An Anti-Lymphocytic Choriomeningitis Virus Ribozyme Expressed in Tissue Culture Cells Diminishes Viral RNA Levels and Leads to a Reduction in Infectious Virus Yield

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Ribozymes, RNA molecules which cleave RNA in a sequence-specific manner, are a promising tool in the development of specific antiviral therapies. The viruses most susceptible to ribozymes may be those in which all aspects of the viral life cycle depend on RNA, with no DNA intermediate. Consequently, we have chosen as a model one such virus, the arenavirus lymphocytic choriomeningitis virus (LCMV), and have previously reported the design of specific anti-LCMV ribozymes (Z. Xing and J. L. Whitton, *J. Virol.* 66:1361-1369, 1992). Here we describe the establishment of several cell lines, each stably expressing an antiarenaviral ribozyme of different specificity. Expression of a single ribozyme leads to a reduction in LCMV RNA levels, and stimulation of ribozyme transcription amplifies the effect. Target site selection may be an important determinant of antiviral effectiveness, since the extent of the antiviral effect, measured by assay of viral RNA, varies with the specificity of the antiviral ribozyme expressed. Furthermore, infectious virus production is reduced approximately 100-fold. This effect is LCMV specific, as yield of a related arenavirus is not similarly curtailed. We are currently investigating the mechanism underlying the ribozyme-mediated antiviral effect.

The development of specific antiviral therapy would be a landmark advance in medical care, but a major stumbling block has been the achievement of specificity; compounds toxic to a virus are most often toxic also to the host in which these obligate intracellular pathogens reside. Recently antisense technologies, in which specificity is conferred at the level of nucleic acid sequence, have been tested for therapeutic potential. One such approach utilizes ribozymes, RNA molecules which cleave other RNAs in a sequence-specific manner. There are several classes of ribozyme, defined by primary (and proposed secondary) structure; one class is the hammerhead, found *in vivo* in plant viroids and virusoids and in transcripts of newt satellite DNA (7, 10, 12, 30). In their natural environments, these enzymes cleave intramolecularly (i.e., in *cis*), cutting 3' to a required sequence, usually GUC. Both of these requirements are less than absolute; hammerhead ribozymes can be made to cut intermolecularly (i.e., in *trans*), and the cleavage site can be UX (where X = A, C, or U [21, 48]). Hammerhead ribozymes contain two functional modules: a catalytic core region which cleaves the target RNA and contains several conserved bases, mutation of which abolishes activity (34); and flanking regions which, by nucleic acid complementarity, direct the ribozyme core to a specific target site. By exploiting the flexibility of these two modular functions, ribozymes can be designed to specifically cleave almost any target RNA molecule (17, 44, 48); the hammerhead catalytic core is flanked by sequences complementary to those adjoining essentially any selected UX target site, resulting in the desired sequence-specific ribozyme.

Although the delivery of ribozymes represents a major hurdle to be overcome in the future, it is vital to first determine whether ribozymes, when introduced into cells, can exert noncytotoxic antiviral effects and, if so, to dissect

and optimize this function. Their potentially great advantage of inbuilt specificity alone mandates careful and thorough research. Such research should be undertaken on several virus families, whose relative susceptibilities may differ depending on their genome structure (DNA/RNA; double or single stranded) and their mode and site of replication. Ribozymes have been shown to exert a limited effect on one animal virus, the human retrovirus human immunodeficiency virus type 1 (HIV-1), in cell culture; an anti-HIV-1 (anti-*gag*) ribozyme stably expressed in a cell line was shown (by using polymerase chain reaction) to cleave target RNA at the appropriate site and to have diminished levels of RNA and protein (39), and two groups have independently shown that ribozymes introduced by using a retroviral vector led to decreased HIV-1 reverse transcriptase activity (8, 45). However, HIV has in its replication cycle a DNA stage, which will be insensitive to ribozyme assault. Postulating that RNA viruses with no DNA stage may be most vulnerable to ribozyme attack, we have begun to use ribozymes directed toward the RNA genome, antigenome, and mRNAs of one such virus family, the arenaviruses.

Arenaviruses, named for their sandy appearance by electron microscopy, are a group of enveloped RNA viruses which replicate in the cytoplasm, have no nuclear phase or DNA intermediate, and present an excellent potential target for ribozyme attack. Lymphocytic choriomeningitis virus (LCMV), the prototype of the family, has a small (ca. 11-kb) bisegmented single-stranded RNA genome. The short (S) segment is ambisense (4), encoding two proteins in convergent transcription units: a 498-amino-acid precursor glycoprotein (GP-C) in the positive sense, and a 558-residue nucleoprotein (NP) in the negative (36). GP-C undergoes posttranslational cleavage to yield the two mature virus polypeptides, GP-1 and GP-2 (6). The long (L) segment also is ambisense and encodes two proteins, the large L protein, the putative viral polymerase (35), and the Z protein, a zinc-containing protein of unknown function (37). We have

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previously described hammerhead ribozymes specific for the S segment of LCMV (48); by using computer analyses, the target sites in the viral molecule were selected for lack of apparent secondary structure and were highly susceptible to ribozyme cleavage *in trans*.

We report here the establishment of stable cell lines, each expressing one of three anti-LCMV ribozymes (Rib3, Rib4, and Rib5), whose precise specificities are described elsewhere (48). Transcription is driven by a promoter which contains a glucocorticoid response element (GRE) and thus can be increased by steroid administration. In all cases, several independently derived, stably transformed clones were obtained, resulting in the establishment of several cell lines for each ribozyme. We have analyzed the effects of the ribozymes on production of LCMV RNA and find that an antiviral effect is clearly demonstrable, that it is increased by increasing the level of ribozyme transcription in the cell lines, and that it is virus specific. Most importantly, we find also that the yield of infectious virus from a ribozyme-expressing cell line is markedly reduced in comparison with the yield from ribozyme-negative cell lines; this antiviral effect is LCMV specific, as production of a related arenavirus is unaffected.

MATERIALS AND METHODS

Cell lines. BHK cells and Vero cells were grown in RPMI with 7% fetal calf serum, L-glutamine, and penicillin-streptomycin. NIH 3T3 cells (ATCC CRL 1658) were obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium supplemented with L-glutamine, 7% calf serum, and penicillin-streptomycin.

Virus infections and titrations. The Armstrong strain of LCMV was used in these studies. Pichinde virus was kindly provided by M. J. Buchmeier (Scripps Research Institute). Plaque-purified viruses were grown in BHK cells. Arenavirus titrations were carried out in Vero cells. Serial dilutions of virus were plated onto Vero cells and covered with an overlay of 1% agarose-1× medium 199; 6 days later, the monolayer was fixed and stained.

Cloning procedures. All cloning was done in the standard manner and in accordance with manufacturers' recommended procedures.

Establishment of cell lines which stably express ribozymes. The DNA sequences encoding the ribozyme were cloned into the vector pMAMneoBLUE (Clontech, Palo Alto, Calif.), which encodes the protein conferring resistance to neomycin analogs, and 5 to 10 µg of the resulting plasmid DNA was introduced into NIH 3T3 cells, using a standard calcium phosphate precipitate procedure. Cells were maintained in normal medium for 3 days, following which they were maintained in medium containing the selective antimitabolite G418 (GIBCO) at a working concentration of 400 µg/ml; 10 to 16 days later, individual colonies could be seen. These were picked and transferred to single wells of a six-well plate, from which they were expanded upon confluence. For each of the three ribozyme constructs, 12 colonies were picked and expanded in this way.

Comparison of LCMV RNA levels in various cell lines. Parental NIH 3T3 cells or ribozyme-expressing cell lines were grown for 5 days in the absence of G418 and then were infected with LCMV at a multiplicity of infection (MOI) of 0.5. At the time points indicated in the individual experiments, cytoplasmic RNA was harvested as described elsewhere (23), and equal quantities of RNA were electrophoresed on a 1.5% agarose-6% formaldehyde gel. Following

transfer to nitrocellulose and baking, the blot was hybridized with probe, washed, and exposed to photographic film. In all cases, probes were made by random priming of LCMV cDNA templates from the 3' half of the viral S segment; such probes, when denatured, will detect NP mRNA and the S (genomic) and S' (genome-complementary) segments; the latter two are of the same size and therefore appear as a single band on a Northern (RNA) blot.

Comparison of yields of LCMV from ribozyme-positive and ribozyme-negative cell lines. Cells were infected as described above, but at 2, 12, 24, 36, 48, and 72 h postinfection 1 ml of the cell supernatant was removed and titrated for virus, using Vero indicator cells. When LCMV was compared with Pichinde virus, the supernatant was harvested and titrated 48 h postinfection.

RESULTS

Targeting of anti-LCMV hammerhead ribozymes. We have previously described (48) the cloning and *in vitro* activity of the three ribozymes used in the present studies. Figure 1A shows the structure of a hammerhead ribozyme; the ribozyme is shown in black type, and the target is shown in gray. Three target sites on the LCMV genome were defined by two procedures. First, the RNA sequence was screened for the dinucleotide UX (X = C, U, or A), the minimal sequence to allow hammerhead ribozyme cleavage (21, 48) (a G would allow base pairing to the adjacent C in the ribozyme and prevents ribozyme cleavage). Second, since secondary structure around the RNA target site can prevent ribozyme cleavage (48), the RNA sequence was analyzed by using a computer-based program (RNAfold) which predicts regions involved in secondary structure; UX sites present in open areas of the virus were selected for further analysis. The locations on the 3,376-base LCMV genome of the three ribozymes sites, along with their sequences, are shown in Fig. 1B. The cleavage sites (UC) are in lowercase type.

Expression of ribozymes in stable cell lines. Each of the three ribozymes was cloned into the expression vector pMAMneoBLUE (Fig. 2), which contains the selectable marker Neo^r and thus allows transformation of cells by a single plasmid carrying both the DNA of interest and the selectable marker. The ribozyme DNAs were cloned into the multiple cloning site of the plasmid, and their orientation was confirmed by DNA sequencing. The ribozyme-containing RNA is driven by the Rous sarcoma virus enhancer coupled to the mouse mammary tumor virus (MMTV) enhancer/promoter; the MMTV enhancer contains a GRE, stimulation of which by certain steroids leads to a 5- to 10-fold elevation in transcription. Northern blot analysis of cytoplasmic RNA from transformed lines, probed with ribozyme-specific sequence, revealed a very faint band of the expected size (not shown).

Stably expressed ribozymes diminish LCMV RNA levels. Ribozyme-expressing cells and the parental (ribozyme-negative) NIH 3T3 cell line were infected with LCMV (MOI of 0.5), and 48 h later RNA was harvested. For all samples, RNA was electrophoresed in a 6% formaldehyde gel, transferred to nitrocellulose, and subjected to Northern blot analysis using a probe specific for the LCMV NP (Fig. 3A). As expected, two bands are detected; the upper band represents the LCMV S and S' segments, and the lower represents the NP mRNA. In comparison with the control, there is a marked diminution in signal in the cell lines expressing ribozymes.

FIG. 4. Time course of viral RNA levels in ribozyme expressing and control cells. Northern blot analysis, using an LCMV NP-specific probe, was carried out on RNA from ribozyme-negative cells (NIH 3T3) or from ribozyme-expressing cells (NIH 3T3-Rib5.12). For each cell line, RNA was harvested at 2, 12, 24, 36, 48, and 72 h postinfection. The positions of 28S and 18S rRNAs are shown, and the S and NP mRNA bands, visible on the autoradiograph, are indicated.

degradation by intracellular nucleases, perhaps proceeding from the newly exposed 5' termini. Several other groups, working mostly with HIV, have also reported difficulty in demonstrating cleavage products within infected cells and have resorted to polymerase chain reaction-based techniques to demonstrate probable cleavage. We have not at this point attempted to detect cleavage products by methods other than Northern blot analysis. The possibility remains that cleavage does not occur and that these ribozyme sequences exert their antiviral effects mainly by an antisense mechanism; we have preliminary data (not shown) that this is not the case, but regardless of the mechanism, the presence of the ribozyme clearly alters viral RNA levels. The extent of the effect appears relatively stable within different subclones of a cell line; four separate subclones of line Rib5.12 were isolated and expanded. Experiments identical to those described above revealed very similar reductions in LCMV RNA levels (Fig. 3B).

Time course of viral RNA levels in ribozyme-expressing and control cells. By using the cell line Rib5.12, an experiment similar to the one described above was carried out, but RNA was harvested at several time points (Fig. 4). The ribozyme prevents the accumulation of viral RNA from an early stage postinfection.

Stimulation of ribozyme transcription leads to further reduction of LCMV RNA levels. The MMTV promoter contains a GRE, and treatment with 10 μ M dexamethasone will stimulate transcription from this promoter. Parental cells or Rib5.12 cells were treated with 10 μ M dexamethasone and then infected with LCMV, and virus RNA levels were measured; the results of Northern blot analyses are shown in Fig. 5. Steroid treatment has a minimal effect on LCMV

FIG. 5. Evidence that dexamethasone treatment leads to further reduction of LCMV RNA levels. Ribozyme-negative cells (NIH 3T3) or cell lines stably expressing ribozyme 5 (NIH 3T3-Rib5.12) were infected with LCMV in the presence or absence of 10 μ M dexamethasone; 48 h postinfection, RNA was harvested and subjected to Northern blot analysis using an LCMV NP-specific probe as before. Locations of the 28S and 18S rRNAs are indicated, as are those of the S genomic RNA and NP mRNA.

RNA levels in parental cells (compare lanes 1 and 3), indicating that virus replication is not directly affected by dexamethasone, but in Rib5.12 cells, the already diminished LCMV RNA levels (lane 4) are markedly decreased in steroid-treated cells (lane 2).

Ribozyme expression leads to a marked diminution in yield of infectious LCMV. To determine whether the reduction in LCMV RNA was reflected in decreased yields of infectious virus, each cell line (parental or ribozyme expressing) was infected with LCMV (MOI of 0.5), and the supernatant was harvested at the time points shown in Fig. 6; for each time point, LCMV was titrated in Vero indicator cells. There is an almost 100-fold reduction in LCMV yield from Rib5.12 cells at 48 h. This experiment has been repeated on three occasions, with essentially identical outcomes.

FIG. 6. Reduction in yield of infectious LCMV from cell lines expressing Rib5. Ribozyme-negative cells (NIH 3T3) or cell lines expressing Rib5 (NIH 3T3-Rib5.12) were infected with LCMV at an MOI of 0.5, and the supernatant was harvested at 2, 12, 24, 36, 48, and 72 h postinfection. The supernatants were titrated on Vero indicator cells under an agarose overlay as outlined in Materials and Methods.

FIG. 7. Virus-specific effect of the anti-LCMV ribozyme. (A) Target sequence for Rib5 on the LCMV S segment and the equivalent region from Pichinde virus, a related arenavirus. The 5'-UC-3' cleavage site on LCMV is shown in lowercase. Identical bases are indicated by asterisks. (B) Yield of infectious LCMV or Pichinde virus 48 h after infection of NIH 3T3 or NIH 3T3-Rib5.12 cells.

The effect of the anti-LCMV ribozyme is virus specific. To determine whether the antiviral effect seen in the experiments described above is specific, a similar experiment was carried out by using Pichinde virus, a related arenavirus. Figure 7A shows a comparison of the Rib5 target sites on LCMV and Pichinde virus. There are eight differences in the sequences which flank the cut site; most probably the anti-LCMV ribozyme would bind poorly, if at all, to Pichinde virus. There is also a ninth difference, which alters the UC cleavage site in LCMV (lowercase in Fig. 7A) to CC in Pichinde virus; such a change would render the latter virus completely insensitive to ribozyme cleavage. Comparison of the growth of Pichinde virus in parental cells and in Rib5.12 cells demonstrates that, in contrast to LCMV, there is no significant difference in virus yield from the two cell lines (Fig. 7B). Thus, the antiviral effect of the ribozyme appears LCMV specific and sequence dependent.

DISCUSSION

The development of specific antiviral treatment would be a boon to medical care, but the question of specificity has been and remains a problem in the development of such compounds. Antiviral specificity can be achieved in at least three ways. First, immunological techniques in which virus proteins are the targets can be used. Vaccination has resulted in remarkable reductions in the incidence of several viral diseases, particularly in the developed nations, and has led to the eradication of one major scourge, smallpox. With the second approach, chemotherapy, success has been

rather more limited, and the specificity barrier has been problematic. For the most part the approach has been empirical, with researchers screening a wide variety of compounds for noncytotoxic antiviral effects; again the targets have been the viral proteins. Recently a third approach, using antisense techniques to achieve specificity; has been taken; here the target is viral nucleic acid. Using the LCMV model, we have utilized the first approach to dissect the immunological requirements for vaccine-mediated antiviral protection (20, 29, 46, 47) and have recently begun to evaluate the third approach (48).

Antisense approaches, using both oligonucleotides (1, 5, 15, 19, 24, 26, 31, 38, 42) and ribozymes (13, 16, 22, 33, 39-41, 48), have shown promise, and with the burgeoning data bases of both virus and human nucleic acid sequences, the time is ripe for a systematic assessment of antisense methods of antiviral prophylaxis and treatment. This growth in sequence data provides two advantages: first, more information about virus sequences increases the available target size against which ribozymes may be designed, and second, with more data from the human genome, it should be possible to more confidently assert that an antiviral ribozyme will not cleave host RNAs.

Reasoning that RNA viruses may be most susceptible to ribozymes, we have designed ribozymes against the arenavirus LCMV, a bisegmented single-stranded RNA virus which replicates in the cytoplasm, with no known nuclear or DNA stage. In vitro, the anti-LCMV ribozymes are efficient and specific in their activities (48); here we extend our observations to a tissue culture model. We have expressed the ribozymes from a standard eukaryotic expression vector and have selected cell lines which stably express the ribozymes. The transformed cells grow at the same rate as, and have plating efficiencies similar to those of, the parental cell line, indicating that ribozyme expression is not toxic to the cell. These cell lines are less able to support LCMV replication, as judged by a marked diminution in LCMV RNA levels, and an approximately 100-fold decrease in the yield of infectious virus.

The first step in designing antiviral ribozymes is the identification of target sites on the viral RNA molecule which are likely to be cleavable and accessible in the infected cell. This itself requires at least two steps. First, the sequence specificity required for ribozyme cleavage must be determined. For hammerhead ribozymes this is 5'-UX-3' (21, 48) rather than the more commonly utilized 5'-GUC-3'. Although this distinction may appear trivial, it can be important, since not all sites selected on the basis of sequence will in fact be cleaved by ribozymes; we have shown that an apparently ideal target sequence (containing 5'-GUC-3') was refractory to cleavage in vitro by a fully functional ribozyme because of the target site's involvement in secondary structure (48). Therefore, the second step that we have employed is to screen the target RNA molecule for potential secondary structure and to consider only those target sites which, with their flanking residues, are in open regions of the target molecule. In the case of the LCMV S and S' segments (a total of 6,752 bases), we found 273 incidences of 5'-GUX-3'; when considered along with their flanking residues, none were in open regions of the RNA (48). Only by reverting to the cut site 5'-UX-3' did we identify several potential open targets, including Rib3, Rib4, and Rib5, which we used in this study.

The mechanism underlying the antiviral effect of these ribozymes in tissue culture remains to be determined. These ribozymes cleave LCMV RNAs with high efficiency in vitro

(48), and it is tempting to conclude that this cleavage is responsible for the antiviral effect seen here. We fail to detect, by Northern blot analysis, the products of target RNA cleavage; however, this mirrors the findings of other groups and may reflect rapid degradation of these products by nucleases acting on the newly generated molecular termini. Although it remains possible that the antiviral effect is mediated at least in part by inactivation of target RNA function through simple hybridization between the ribozyme and target RNAs and that cleavage does not occur, it seems unlikely that hybridization would take place in the absence of cleavage; our *in vitro* data show that these ribozymes cleave target molecules very rapidly (48), and others have pointed out that ribozyme cleavage is extremely efficient and that the rate-limiting step in ribozyme activity is not cleavage but rather either binding of ribozyme to target or the release of the cleaved products by the ribozyme (27, 49). Nevertheless, the situation may differ in tissue culture cells, and it remains possible that the anti-LCMV effect seen here is attributable to both cleavage and hybridization mechanisms. We have preliminary data (not shown) that a negative-sense ribozyme, which has no cleavage activity but is still complementary to LCMV RNAs, has no detectable antiviral effect, suggesting that cleavage may be important. The absence of effect on Pichinde virus does not address this issue, since there are marked differences in the flanking regions which would probably reduce hybridization of the anti-LCMV ribozyme to the Pichinde virus RNA; thus, neither mechanism is available.

Many questions remain concerning optimization of ribozyme structure. For example, what is the optimal length of the flanking regions which bind ribozyme and target? If the flanking region which targets the ribozyme is too short, there is a risk that the ribozyme may perfectly match a host RNA, with toxic consequences. This risk may be overcome by increasing the length of the flanking sequences, but this may itself cause two other problems. First, if the ribozyme-substrate complex is too stable, the cleavage products may not dissociate, and in consequence the ribozyme will be unable to inactivate more than one target molecule; the dissociation step has been identified as the rate-limiting step in ribozyme activity (27). Second, if the flanking sequences are too long, then hybridization with imperfectly matched sequences may be sufficiently stable to allow cleavage to occur; this would reduce the effective specificity of the molecule. Thus, the optimal length of flanking sequences lies somewhere between these two extremes; this complex problem is more fully discussed elsewhere (18). In the ribozymes described here, the flanking sequences are 16 bases in length (8 to either side of the dinucleotide cleavage site). We have not yet assessed the effects of altering this variable. A further structural question is whether ribozymes are more effective when expressed as short free RNAs or when contained within relatively lengthy polyadenylated transcripts. The effects of long sequences flanking the ribozyme are unknown; they could reduce ribozyme effectiveness by decreasing the ability of the ribozyme to gain access to its target, or conversely, the long flanking sequences may stabilize the ribozyme, prolonging its half-life and hence augmenting its effectiveness. In the constructs described herein, the ribozymes are contained within a transcript of approximately 650 bases but nevertheless exert a marked anti-LCMV effect. We are presently evaluating the antiviral efficacy of Rib5 when expressed as a free molecule, with minimal foreign flanking sequences. Furthermore, the effect of dose must be analyzed. We show here that dexametha-

sone treatment has no effect on LCMV RNA levels in ribozyme-negative cells but results in a further decrease of LCMV RNA levels in ribozyme-expressing cells, in which stimulation of the GRE within the MMTV promoter will up-regulate transcription of the ribozyme-containing mRNA. These data are consistent with, though they do not prove, a dose-response relationship. In this model, we have used an RNA polymerase II promoter to drive ribozyme transcription; a polymerase III promoter may offer the chance of higher (and ubiquitous) expression levels. Other workers have used a polymerase III system to express antisense molecules and have demonstrated significant inhibition of viral replication (43). Thus, the optimal delivery system, even in a relatively simple tissue culture model, remains unresolved.

Having designed effective ribozymes and having determined how best to deliver them within an infected cell, one must next consider the optimization of their antiviral effect. One potential problem is the emergence of ribozyme-resistant viruses. For other antiviral agents, the appearance of resistant variants is well documented; examples include zidovudine- and ddI-resistant HIV (11, 14, 32) and herpesviruses resistant to acyclovir or ganciclovir (3, 9, 25). A similar problem may exist for ribozymes, particularly since RNA viruses have a high mutation rate (around 10^{-4} to 10^{-5} per base per generation), which may allow them to evolve more rapidly into escape variants. Thus, the RNA viral genome could undergo mutation at or around the target site, allowing escape from the unwelcome attentions of the catalytic RNAs. The extent of flanking region mutation required to abrogate ribozyme activity is unknown; however, recent studies showed that a synthetic ribozyme, which shared a 22-base complementarity with its target, lost its ability to cleave a target RNA which differed at 4 of the 22 bases (2). If virus escape can indeed occur, it might be minimized in at least two ways. First, virus escape could be diminished by targeting a single ribozyme to a conserved region of the viral genome, in which mutation might be lethal. In these studies, we have expressed in each cell line a single ribozyme specificity, against regions of the genome not known to be particularly highly conserved. Although we find a marked reduction in virus yield, significant numbers of LCMV are still produced. We are currently determining whether the viruses which emerge from the Rib5.12 cell line are ribozyme-resistant variants or are simply leak-through of wild-type LCMV. A second method to minimize virus escape may be the simultaneous expression of several ribozymes, each specific for a different cleavage site on the virus; to allow virus escape, several mutations would have to appear essentially simultaneously on a single viral genome. This is similar in principle to combination drug therapy, which has been used to suppress the emergence of resistant HIV (reviewed in reference 28). From the foregoing discussion, it is clear that there are several possible explanations for our observation (Fig. 3A) that the three ribozymes have different effects on LCMV RNA levels. For example, it could reflect different activities due to different cleavage site accessibility, different binding efficiencies due to the different compositions of the flanking regions, or different rates of escape mutants at the different target sites. These and other possibilities are being pursued.

In summary, although the precise mode of action of these ribozyme sequences remains to be determined, it is clear that they exert a marked antiviral effect. In tissue culture, the expression of a single antiviral ribozyme contained in a longer mRNA molecule is sufficient to diminish LCMV RNA

levels and to reduce yield of infectious virus by approximately 100-fold. Given that, as outlined above, many steps remain to be taken in optimizing the system, our results suggest that ribozymes may become useful tools in prevention and perhaps treatment of diseases caused by RNA virus infection.

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