# Inhibition of Viral Multiplication by Hammerhead Ribozymes Targeted against the Polymerase Gene of Mouse Hepatitis Virus

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ABSTRACT. We designed and constructed two hammerhead ribozymes targeted against the polymerase gene of mouse hepatitis virus (MHV). They consisted of a 22-nucleotide (nt) ribozyme core sequence and antisense sequences of different lengths, 243-nt (S-ribozyme) and 926-nt (L-ribozyme). In cell-free reactions, the constructed ribozymes cleaved the target RNA at a specific site. Vectors that directed the expression of ribozymes by a promoter of human elongation factor 1$\alpha$ were introduced into DBT cells, and the resulting several cell lines constitutively expressing the ribozymes were selected by Northern blot analysis and examined for intracellular multiplication of MHV. The production of infectious progeny virus particles was significantly reduced in the transfected cell lines expressing either S-ribozyme or L-ribozyme. Although the *in vitro* cleavage process of L-ribozyme was slower than that of S-ribozyme, no difference was observed in inhibitory effects on MHV multiplication between S- and L-ribozymes in the transfected cells.—KEY WORDS: antiviral agent, hammerhead ribozyme, mouse hepatitis virus (MHV).

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Mouse hepatitis virus (MHV) is a member of the *Coronaviridae* family that causes a variety of diseases, including encephalomyelitis, hepatitis and gastroenteritis in laboratory mice [1, 30]. In the replication cycle of MHV, virion RNA is initially transcribed into a full-length negative-stranded RNA by RNA-dependent RNA polymerase encoded at the 5' portion of virion RNA. In MHV-infected cells, three major structural proteins, the nucleocapsid protein (N) transcribed from mRNA7, the glycosylated transmembrane protein (M) from mRNA6 and spike glycoprotein (S) from mRNA3 [26] have been detected. The remaining viral proteins are RNA-dependent RNA polymerase [2] and other nonstructural proteins whose functions remain unknown [3]. The genomic RNA of MHV encodes RNA-dependent RNA polymerase, which plays roles in the synthesis of the genome-sized negative-stranded RNA at an initial stage of the viral replication cycle as well as of a full-length positive-stranded genomic RNA and subgenomic mRNA [2]. Therefore, the RNA polymerase is critical for viral multiplication.

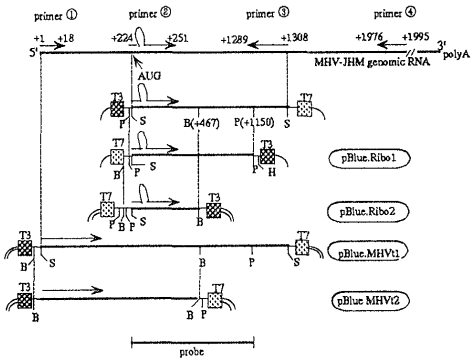
Ribozymes, antisense agents, are catalytic RNAs that are able to cleave the phosphodiester bond of the target RNAs in a sequence-specific manner [4]. The hammerhead ribozymes in plant viroids and virusoids contain two separable functional regions: a catalytic core region containing several conserved bases, which cleaves the target RNA, and flanking regions, which direct the ribozyme core to the specific target site by nucleic acid complementarity [9, 28]. Therefore, by attaching the core regions to sequences complementary to those flanking the selected target site, NUX (where N is any base and X is U, C or A), ribozymes can be designed to specifically cleave any target RNA molecules. Although ribozymes are worthy of evaluation as potential antiviral agents, they have been shown to exert an effect on only a few animal viruses, for instance, human immunodeficiency virus (HIV) [7, 22], arenavirus [31, 32], and prions [6].

To see the effects of ribozymes on viral multiplication, we constructed two hammerhead ribozymes with antisense sequences of different lengths that were expected to cleave the positive-stranded genomic RNA at the 5' portion. In the present study, we report that stable expression of hammerhead ribozymes targeted against the MHV polymerase gene led to effective inhibition of MHV multiplication.

## MATERIALS AND METHODS

*Cell and virus*: DBT cells [11] were cultured in Eagle's minimum essential medium (MEM) with 5% calf serum (CS) at 37°C in an atmosphere of 5% $CO_2$. The JHM strain of MHV [17] was propagated in DBT cells.

*cDNA synthesis and polymerase chain reaction (PCR) amplification*: The oligonucleotides used in this study were synthesized using the phosphoramidate method on a Beckman system Plus-1 DNA Synthesizer and purified by high-performance liquid chromatography (HPLC) (Fig. 1). First-strand cDNA was synthesized from virion RNA isolated by the method of Robb and Bond [21], using a primer oligonucleotide ④. Briefly, the RNA was denatured at 65°C for 15 min, chilled on ice for 3 min, and then incubated at 42°C for 120 min in 50 $\mu l$ of a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, four deoxyribonucleoside triphosphates (0.5 mM each), 4 mM DTT, 40 U of RNasin, 100 U of avian myeloblastosis virus reverse transcriptase (Promega), 5 $\mu$g of virion RNA and 5 $\mu$g of oligonucleotide ④. The cDNA was then used as a template for PCR amplification. Ten-microliters of the reverse transcriptase reaction mixture was mixed with 50 $\mu l$ of PCR buffer (Takara) containing 1U of Taq DNA polymerase, 1 $\mu$M each of four deoxyribonucleoside triphosphates, and 1 $\mu$M oligonucleotide primers either ② and ③, or ① and ③




| primer | sequence | polarity | corresponding site of MHV |
|---|---|---|---|
| ① | TATAAGAGTGATTGGCGT | + | 1-18 |
| ② | ATGGGCAAATACGGTTTCGTTCCT CACGGACTCATCAGTCGGCTTCAAAT | + | 224-251 |
| ③ | GAATCCGTATACAGCATAGT | - | 1289-1308 |
| ④ | AAAGCTTGACCACTCTTAAT | - | 1976-1995 |

Fig. 1. Construction of the ribozyme against the MHV polymerase gene. Oligonucleotide ② contained a hammerhead ribozyme core sequence (underlined). pBluescript II SK(+) (double line) and pBluescript II KS(+)(single line). The restriciton sites, B, *Bam*HI, S, *Sma*I, P *Pst*I, H, *Hin*dIII. AUG represents the putative initiation codon of the RNA polymerase gene. T3 and T7 represent the promoter regions of RNA polymerases T3 and T7, respectively. + and − polarity represent the sense and antisense directions of oligonucleotides toward the genomic RNA of MHV.

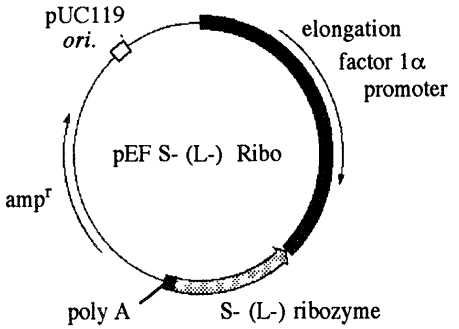



Fig. 2. Construction of expression vector of ribozymes, pEF S-Ribo and pEF L-Ribo. pEF 321 was ligated with S-ribozyme and L-ribozyme, which contained antisense sequences from +224 to +467-nt and from +224 to +1150-nt of the RNA polymerase gene of MHV-JHM, respectively. Poly A represents the SV40 poly A signal.

on Fig. 1.

*Construction of expression vectors for L-ribozyme, S-ribozyme and target RNAs*: To compare the cleavage efficiency of ribozymes with different lengths, we constructed vectors which expressed the ribozymes containing the 243- and 926-nt antisense sequences, referred to as S-ribozyme and L-ribozyme, respectively. To construct the L-ribozyme expression vector, the PCR product using primer oligonucleotides ②, containing the catalytic domains of hammerhead ribozymes, and primer ③ was cloned into pBluescript II KS(+) and the *Pst*I fragment of this plasmid was subsequently recloned into pBluescript II KS(+), pBlue.Ribo1 (Fig. 1). After the digestion of pBlue.Ribo1 with *Hin*dIII and *Sma*I, or *Bam*HI and *Sma*I, the 945-base pair (bp) and 267-bp fragments containing ribozyme sequences, were electrophoretically separated on agarose gel. Both fragments were filled in and then ligated into the expression vector, pEF321-T [13] after the removal of the SV40-largeT gene by digestion with *Hin*dIII-*Hpa*I and the formation of a blunt-end with a fill-in reaction. The constructed plasmids were referred to as pEF L-Ribo and pEF S-Ribo, respectively (Fig. 2). Another vector, which expressed the 243-nt antisense RNA and contained no ribozyme sequences, was constructed (pEF AS). For the synthesis of S-ribozymes *in vitro*, pBlue.Ribo1 was digested with *Bam*HI, and a 267-bp fragment was recloned into the *Bam*HI site of pBluescript II SK(+) in the antisense direction toward the T3 RNA polymerase promoter (pBlue.Ribo2) (Fig. 1). For construction of the vector which expressed the target RNAs, the PCR product using oligonucleotides primers ③ and ① was cloned into the *Sma*I site of pBluescript IISK(+), pBlue.MHVt1, and it was digested with *Bam*HI, and a 478-bp fragment was recloned into the *Bam*HI site of pBluescript II SK(+), pBlue MHVt2.

*In vitro transcription*: To synthesize target RNAs, 543-nt and 1214-nt target RNA (named S- and L-target RNA), pBlue.MHVt2 and pBlue.MHVt1 were linearized by *Pst*I digestion. To synthesize S-and L-ribozymes *in vitro*, pBlue.Ribo2 and pBlue.Ribo1 were linearized by *Pst*I and *Sma*I, respectively. The linearized DNA was extracted twice with phenol-chloroform (1:1) and then used as a template for RNA synthesis *in vitro*. The ribozyme RNAs were synthesized in a mixture contained 1.0 $\mu$g of linearized template DNA, 40 U of RNasin, ATP, GTP, CTP and UTP (10 mM each), 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 30 mM DTT, and 25 U of T3 RNA polymerase. To synthesize the radiolabeled target RNA, 1.85 MBq of [$\alpha$-$^{32}$P] UTP (specific activity 24.1 TBq/mmol) (ICN Biomedicals, Inc.) was used instead of 10 mM UTP. The mixture was incubated at 37°C for 30 min. The products were treated with 10 U of RQ1 RNase-free DNase (Promega) followed by phenol-chloroform (1:1) extraction and ethanol precipitation.

*Cleavage reactions in vitro*: The ribozymes and the target RNAs (0.01 $\mu$M) were mixed in a 12.5-$\mu$l reaction solution containing 50 mM Tris-HCl, pH 7.5. The RNAs were denatured at 95°C for 1 min and then chilled on ice for 3 min. The reaction mixtures were pre-warmed at 37°C for 3 min and $MgCl_2$ was added to the mixtures at final concentrations from 0 to 30 mM. The reactions were carried out at 4, 37 and 42°C for various incubation times as described in the figure legends. The reactions were stopped by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bro-

mophenol blue and 0.05% xylene cyanol), heating at 95°C for 1 min. The cleaved products were analyzed by electrophoresis on a 6% polyacrylamide gel containing 8.24 M urea [3]. Autoradiography was carried out with New RX Fuji Medical X-ray film.

*Transfection of the cells*: DBT cells ($1\times10^6$) were cotransfected with 10 $\mu$g of pEF S-Ribo, pEF L-Ribo or pEF AS, and $pSV_2$-Neo DNA by the standard calcium phosphate precipitation procedure [8]. One day after transfection, cells were trypsinized and transferred to several 60-mm tissue culture dishes and cultured in MEM containing 5% CS and G418 (1 mg/m*l*).

*Northern blot hybridization*: Cytoplasmic RNAs were extracted from DBT and each transfected cell lines according to the method of Silver *et al.* [24]. Ten microgram RNA samples were dissolved in a solution of 25 mM EDTA and 0.1% SDS, and these solutions were mixed with loading solution consisting of 42.5% deionized formamide, 4.6% formaldehyde, 2.5 mM morpholinepropanesulfonic acid (MOPS), 6.4 mM sodium acetate and 1.25 mM EDTA. After electrophoresis was performed on 1.0% agarose gel containing 5.5% formamide, the RNA molecules were transferred onto nitrocellulose membranes by the method of Maniatis *et al.* [16]. As a probe, the *Pst*I fragment of pBlue.Ribo1 containing cDNA corresponding to nucleotides 224 to 1150 of MHV genomic RNA and the ribozyme core sequence, was radiolabeled with [$\alpha$-$^{32}$P] dCTP by nick-translation [20]. Prehybridization, hybridization and washing were carried out as described previously [18].

*MHV-infection and plaque assay*: The DBT and transfected cells were seeded confluently on 96-well culture plates and washed twice with CS-free MEM, and were infected with MHV-JHM at a multiplicity of infection (m.o.i.) of 0.1 to 5.0 for 1 hr at 37°C. After washing twice with CS-free MEM to remove unadsorbed virus particles and adding MEM with 5% CS, the infected cultures were assayed for infectious progeny viruses by plaque assays at each time of postinfection according to the method of Hirano *et al.* [11].

## RESULTS

*Specific cleavage of MHV-RNAs by ribozymes in a cell-free system*: In the present study, hammerhead ribozymes were designed to cleave the GUC sequence at nucleotide 238 in MHV-genomic RNA encoding RNA-dependent RNA polymerase [2]. The ribozymic cleavage of the target MHV-RNA was confirmed by synthesis of the S-ribozyme (341-nt) consisting of a 22-nt catalytic domain flanked by complementary sequences against MHV-genomic RNA and the template plasmid sequences on both sides: 15-nt (+224 to +239 of genomic RNA of MHV) and 64-nt (pBluescript II sequences) at the 5' region, and 228-nt (+240 to +467 of genomic RNA of MHV) and 12-nt (pBluescript II sequences) at the 3' region, respectively (Fig. 1). We also transcribed the 543-nt S-target RNA corresponding to +1 to +467 of MHV genomic RNA flanked by template plasmid sequences on both sides: 64-nt (the 5' region) and 12-nt (the 3' region). When the mixture of substrate RNA and S-ribozyme were incubated at physiological pH and temperature in the presence of $Mg^{2+}$, two RNA bands with expected sizes, a 302-nt of the 5'-fragment (P1) and a 241-nt of the 3'-fragment (P2) were observed (Fig. 3). Increasing in the amounts of S-ribozyme relative to target RNA, the cleavage of the target RNA progressed. The cleavage reaction was dependent on the $Mg^{2+}$ concentration and temperature. The RNA target almost disappeared during incubation for 1 min at a 1:1 ratio of ribozyme to substrate in the presence of 20 mM $MgCl_2$ at 37°C (Fig. 4). In case of L-ribozyme, which contained a sequence complementary to +224 to +1150-nt of MHV genomic RNA and the plasmid sequence (64-nt at 5' portion), a specific cleavage of the substrate RNA was observed, while slower than in case of S-ribozyme. A

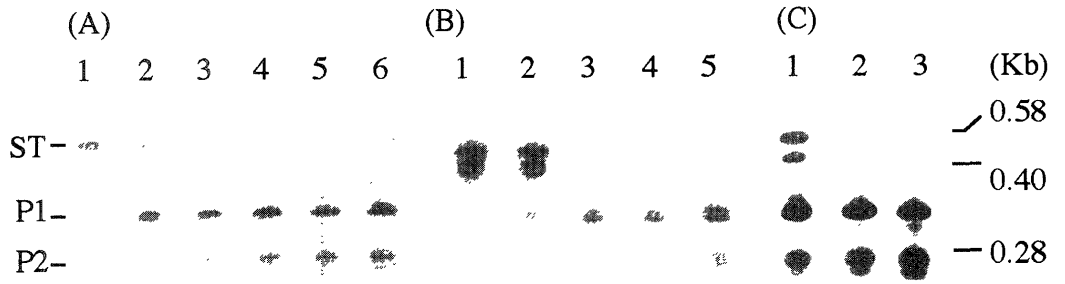



Fig. 3. Cleavage activity of S-ribozyme. S-target RNA (ST) (543-nt) was cleaved into two products by S-ribozyme. The 5' (P1) and 3' (P2) fragments were 302-nt and 241-nt long, respectively. The target RNA was labeled with [$\alpha$-$^{32}$P]UTP. (A) Cleavage reaction with the increasing molar ratio of ribozyme to target RNA, for 6 hr at 37°C in the presence of 20 mM $MgCl_2$. Lane 1, substrate RNA only; lane 2, molar ratio of ribozyme (R): target RNA (T) is 0.5:1; lane 3, 1:1; lane 4, 2:1; lane 5, 4:1 and lane 6, 8:1. (B) $Mg^{2+}$ dependence of ribozyme reactions. A mixture of equal amounts of ribozyme and target RNA was incubated for 6 hr at 37°C at concentrations of 0 mM (lane 1), 5 mM (lane 2), 10 mM (lane 3), 20 mM (lane 4) and 30 mM (lane 5) $MgCl_2$. (C) Temperature dependence of ribozyme reactions, for 6 hr with 20 mM $MgCl_2$ in the presence of equal amounts of ribozyme and target RNA at 4°C (lane 1), 37°C (lane 2) and 42°C (lane 3). The size of RNA was determined using an RNA marker (GIBCO BRL).

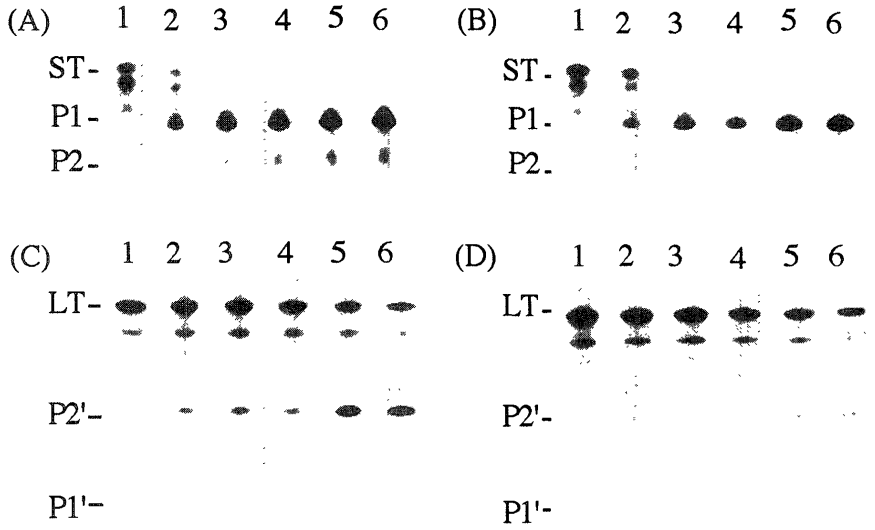



Fig. 4. Time kinetics of ribozyme reactions. The reaction mixtures of S-ribozyme (SR) and an equal amount of radiolabeled S-target RNA (ST) (A), L-ribozyme (LR) and radiolabeled ST (B), SR and radiolabeled L-target (LT) (C) or LR and radiolabeled LT (D), were incubated at 37°C in the presence of 20 mM $MgCl_2$ for 0 min (lane 1), 0.5 min (lane 2), 1 min (lane 3), 5 min (lane 4), 15 min (lane 5), or 30 min (lane 6). P1: 5'-cleavage product of ST (302-nt), P2: 3'-cleavage product of ST (241-nt), P1': 5'-cleavage product of LT (302-nt), P2': 3'-cleavage product of LT (912-nt).

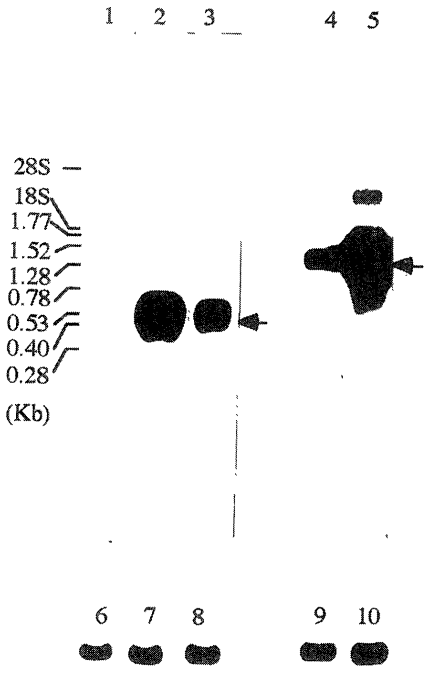



Fig. 5. Expression of S-ribozyme and L-ribozyme in the transfected cell lines. The cloned cell lines SR-1 and SR-2 were transfected with pEF S-Ribo, and LR-1 and LR-2 with pEF L-Ribo. Cytoplasmic RNAs were isolated from control DBT cells (lanes 1 and 6), SR-1 (lanes 2 and 7), SR-2 (lanes 3 and 8), LR-1 (lanes 4 and 9) and LR-2 (lanes 5 and 10). The RNA samples (10 $\mu$g) were analyzed by Northern blot hybridization using the *Pst*I fragment of pBlue.Ribo1 (Fig. 1) as a probe (lanes 1 to 5). Lanes 6 to 10 were rehybridized with $\beta$-actin cDNA as a probe after dehydridization of the filter. The size of RNA is shown on the left. The arrows indicate the ribozymes expressed in the transfected cells.

substantial amount of uncleaved RNA remained after incubation for 5 min. The cleavage process of the L-target RNA as well as the case of S-target RNA by L-ribozyme was also slower than that by S-ribozyme.

*Establishment of transfected DBT cell lines expressing ribozymes against MHV*: Vectors pEF S-Ribo and pEF L-Ribo, which expressed S-ribozyme and L-ribozyme, respectively, under the control of human elongation factor 1$\alpha$ promoter [13], were constructed (Fig. 2). DBT cells were co-transfected with pEF S-Ribo or pEF L-Ribo and $pSV_2$-neo, and G418-resistant clones were isolated. The clones that expressed ribozyme RNA were subsequently selected by dot blot hybridization (data not shown). Cytoplasmic RNAs were extracted from the selected

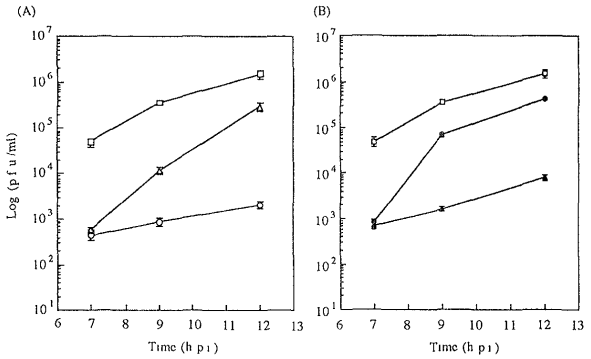



Fig. 6. Inhibition of virus production in the transfected cell lines expressing S-ribozyme (A) and L-ribozyme (B). Parental DBT cells (□) and transfected cell lines, SR-1 (○), SR-2 (△), LR-1 (●) and LR-2 (▲) were infected with MHV-JHM at an m.o.i. of 1.0 at 37°C. At 7, 9 and 12 hr p.i., the number of infectious progeny viruses in each culture medium was determined by plaque assay. Each point with bar represents average value with the standard deviation of four separate experiments.

clones, SR-1 and SR-2 cells transfected with pEF S-Ribo, and LR-1 and LR-2 cells transfected with pEF L-Ribo, and they were analyzed by Northern blot hybridization using the *Pst*I-fragment of pBlue.Ribo1 as a probe (Fig. 1), giving expected size of RNAs, of which the expression levels were different among the clones (Fig. 5). The AS cells, which were transfected with pEF AS, were also isolated.

*Challenge with MHV-JHM*: The transfected cell clones that expressed S-ribozyme or L-ribozyme were infected with MHV-JHM at a m.o.i. of 1.0, and the supernatant fluid was assayed for progeny virus by plaque formation at 7, 9 and 12 hr postinfection (p.i.) (Fig. 6). The yield of infectious progeny viruses was markedly lower in the transfected cell clones as compared with that in the untransfected DBT cells. Syncytia formation was also inhibited in the transfected cells, being undetectable at 9 hr p.i. (Fig. 7).

In the next experiments, each transfected cell clone was plaque-assayed the yield of the progeny viruses at 12 hr p.i. with MHV-JHM at m.o.i. of 0.1, 1.0 and 5.0 (Table 1). At 0.1 and 1.0 m.o.i., the yield of infectious progeny

Table 1. Effect of m.o.i. of MHV on inhibitory effect of ribozymes

| Cell | Yields of virus particles (p.f.u./ml*) | | |
|---|---|---|---|
| | m.o.i. | | |
| | 0.1 | 1.0 | 5.0 |
| DBT | $2.74\times10^6$ | $1.49\times10^6$ | $1.79\times10^6$ |
| SR-1 | $1.69\times10^4$ | $2.15\times10^4$ | $3.16\times10^5$ |
| SR-2 | $1.08\times10^5$ | $2.97\times10^5$ | $1.61\times10^6$ |
| LR-1 | $1.75\times10^5$ | $4.13\times10^5$ | $4.47\times10^6$ |
| LR-2 | $6.22\times10^4$ | $7.95\times10^3$ | $2.13\times10^4$ |
| AS | $3.20\times10^5$ | $1.18\times10^6$ | $1.13\times10^6$ |
| cont. | $2.54\times10^6$ | $3.96\times10^6$ | $4.08\times10^6$ |

*P.f.u/ml of the culture medium of each cells that infected with MHV-JHM was assayed at 12 hr p.i. Each value represents average of four separate experiments.

viruses was significantly lower in the transfected cell clones with the ribozyme sequences than in DBT cells and in those expressing the antisense RNA without ribozyme sequences. No inhibitory effect on MHV multiplication was shown in the control cell line, cont., which was transfected with pEF-321 containing neither ribozymes nor antisense sequences. At 5.0 m.o.i., the inhibitory effect of ribozymes on MHV multiplication was observed in SR-1 and LR-2 cells, but not in SR-2 and LR-1.

## DISCUSSION

Although the molecular mechanisms are still unclear, antisense oligonucleotides and RNAs have been used to study the functions of specific gene products [5], and antisense nucleic acids have been applied to control some viral infections, for example, with HIV [29], HSV [25], RSV [33], VSV [23], and MDV [12]. Recently, Mizutani *et al.* [18, 19] reported effective inhibition of viral multiplication by antisense oligonucleotide against MHV-leader RNA as well as antisense RNA against MHV-mRNA7 that encoded N protein. The antisense nucleic acids may inactivate the target molecules at best with 1:1 stoichiometry.

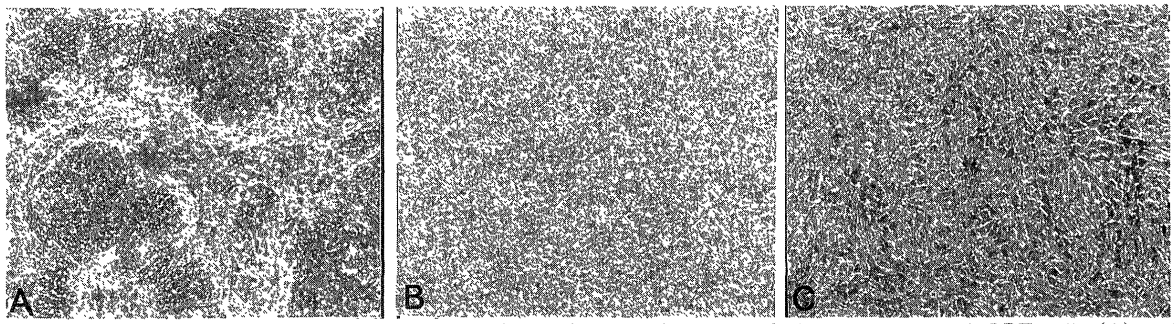



Fig. 7. Syncytia formation of MHV-JHM-infected cell lines which expressed ribozyme. Parental DBT cells (A) and transfected cell lines, SR-1 (B) and LR-2 (C) were fixed with ethanol at 9 hr p.i. with MHV-JHM at an m.o.i. of 0.1, stained with Giemsa and then photographed on a phase-differential microscope (× 10).

Since ribozymes catalyze RNA cleavage in a sequence-specific manner [6], they might be able to be more potent antiviral agents, as reported with HIV [7, 22], arenavirus [31], and prions [6]. The inhibitory effects of ribozymes on viral multiplication might be dependent on the type of viruses, target genes, length of antisense sequences against the target molecules, expression levels of the ribozymes and mode of infections. In the present study, the translation initiation site of the MHV RNA-dependent RNA polymerase gene was selected as a target of the ribozyme cleavage site, since the RNA polymerase synthesized at an initial stage of the MHV replication cycle, plays a role in both early and late transcriptions. In the transfected and ribozyme-expressing cell lines, the yield of infectious progeny viruses was markedly reduced, compared with that in untransfected DBT cells and those transfected with pEF-321. Therefore, the RNA polymerase gene might be an appropriate target of ribozymes for the inhibition of MHV multiplication.

The results in cell-free reactions showed that the specific cleavage of the substrate RNAs by the synthesized S-ribozyme depended on the ratio of ribozyme to substrate, $MgCl_2$ concentration and reaction temperature, in agreement with Xing and Whitton [31] and Kobayashi *et al.* [14]. The rate-limiting step in ribozyme activity appears to be unlike the cleavage step [4, 15]. The catalytic activity of ribozymes is influenced by a length of the flanking sequence complementary to the substrate RNA. Ribozymes with long antisense sequences might bind more effectively to the specific target site. However, a hybrid with a long complementary sequence between a ribozyme and its target RNA may result in suppressed cyclization of the ribozymic catalysis. Furthermore, a long ribozyme molecule may be liable to form a secondary complex structure, causing difficulty in evaluation of the interaction between substrate RNA and the ribozyme.

Although ribozyme activity was shown to increase with decreasing lengths of flanking sequences in cell-free reactions [10], Dropulic *et al.* [7] reported that the cleavage efficiency of the target RNA was the same between two ribozymes, containing either 90- or 840-nucleotide complementary sequences against the HIV1 gene. In the present study, we constructed S- and L-ribozymes with antisense sequences of 243-nt and 926-nt, respectively, against the MHV RNA polymerase gene. Although the cleavage process of L-ribozyme was slower than that of S-ribozyme in the cell-free reaction, no difference was observed in inhibitory effects on MHV multiplication in transfected cells between the two, suggesting that the inhibition of viral multiplication in infected cells did not depend on the length of the antisense sequence, but on other factors such as the amount and stability of intracellular ribozymes and their cytotoxicity, while toxicity of ribozymes was not observed in this study (data not shown). A high level of ribozyme expression may be necessary for the effective inhibition of viral multiplication [27]. We used human elongation factor $1\alpha$ promoter, which directs high levels of RNA expression in different cell types [13]. In the present study, the yield of progeny viruses was significantly lower in SR-1 and LR-2 cells expressing a high level of ribozymes than in SR-2 and LR-1 cells expressing lower level of ribozymes, indicating that the inhibitory effect on viral multiplication depended upon the amounts of the ribozymes.

The multiplication of MHV, a rapid-growing virus, was shown to be inhibited in the transfected cells that expressed ribozymes directed against the 5'-end of genomic RNA. Although the ribozymes cleaved the expected site of the target RNA in cell-free reactions, cleavage products was undetectable in the transfected cells infected with MHV (data not shown). Although possibility that the antisense sequence *per se* inhibits the expression of the RNA polymerase gene could not be excluded, viral multiplication in transfected and ribozyme-expressing cells was inhibited more efficiently than in the antisense RNA-expressing cells that contained no ribozyme core sequence, which is suggested to provide the catalytic cleavage activity with the antisense sequence in infected cells resulting in additional inhibition of MHV multiplication.

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