR confirmed cleavage of the target RNA. The cycle threshold (ct) values for the RT-PCR analysis were as follows: 0 hr = 5.54, 1 hr = 5.93, 2 hr = 6.95, and 3 hr = 6.97. A second time course assay was performed using approximately fivefold less target RNA and a final ribozyme concentration of 10.0 μM. Both agarose gel analysis (Fig. 3) and relative quantitative RT-PCR using a primer set that flanked the ribozyme target site (Fig. 4A) showed a continual decline of full-length target RNA over time. The ct values for the RT-PCR analysis were as follows: 0 hr = 7.94, 1 hr = 9.82, 2 hr = 10.92, 3 hr = 11.56, 4 hr = 11.82, and 5 hr = 12.79. Using primers that did not flank the ribozyme target site, no difference in the amount of target RNA was observed by relative quantitative RT-PCR (Fig. 4B), indicating that the target RNA was specifically cleaved and not degraded.

DISCUSSION

In this study, we designed, synthesized, and tested a hammerhead ribozyme targeted against the nucleocapsid mRNA of IBV. During the course of our study, only one ribozyme was synthesized and analyzed. We believe that the MFOLD software program and careful analysis of the secondary structure prediction data was key in design and synthesis of an effective hammerhead ribozyme.

Cleavage at the proper target site was monitored by visualization of correctly sized cleavage products on an agarose gel. Further confirmation of cleavage at the target site was provided by relative quantitative RT-PCR using two different primer sets. One primer set flanked the cleavage site and showed a continual decline of full-length template RNA over time. The other primer set, which amplified a segment in the 3′ end of the template RNA, showed no decline over time. The second primer set provided proof that amplification differences observed for the first primer set flanking the IBV-N-Rz target site were due to cleavage and not template RNA concentration differences or degradation.

Using two different IBV-N-Rz concentrations (0.5 and 10.0 μM), target RNAs were effectively cleaved. Two different time course assays were performed using the two different target RNA and IBV-N-Rz concentrations. In the initial time course assay, the ct difference between the 0 time point and the 3-hr time point equaled 1.43. In the second time course assay, the ct difference between the 0 time point and the 3-hr time point equaled 3.62. This equates to approximately 30% more cleavage of the target RNA in the second time course assay within a 3-hr period. When comparing the two time course assays, there was a 100-fold increase of IBV-N-Rz to target RNA in the second assay, which explains the increased cleavage that was observed.

Although the optimal reaction conditions for the IBV-N-Rz were not explored, it appears that the association/cleavage rates of the ribozyme are high. As an example, cleavage of the target RNA was observed at time point 0 during the second time course experiment. Only 1 min passed between the addition of the IBV-N-Rz and

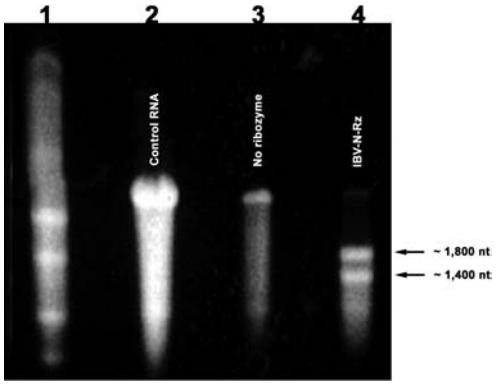

Fig. 2. Cleavage of membrane gene target RNA by IBV-N-Rz. Lane 1 = RNA ladder with bands of 9000, 7000, 5000, 3000, 2000, 1000, and 500 nt; Lane 2 = Control RNA, kept on ice with no other chemicals added; Lane 3 = Target RNA incubated at 37 C for 1 hr with no ribozyme added; Lane 4 = Target RNA incubated at 37 C for 1 hr with IBV-N-Rz added.

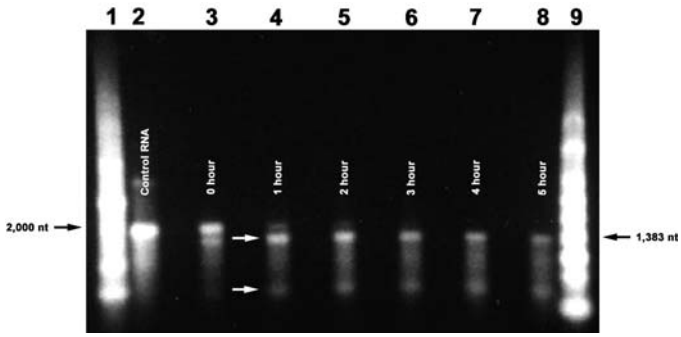

Fig. 3. Visualization of IBV-N-Rz reaction over time. White arrows indicate cleavage products. Lane 1 = RNA ladder, see Fig. 1 for band sizes (New England Biolabs, Inc.); Lane 2 = Control RNA; Lanes 3–8 = Target RNA incubated with IBV-N-Rz for increasing amounts of time; Lane 9 = RNA ladder with bands of 6583, 4981, 3638, 2604, 1908, 1383, 955, 623, and 281 nt (Promega Corp., Madison, WI).

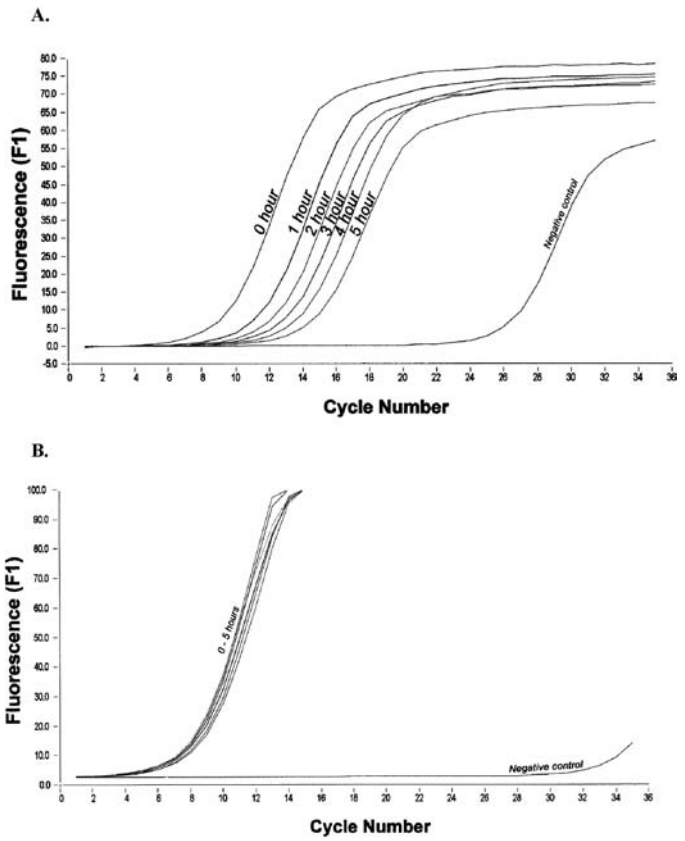

Fig. 4. Relative quantitative RT-PCR using template RNA taken from each time point of a time course assay. (A) Amplification graph of template RNA taken from each time point using primer set M41NF1,R1 that flanks the IBV-N-Rz target site. (B) Amplification graph of template RNA taken from each time point using primer set M41NF2,R2 that does not flank the IBV-N-Rz target site. Fluorescence for each reaction was measured after each PCR cycle by the LightCycler™ instrument (Roche Diagnostics Corp.). Amplification signal from correct product and primer dimer (negative control signal) was verified by melting curve and agarose gel analysis (data not shown).

inactivation by addition of EDTA to the tube. Therefore, 1 min was enough time for the IBV-N-Rz to associate and catalyze cleavage of the target RNA such that it could be discerned by visual examination on an agarose gel.

It is reasonable to believe that the IBV-N-Rz may have some level of antiviral activity *in vivo*. Since *Coronaviruses* have a 3′ co-terminal nested set of mRNAs, the IBV-N-Rz should be able to cleave all of the IBV mRNAs, including the full-length viral genome. Indeed, the IBV-N-Rz effectively cleaved the three different RNA substrates tested (nucleocapsid gene RNA runoffs, membrane gene RNA runoffs, and full-length genomic RNA extracted from virus in allantoic fluid). Cleavage of the nucleocapsid and other IBV mRNAs may hinder IBV replication, since nucleocapsid protein is reported to play a role in replication (4,9,10) and interacts with viral RNAs (27). In addition, the viral transcription recognition sites are located in the UTR at the extreme 3′ end of the viral genome (11).

Despite stability and delivery problems, ribozymes show great potential as antiviral therapeutic molecules (6). Published reports have shown that hammerhead ribozymes inhibited viral replication for three RNA viruses from different virus families; lymphocytic choriomeningitis virus (LCMV, *Arenaviridae*), mouse hepatitis virus (MHV, *Coronaviridae*), and human immunodeficiency virus (HIV, *Retroviridae*) (16,21,22,26). Replication of LCMV in cell culture was inhibited by the stable expression of a hammerhead ribozyme targeted to the S segment of the viral genome. The hammerhead ribozyme decreased the production of infectious virus by 100 times, and the antiviral activity was shown to be specific for LCMV (26). Replication of MHV in cell culture was inhibited by the stable expression of either of two different hammerhead ribozymes targeted to the polymerase gene. Inhibition of acute and chronic viral infection was shown (21,22). Lastly, replication of HIV in cell culture was inhibited by the stable expression of a hammerhead ribozyme targeted to the tat gene. Specifically, CD4+ cells stably transduced by a pseudotyped retroviral vector containing the anti-tat hammerhead ribozyme were able to resist HIV infection for up to 20 days (16). Although many hurdles remain, the promise of ribozymes as effective antiviral molecules makes them an attractive research area, and hopefully their full potential will be realized in the future.

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