Efficient Hammerhead Ribozymes Targeted to the Polycistronic Sendai Virus P/C mRNA

STRUCTURE-FUNCTION RELATIONSHIPS*

Denise K. Gavin‡§ and Kailash C. Gupta¶
From the Department of Immunology/Microbiology, Rush Medical College, Chicago, Illinois 60612

The Sendai virus polycistronic P/C mRNA encodes the P and C proteins from alternate overlapping reading frames. To determine the functions of these proteins in virus replication, hammerhead ribozymes were targeted to cleave the 5’-untranslated region of the P/C mRNA. Both cell-free and intracellular assays were employed to determine ribozyme efficacy. To appropriately compare activities between cell-free and intracellular assays, identical ribozymes were synthesized in vitro as well as expressed in cells. Ribozyme parameters, namely hybridization arm length (HAL) and nonhybridizing extraneous sequences (NES), were found to have rate-determining properties. In cell-free reactions, ribozymes with 13-mer HAL were up to 10-fold more efficient than those with 9-mer HAL. Ribozymes with 9-mer HAL were relatively ineffective in transfected cells. Minimizing the number of NES increased ribozyme efficiency in vitro. However, ribozymes with minimal NES were essentially inert intracellularly. The NES at the termini of the most effective intracellular ribozyme, Rz13st (~95% inhibition of the p gene expression), were predicted to fold into stem-loop structures. These structures most likely increase ribozyme stability as evidenced by the 8-fold higher resistance to ribonuclease T2 digestion of Rz13st compared with Rz13B.

Our results suggest that when designing effective intracellular ribozymes, parameters that enhance formation of productive ribozyme-substrate duplexes and that increase RNA stability should be optimized.

Sendai virus is a prototypic paramyxovirus that replicates exclusively in the cytoplasm. The single strand negative-sense RNA genome of the Sendai virus encodes at least six genes, np, p, m, f, hn, and l (1). The p gene is transcribed into two polycistronic mRNAs, P/C and V/C (2). The polycistronic P/C mRNA is translated to synthesize the P, C, Y1, and Y2 proteins from independent start sites in two overlapping reading frames. Although the C protein is expressed at levels comparable with P in infected cells (3), it is a minor component of virions (4). The other proteins, C’, Y1, and Y2 are expressed at relatively low levels in virus-infected cells (3). Although the P protein is required for viral transcription and replication (1, 5), the functional significance of the C protein is not precisely defined. A recent study has suggested that the C protein may be involved in the regulation of viral transcription (6). To define further the functions of the P and C proteins, we have developed ribozymes to block the P/C mRNA expression in virus-infected cells.

Ribozymes are RNA molecules with self-cleaving enzymatic activities (7, 8). Ribozymes can be designed to act in trans to specifically cleave virtually any RNA molecule (9), making them particularly attractive as antiviral agents (10–14) and as tools for studying gene function (15, 16). However, the factors that influence ribozyme and substrate interactions in vitro are not well defined. In many in vivo studies, very high ribozyme:substrate ratios were necessary to detect ribozyme activity (17–20). Ribozymes to be used intracellularly are generally optimized using cell-free cleavage assays. Considerable disparity appears to exist between in vitro kinetics and intracellular activities (18, 19). This could be due to differential intracellular structural properties of the ribozyme and/or substrate and/or to RNA-protein interactions. These discrepancies reflect that in vitro kinetics are frequently determined with synthetic model ribozymes that do not accurately represent the ribozymes that would be synthesized in vivo from expression vectors. Ribozymes expressed intracellularly from expression vectors contain modifications to their basic structure due to the incorporation of vector sequences. These changes may influence appropriate folding and thus inherently affect activity. Consequently, cell free kinetics would be more predictive of intracellular ribozyme activity if they were performed with RNA transcripts containing comparable structural elements as those synthesized intracellularly.

In the present study, we compared the cell-free and intracellular activities of several hammerhead-type ribozymes to develop the most effective intracellular ribozymes targeted to the Sendai virus P/C mRNA. Ribozymes were designed such that their in vitro and in vivo structural elements would be essentially identical. We examined the influence of parameters effecting ribozyme-substrate interactions, namely hybridization arm length (HAL) and nonhybridizing extraneous sequences (NES) on ribozyme activity. Manipulating these parameters, we generated ribozymes that inhibited p/c gene expression by nearly 95% in transiently transfected cells at a low ribozyme:substrate molar ratio. These results indicate that the ribozyme strategy is amenable for studying the function of p gene encoded proteins. Moreover, our study shows that efficient ri.

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* This work was supported by Research Grant AI30517 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Portions of this work will be submitted in partial fulfillment of the requirements for the Ph.D. degree from the Graduate College of Rush University. Present address: Gene Therapy Center, University of North Carolina, Chapel Hill, NC 25399.

§ To whom correspondence should be addressed: Dept. of Immunology/Microbiology, Rush Medical College, 1653 West Congress Pkwy., Chicago, IL 60612. Tel.: 312-942-7768; Fax: 312-226-6020.

The abbreviations used are: HAL, hybridization arm length; NES, nonhybridizing extraneous sequences; STO, single turnover; MTO, multiple turnover; UTR, untranslated region; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; STO, satellite RNA of tobacco ring spot virus; sRz, self-cleaving ribozyme; tt, transcription terminator.
bozymes can be expressed intracellularly by manipulating their structural features.

MATERIALS AND METHODS

Construction of Plasmids—All recombinant DNA manipulations were performed following standard techniques (21). Sense and antisense ribozyme plasmid constructs, pcRz1 and pcRz2 (Fig. 2A), respectively, were generated from two synthetic complementary oligodeoxynucleotides (oligonucleotides, Table I). The oligonucleotides contained the 22-nucleotide (nt) catalytic region from the satellite RNA of the tobacco ring spot virus (tobRsv) (22), flanked on either side by 13 nt complementary to the 5′-UTR of the PC mRNA (between nt 58–70 and 72–84) (Fig. 1A). BamHI sites (Table I) were designed at the termini of the duplex for cloning into the BamHI site of the eukaryotic expression vector, pcDNAI/amp (Invitrogen). Equivalent amounts of complementary oligonucleotides were mixed, heated to 95 °C for 3 min, and annealed by slowly cooling to room temperature. Annealed oligonucleotides (duplexes) were then digested with BamHI, phenol/chloroform extracted, ethanol-precipitated, and ligated to the BamHI-digested and alkaline phosphatase-treated pcDNAI/amp.

In addition to pcRz1 and pcRz2 several other ribozyme containing plasmids were constructed from oligonucleotides designated as Rz13, Rz9, and anti-PC (Table I). Upon annealing, these oligonucleotides formed duplexes with cohesive restriction sites at both termini for their directed ligation into the HindIII and BamHI (H/B) sites of pcDNAI/amp. Upon ligation of duplexes to pcDNAI/amp, the following plasmids were generated, pcRz13, pcRz9, and pcanti-PC (Fig. 2B). The antisense duplex (anti-PC) contained 27 nt complementary to the PC mRNA from nt 58–84, without the catalytic core (Table I). Additional duplexes (Table I) were also synthesized which contained the sequences 5′-GGCACCGACAGAGCTGACGACCCACACcctag-3′ and 5′-atccTGATGAGTCCGAGAGGACGAAACTGCTGGGg-3′.

Ribozymes were designated based on the structural characteristics of their terminal region of the P/C mRNA (Fig. 1). BamHI sites (Table I) were designed at the termini of the duplex for cloning into the BamHI site of the eukaryotic expression vector, pcDNAI/amp (Invitrogen). Equivalent amounts of complementary oligonucleotides were mixed, heated to 95 °C for 3 min, and annealed by slowly cooling to room temperature. Annealed oligonucleotides (duplexes) were then digested with BamHI, phenol/chloroform extracted, ethanol-precipitated, and ligated to the BamHI-digested and alkaline phosphatase-treated pcDNAI/amp.

In Vitro Versus in Vivo Ribozymes

Table I

| Rz1<sup>a</sup> | 5′-CTGgaattcccGGCGGCTGTCTCTGATTGAGCGAAGAGAAACTGCTGGTGGAAGgc-3′ | 3′-GAGcttGGCAGCACAGGACACATCCTAGGCTCTTTGAGACAAGCCCACACGcctag-5′ |
| Rz2<sup>a</sup> | 5′-CTGgaattcccGGCGGCTGTCTCTGATTGAGCGAAGAGAAACTGCTGGTGGAAGgc-3′ | 3′-GAGcttGGCAGCACAGGACACATCCTAGGCTCTTTGAGACAAGCCCACACGcctag-5′ |
| Rz9 | 5′-gtccGAGAGGACGAAACTGCTGGGg-3′ | 3′-agcttGGCTGTCTCCTGATGAGTCCGAGAGGACGAAACTGCTGGGg-3′ |
| antiPC | 5′-gtccGAGAGGACGAAACTGCTGGGg-3′ | 3′-agcttGGCTGTCTCCTGATGAGTCCGAGAGGACGAAACTGCTGGGg-3′ |
| sRz | 5′-gtccGAGAGGACGAAACTGCTGGGg-3′ | 3′-agcttGGCTGTCTCCTGATGAGTCCGAGAGGACGAAACTGCTGGGg-3′ |

<sup>a</sup>The same pair of oligonucleotides was used to clone Rz1 (sense orientation) and Rz2 (antisense orientation); lowercase sequences, restriction sites; underlined sequences, hybridization arms complementary to PC mRNA; sRz, self-cleaving ribozyme cassette from tobRSV, site of tobRSV self-cleavage indicated in bold italics; tT, bacteriophage T7 RNA polymerase terminator sequence.

Cleavage Reactions—Rate constants for the cleavage of PC mRNA (P274) were determined under single turnover (STO) conditions with excess ribozyme or under multiple turnover (MTO) conditions with excess substrate. The STO reactions were performed using 2–10-fold molar excess of ribozyme. Substrate concentration [S] was kept at 80 nM while ribozyme concentrations [R] ranged from 160 to 800 nM. MTO reactions were performed with 5–20-fold molar excess of the substrate. [S] ranged from 50 to 200 nM with a constant [R] of 10 nM. Substrate and ribozyme transcripts were combined in cleavage buffer (50 mM Tris-Cl, pH 7.4 and 1 mM EDTA) and allowed to anneal for 5 min at 37 °C prior to initiation of the cleavage. An additional set of reactions was performed to determine the influence of preformed secondary structures on ribozyme activity. For these reactions, ribozyme (160 and 400 nM) and the substrate P274 (80 nM) were preincubated at 95 °C for 3 min and annealed by slowly (over 30 min) cooling to the reaction temperature of 37 °C. All cleavage reactions were initiated by adjusting the MgCl<sub>2</sub> concentration to 20 mM and incubated at 37 °C in a total volume of 20–30 μl. Reactions were quenched at various times by mixing with an equal volume of stop solution (40 mM EDTA, 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol) and then immediately frozen to −70 °C. Heat-denatured reaction products were resolved by denaturing polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide, 7 M urea) followed by autoradiography. The extent of substrate cleaved was quantified from autoradiographs scanned by a laser densitometer (Molecular Dynamics).

Initial cleavage rates (<i>v</i><sub>obs</sub>) were determined from logarithmic plots of the fraction of substrate remaining (<i>v</i><sub>obs</sub>) versus time during the earliest phase of the reactions when product formation was linear with time. For the STO reactions, several ribozyme concentrations [R] were used and the rate constant was obtained from Eadie-Hofstee plots of the <i>k</i><sub>i</sub> versus <i>v</i><sub>obs</sub>[R] (26) using a linear curve fit analysis (Kaleidagaph, Synergy Software). The “pseudo-Michaelis constant,” <i>K</i><sub>pseudo</sub>, and the initial cleavage rate (<i>k</i><sub>i</sub> values were obtained from the slope and Y intercept, respectively, <i>K</i><sub>pseudo</sub> values describe the ribozyme concentration [R] at which the rate (<i>k</i><sub>i</sub> = <i>V</i><sub>max</sub>/2). The MTO constants were similarly obtained from Eadie-Hofstee plots of the initial velocity (<i>v</i><sub>obs</sub>) versus (<i>v</i><sub>obs</sub>) [S] (27, 28). Initial velocities were obtained from the early phase of the reactions when less than 10% of the substrate [S] had been converted to product. For the MTO reactions, the <i>K</i><sub>i</sub> value reflects the substrate concentration [S] at which the reaction rate is half-maximal (<i>V</i><sub>max</sub>/2).

Intracellular Expression of Ribozyme—CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum in a moist atmosphere with 5% CO<sub>2</sub> at 37 °C in 35-mm dishes. Subconfluent CV-1 cells were infected at a multiplicity of infection of 5 with vTF7–3, a recombinant vaccinia virus that expresses T7 RNA polymerase in the cytoplasm of infected cells (29). Supernatant pcPC was transfected alone or cotransfected with a 5-fold molar excess of linearized or supercoiled ribozyme (or antisense) plasmid, 15 μg of per pcRz or pcanti-PC using 10 μl of Lipofectin (Life Technologies, Inc.). DNA-Lipofectin complexes were added to cells 30 min after vTF7–3 infection and incubated at 37 °C. At 14 h postinfection, cells were washed with phosphate-buffered saline containing Mg<sup>2+</sup> and Ca<sup>2+</sup> (phosphate-buffered saline<sup>+</sup>) and lysed with 500 μl of RIPA buffer (150...
Characteristics of the P/C mRNA-specific ribozymes and antisense RNA and their predicted interactions

**TABLE II**

| Ribozyme | HAL | NES | R-R ΔG | R-P_{PT} ΔG^\text{a} |
|----------|-----|-----|---------|---------------------|
|          | nt  | nt  | kcal/mol| kcal/mol            |
| Rx1E     | 13  | 35  | 1       | 11.5                |
| Rx13B    | 13  | 17  | 1       | 9.5                 |
| Rx13E    | 13  | 17  | 1       | 16.1                |
| Rx13s(Rx13st)^b | 13  | 17  | 1       | 14.7                |
| Rx9B     | 9   | 17  | 1       | 7.4                 |
| Rx9E     | 9   | 17  | 31      | 12.0                |
| Rx9s(Rx9st)^b | 9   | 17  | 17      | 9.1                 |
| Anti-PCs | 26  | 17  | 17      | 7.5                 |

^a Free energy of the intramolecular interactions (R-R) within the ribozyme transcripts.
^b Free energy of intermolecular interactions (R-S) between the hybridization arms and substrate (P_{PT}).

*In Vitro Versus in Vivo Ribozymes*

Results

Ribozyme Target Site in P/C mRNA—Our previous studies have shown that relatively low amounts of antisense oligonucleotides (50–100 fmol/mg mRNA) directed to the 5′-UTR of the P/C mRNA efficiently inhibited cell-free synthesis of all the P/C mRNA-encoded proteins (34). In addition, both the RNAFOLD predicted (Fig. 1B) and the experimental determination of the secondary structure (35) suggested that this region lacked strongly folded structures. Thus, we suspected this region of the 5′-UTR would be readily accessible for ribozyme interaction. To target this region, we constructed ribozymes containing the catalytic domain of sTobRSV (22) flanked by 18 (9-mer ribozymes) or 26 (13-mer ribozymes) nt complementary to the P/C mRNA at bases 62–80 and 58–84, respectively (Fig. 1A). The GUC cleavage site at nt 71 is located upstream of all the translation start sites (36). Thus, the cleavage at this site would abrogate the synthesis of all the p gene encoded proteins. Therefore, we targeted only one site for ribozyme interaction.

Ribozyme Structure—Several hammerhead ribozymes were compared to assess the influence of hybridization arm length (HAL) and nonhybridizing extraneous sequences (NES) on the cleavage of the substrate P274. Ribozymes contained HAL of 13 nt (% GC = 65) or 9 nt (% GC = 67) each with varying NES length (Figs. 2 and 3; Table II). NES are sequences transcribed along with the ribozyme which are not part of the hybridizing arms or the catalytic core. The NES for our ribozymes contained vector-derived sequences generated during run-off transcription of plasmids linearized at EcoRI site (E) (31 nt 3′-NES) or BamHI site (B) (17 nt 3′-NES) or contained sequences from intact plasmide containing the cis-acting self-cleaving ribozyme (sRz) and the T7 RNA polymerase terminator (tT) (designated as st; 17 nt 3′-NES). Although the efficacy of sRz self-cleavage in cells was not determined, efficient self-cleavage was observed in vitro especially when the sRz was upstream of the T (data not shown).

RNAFOLD predicted secondary structures for ribozyme RNA in the absence of substrate (left panel), and the structures predicted for the ribozyme-substrate interactions (R-S = r = P_{PT} (right panel) are shown in Fig. 3, A and B. The structures of ribozymes (Rx13s and Rx9s) that would be putatively generated following self-cleavage of sRz from the larger precursors (Rx13st and Rx9st) are shown in Fig. 3B. Similarly, the predicted secondary structures of the antisense RNA molecules, anti-PCs, generated from the anti-PCst precursors are presented in Fig. 3B. The thermodynamic stabilities of the individual ribozymes and the ribozyme-substrate (R-S) complexes are presented in Table II.

Ribozyme Activity in Cell-free Reactions—For cell-free cleavage reactions to be predictive of intracellular ribozyme activity, they need to be conducted under conditions that approximate those found in vivo. However, in several previous studies, ribozyme and substrate RNAs were preincubated at high temperature and then snap-cooled or slowly cooled to reaction temperature. Elevated temperatures and pH and denaturants such as urea and formamide have all been used to enhance ribozyme activity. These conditions favor the formation of R-S duplexes by removing preformed intramolecular secondary
structures. Since these situations would not be available intracellularly, we evaluated ribozyme activities under nearly physiological conditions as described under "Materials and Methods." All of the ribozymes cleaved the substrate (P274) yielding the expected sized products, P1 and P2 (Fig. 4). To determine the relative efficiencies of various ribozymes, rate constants for the cleavage of the substrate (P274) were determined under single turnover (STO) and multiple turnover (MTO) conditions (described below). Overall, the experimentally determined rate constants were consistent with the predicted secondary structures. The rate constants observed for the various ribozymes correlated with the thermal stabilities of their predicted secondary structure and with their predicted ability to interact with the substrate to form an active hammerhead complex (Fig. 3, right panel). In essence, ribozymes that were predicted by RNAFOLD to interact productively with the target region cleaved substrate more efficiently than those not predicted to interact productively.

**Single Turnover Kinetics**—Rate constants were obtained under STO conditions from pseudo-first order reactions where ribozymes were in excess of the substrate (P274). In STO experiments, rate constants are derived from the first turnover, and therefore, they reflect the rate-determining step(s). Large differences in the initial cleavage rates \( k_2 \) were observed for ribozymes differing only in HAL and NES (described below). Since all the ribozymes had the same catalytic core and were targeted to the same site, the differences in initial cleavage rates most likely reflect the rate of R-S interaction and suggest the inherent ability of each ribozyme to interact with the substrate rather than the rate of chemical cleavage step (26).
Effect of HAL on Ribozyme Activity—To more accurately determine the influence of HAL (13 versus 9 nt) on ribozyme activity, we examined reaction kinetics of ribozymes with a minimum of NES (Rz13B versus Rz9B) under STO conditions. The initial cleavage rates ($k_2$) differed by about 7-fold with Rz9B being less efficient (Table III). The lower efficiency of Rz9B compared with Rz13B was consistent with its lower energetic favorability for R-S interactions (Table II). The free energy ($\Delta G$) of the R-S interactions for Rz13B (−37.6 kcal) was nearly 3-fold greater than that of the Rz9B (−13.7 kcal) (Table II). In addition, the RNAFOLD analysis indicated that intramolecular interactions (R-R) within the 9-mer ribozymes were more favorable than intermolecular interactions with the substrate (R-S) (Fig. 3A; Table II). Thus, the intramolecular interactions within the 9-mer ribozymes are more likely to interfere with the R-S interactions than those within 13-mer ribozymes. This observation could explain the differences in ribozyme activity. However, these predictions would need experimental validation.

Effect of NES on Ribozyme Activity—The computer-predicted folding patterns of Rz13 and Rz9 (Fig. 3) illustrate that ribozyme structures change depending on the extent of NES, which in turn may influence ribozyme activity. The influence of NES on ribozyme activity was determined by comparing the kinetics of ribozymes with varying NES length (Fig. 3). Under STO conditions, there was an apparent delay in the initiation of cleavage for all of the ribozymes relative to Rz13B (Fig. 5A). These delays resulted in large differences in the $k_2$ values (Table III). The differences were greater among the 13-mer ribozymes than among the 9-mer ribozymes, probably due to the reduced efficiency of the 9-mer ribozymes. The $k_2$ values for Rz13E, Rz1E, and Rz13st were 3-, 9-, and 15-fold, respectively, lower than Rz13B, whereas the $k_2$ values among 9-mer ribozymes differed by about 2-fold. The $k_2$ values were inversely related to $K_{m_{\text{high}}}$ values, suggesting that the NES affect R-S interaction. The differences in catalytic efficiency ($k_2/K_{m_{\text{high}}}$) were even more pronounced, with Rz13B >100-fold more efficient than Rz13st (Table III). These results clearly suggest that the length and strength of the intramolecular interactions have a substantial effect on the R-S interaction. One explanation for this could be that the NES cause the ribozymes to adopt alternative secondary structures which may not allow ribozyme to readily interact with the substrate. Alternatively, the ribozymes may initially interact with the substrate (i.e., formation of one hybridization arm only) but must undergo a conformational change after binding to generate an active hammerhead complex.

To determine whether preformed structures within the ribozymes (R-R) hindered the R-S interactions, ribozyme and substrate RNAs were heat-denatured prior to annealing and allowed to cool slowly to 37°C. This step was presumed to remove most of the secondary structures within the RNAs and allow the most energetically favored intramolecular (R-R) as well as intermolecular (R-S) interactions to occur. Following heat denaturation and slow renaturation, dramatic increases in initial cleavage rates and efficiencies were observed for most of the ribozymes (Table III). Interestingly, the magnitude of the increases correlated with the strength of the structural elements predicted to exist in the 3′-NES of the ribozymes (Fig. 3, left panel; Table II). Cleavage efficiency ($k_2/K_{m_{\text{high}}}$) increased by −12- and −100-fold for Rz13E and Rz13st, respectively, whereas the efficiency of Rz13B increased only marginally. Under these conditions the catalytic efficiencies of the various 13-mer ribozymes were essentially the same. Heat denaturation also increased the cleavage rates for the 9-mer ribozymes, Rz9B and Rz9st, but not Rz9E (Table III). Curiously, cleavage
by Rz9E remained relatively linear compared with the other ribozymes (Fig. 5B). These results suggested that the R-R interactions within Rz9E may be in equilibrium with the R-S interactions.

After the initial burst of cleavage, there was a second slower phase of continuing cleavage with the other ribozymes, except Rz13B. This observation suggested that more than one ribozyme conformation may form following renaturation and presumably during transcription. It is likely, therefore, that additional ribozyme conformations exist in RNA solutions and
that some may have suboptimal activity. Therefore, the delay in the initial cleavage rates relative to Rz13 are probably due to the time required to acquire necessary conformational changes to form an active hammerhead complex with the substrate.

**Multiple Turnover Kinetics**—MTO reactions were performed with 5–20-fold molar excess of the substrate (P274) to determine the efficiency of ribozyme turnover. Initial velocities were determined during the earliest phase of the reactions when the rate of the substrate cleavage was linear with time. Consistent with the Michaelis-Menten model for enzyme kinetics (27), initial velocities increased as substrate concentration [S] increased. Turnover numbers \( k_{\text{cat}} \) for the various ribozymes were relatively low and ranged from 0.004 to 0.025 per min, the lowest being for the ribozyme, Rz13E (Table IV). \( K_m \) values on the other hand varied by more than 15-fold (22–395 nM). Consistent with the STO reactions, the \( K_m \) values increased in direct proportion to the complexity of the predicted secondary structures within the 3'-NES of the ribozymes (Fig. 3). The increases in \( K_m \) values were also accompanied by decreases in the catalytic efficiencies \( (k_{\text{cat}}/K_m) \). The ribozymes Rz13E and Rz13st showed 40- and 25-fold lower \( k_{\text{cat}}/K_m \) values, respectively, than Rz13B which lacked 3'-secondary structure (Fig. 3). The catalytic efficiencies of the 9-mer ribozymes differed by \(-10\)-fold, and the values were related to their \( K_m \) values as observed for 13-mer ribozymes. Because \( K_m \) values reflect the ability of enzyme and substrate to interact, these results further suggest that the presence of NES slowed the rate of association between the ribozyme and substrate (R-S). These observations further indicated that the NES have a rate-determining effect on ribozyme efficiency and that the rate-limiting step in these reactions was the R-S association.

On the other hand, while the intramolecular interactions within the ribozymes may hinder R-S interaction, they could also enhance turnover. Intramolecular interactions may help to increase turnover by destabilizing the intermolecular interaction between ribozyme and substrate. Due to the length of the hybridization arms (9 or 13 nt each) and rather high binding energy between the ribozyme and substrate (Table II), high catalytic turnover was not expected for these ribozymes. However, all of the ribozymes cleaved more than 1 eq of substrate and so were truly catalytic (Fig. 6).

In sum, the \( k_2 \) values from the STO reactions (Table III) were much more rapid than the \( k_{\text{cat}} \) values derived from MTO reactions (Table IV) for the same ribozymes. The initial cleavage rates \( (k_2) \) obtained under excess ribozyme conditions reflect the rate-determining step(s) prior to product release, whereas the \( k_{\text{cat}} \) values represent the rates of all phases of the reaction (27). Thus, the low \( k_{\text{cat}} \) values appear to be due to a slow product release step. The \( k_{\text{cat}} \) and \( k_2 \) values will be equivalent only if the binding steps (association and dissociation) were much faster than the cleavage step of the reactions (27). The observation made with the STO reactions that ribozyme structural elements affected its efficiency was also borne out by the MTO reactions. The rate constants observed for Rz13B (Tables III and IV) were comparable with the \( k_{\text{cat}} \) and \( K_m \) values reported for similar types of ribozymes (19, 28, 37, 38), suggesting the general validity of our observations.

**Ribozyme-mediated Inhibition of P and C Protein Expression in CV-1 Cells**—To determine the intracellular efficacy of ribozymes, pcPC and pcRz constructs were cotransfected (at a
molar ratio of 1:5) into CV-1 cells that had been previously infected with the recombinant vaccinia virus, vTF7–3. vTF7–3 expresses T7 RNA polymerase in the cytoplasm (29) of the infected cells. We chose this system because both P/C mRNA and ribozymes could be expressed in the cytoplasm where Sendai virus replication naturally occurs. Ribozyme constructs were transfected after linearization at BamHI site (B) or EcoRI site (E) or transfected as intact circular/supercoiled molecules. Similarly, antisense constructs were also cotransfected with pCPC to determine whether the intracellular ribozymes were more efficient than antisense RNAs. With this system, transfected ribozyme and antisense constructs would generate transcripts identical to the RNA molecules synthesized in vitro. Therefore, a correlation was expected between in vitro cleavage results and the inhibition of p gene expression in cells.

The in vitro results in conjunction with RNAFOLD analysis of the 9-mer and 13-mer ribozymes indicated that the 13-mer constructs would be more effective in vivo. This was essentially the case as the 9-mer ribozymes were relatively inert in transiently transfected cells compared with the 13-mer ribozymes (Fig. 7).

To determine the influence of NES on the intracellular ribozyme activity, we compared ribozymes with variable NES length. To determine the influence of 5’-NES, we examined ribozymes with 35 nt (Rz1E) and 17 nt (Rz13E) of 5’-NES. Rz13E was 20% more effective in inhibiting p gene expression than Rz1E (Fig. 7). Plasmids were also linearized at different restriction sites which on expression in cells yielded variable lengths of 3‘-NES. Contrary to the cell-free results, none of the constructs with 1 nt of 3‘-NES (Rz13B, Rz9B, or anti-PCB) were effective in cells. The extra 31 nt on the 3‘-termini of Rz13E and anti-PC (but not Rz9E) enhanced activity in cells (50% inhibition of P expression), perhaps by rendering these transcripts more resistant to intracellular nucleases.

As an alternative to linearizing plasmids to control the length of 3‘-NES, we incorporated a cis-acting self-cleaving ribozyme (sRz) and a T7 terminator (tT) into the ribozyme constructs (Fig. 2). Because these constructs were intact supercoiled molecules, they had increased transfection efficiency over linear plasmids. In addition, it has been observed that transcription by T7 RNA polymerase is up to 10-fold more efficient with intact than with linear plasmids (39). As expected, expression of ribozymes (and antisense RNAs) from these intact constructs resulted in substantially more inhibition of p gene expression in cells. Rz13st and anti-Pc st inhibited P/C expression by about 70% in transfected cells (Fig. 7B), whereas supercoiled ribozyme constructs without these cassettes had no detectable effect on P/C expression (data not shown). Clearly, the incorporation of the sRz and tT cassettes into the constructs resulted in increased effectiveness of the T7 expression system and the increased efficiency of ribozyme- and antisense-mediated inhibition of p gene expression.

To determine the ratio of ribozyme to P/C mRNA necessary for efficient intracellular ribozyme activity, plasmids (pCz13st, pcanti-Pc st, and pCPC) were cotransfected at R:S molar ratios of 1:1, 2.5:1, 5:1, 10:1, or 20:1. However, the ratio of the intracellular ribozyme and substrate is only implied. While efficient inhibition of p protein expression was observed when pCz13st and pCanti-Pc st were cotransfected with pCPC at a 5:1 R:S ratio (Figs. 7 and 8), at R:S ratios of 2.5:1 much less

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**Table III**

Single-turnover kinetics for Sendai virus P/C mRNA-specific ribozymes

| Ribozyme | \( k_a^a \) | \( k_a^{b} \) | \( k_{a}^{b}/K_m^{a} \) | \( k_a^{b}/K_m^{b} \) |
|----------|------------|------------|----------------|----------------|
| Rz1E     | 0.16       | 797        | 0.20           | ND             |
| Rz13B    | 1.48       | 135        | 11             | 6.9            |
| Rz13E    | 0.27       | 365        | 0.74           | 3.4            |
| Rz13st   | 0.087      | 949        | 0.09           | 4.5            |
| Rz9B     | 0.21       | 182        | 1.2            | 2.9            |
| Rz9E     | 0.13       | 421        | 0.3            | 0.21           |
| Rz9st    | 0.086      | 641        | 0.13           | 1.5            |

\(^a\) Single turnover kinetics measured with ribozyme [R] in excess of substrate (80 nM). Values obtained from Eadie-Hofstee plots of cleavage rates \( (k_{a}) \) versus [R] with [R] of 160, 320, 640 nM. \( k_{a}^{b} \) values were obtained from graphs of the ln fraction of substrate remaining (ln fracS) versus time as described in text.

\(^b\) Single turnover kinetics were determined following removal of preformed secondary structures by heat denaturation. Ribozyme (160 and 400 nM) and substrate (80 nM) were preincubated at 95°C for 3 min and slowly cooled to reaction temperature of 37°C prior to addition of MgCl₂, ND, not determined.
of an effect was seen, especially for the antisense construct (Fig. 8). Increasing the molar ratio of R:S to 10:1 and 20:1 resulted in increasing the inhibition of P/C expression to about 95% (Fig. 8). For these transfections 5-fold more lysate was required to detect the P protein at the 20:1 R:S ratio (Fig. 8 B).

The reduced activity observed at the low R:S ratios could be explained by the Poisson distribution of the transfected plasmids into cells. Overall, the results showed that both Rz13st and anti-PCst inhibited the expression of P and C proteins with approximately equal efficiency, albeit at the higher R:S ratio. Nonetheless, the effect of the ribozyme was about 20% greater than the antisense RNA (Fig. 8 B) at the lower R:S ratio. In addition, the dose-response relationship indicated that the inhibition of p gene expression was due to ribozyme or antisense activity and not due to other factors such as transfection efficiency.

Stability of Ribozyme RNA—We alluded previously that NES may help to stabilize ribozymes. To test this directly, gel-purified 32P-labeled ribozyme transcripts Rz13B (1 nt 3'-NES), Rz13E (31 nt 3'-NES), and Rz13s (17 nt 3'-NES, following self-cleavage of precursor Rz13st) were incubated with a single strand-specific ribonuclease, RNase T2, to determine the effect of 3'-NES on RNA stability (all of these ribozymes had identical 5'-NES). Ribozyme bands were purified to separate Rz13s from the precursor Rz13st and the products generated following self-cleavage of sRz. All ribozymes were purified to ensure similar treatment for each transcript. Rz13s was shown to be 8-fold more resistant to nuclease digestion than Rz13B and about 3-fold more resistant than Rz13E. Rz13E was also almost three times more stable than Rz13B (Fig. 9). RNAFOLD analysis predicted that the 3'-termini of Rz13E and to a greater extent Rz13st would fold into relatively stable stem-loop structures (Fig. 3, left panel). Thus, these results suggest that ribozymes whose termini are predicted to contain stable secondary structures have a better chance to survive and act in the cellular milieu.

DISCUSSION

To create effective intracellular ribozymes targeted to the Sendai virus P/C mRNA, critical parameters that influence ribozyme efficiency were examined. Use of cell-free cleavage reactions and a vaccinia virus-T7 RNA polymerase expression system (29) enabled us to analyze several ribozymes. With this system the ability of a ribozyme to interact with the substrate in the complex milieu of the cytoplasm can be determined rather quickly. The benefits of this system for ribozyme analysis have been previously described (19). In vitro assays to optimize ribozymes for intracellular use have often produced inconsistent results. For the current study cell-free cleavage assays were performed under physiological conditions of temperature and pH with ribozyme transcripts which were representative of those that would be generated in vitro. Under these conditions in vitro kinetics were generally consistent with the intracellular results. Significantly, the experimental results were consistent with the computer-generated secondary structure predictions. This suggests that structural analyses when
combined with experimental analysis should prove useful for designing effective intracellular ribozymes.

Accessibility of the target site (19, 28, 40), the length of hybridization arms (HAL) (26, 37, 41, 42), and the extent of nonhybridizing extraneous sequences (NES) (9, 19, 22, 43) are some of the parameters that can affect the interaction between the ribozyme and the substrate. As predicted from our previous studies (34, 35), the targeted cleavage site in the 5'-UTR was shown to be accessible to all of the ribozymes examined. In addition, all ribozymes exhibited catalytic activity, cleaving more than one molecule of substrate per molecule of ribozyme. However, large differences in catalytic efficiency were found between ribozymes differing only in their HAL and NES. Differences in ribozyme activity were consistent with the RNAFOLD-predicted ability of the ribozymes to interact with the substrate. Both the kinetic data and intracellular results suggest that HAL and NES of the P/C-specific ribozymes modulated ribozyme-substrate interaction in a rate-determining manner.

Hybridization arm length (HAL) can have a significant effect on ribozyme activity, not only by providing specificity but also by determining the dynamics of the R-S interaction. In vitro kinetic studies have suggested that hammerhead ribozymes

### TABLE IV

**Multiple turnover kinetics for Sendai virus P/C mRNA-specific ribozymes**

Multiple turnover kinetics were measured with substrate in excess of ribozyme. Substrate concentrations [S] ranged from 50 to 200 nM with ribozyme concentration kept at 10 nM.

| Ribozyme  | k<sub>cat</sub> | K<sub>m</sub> | k<sub>cat</sub>/K<sub>m</sub> |
|-----------|---------------|-------------|-----------------|
| Rz1E<sup>a</sup> | 0.008 min<sup>-1</sup> | 58 nM | 0.14 μM<sup>-1</sup> min<sup>-1</sup> |
| Rz13B | 0.025 | 22 | 1.1 |
| Rz13E | 0.004 | 107 | 0.028 |
| Rz13st | 0.013 | 289 | 0.045 |
| Rz9B | 0.013 | 37 | 0.35 |
| Rz9E | 0.022 | 385 | 0.055 |
| Rz9st | 0.011 | 133 | 0.082 |

<sup>a</sup> [R] at 20 nM. Values were obtained from Eadie-Hofstee plots of cleavage velocity (v) versus v/[S].
with hybridization arms >5 nt are limited by their ability to dissociate from their substrate (26, 41). Consequently, long hybridization arms could inhibit ribozyme turnover. Reducing the hybridization arm length which subsequently reduces the binding energy (ΔG) between the R-S interaction should promote better release of ribozymes. However, this could also diminish the potential association between the ribozyme and substrate, which appears to be the rate-limiting step in the cleavage of long substrates (22, 37, 38). Ribozymes with shorter arms are also more likely to have reduced intracellular cleavage of long substrates (22, 37, 38). Ribozymes with shorter substrate, which appears to be the rate-limiting step in the increase turnover. However, these constructs failed to increase ribozyme activity when compared with pcPC alone (−) or pcPC cotransfected with negative control, pcRe2.

![Image](image_url)

**Fig. 8.** Dose-response relationship of ribozyme and antisense RNA activity in transiently transfected cells. pcPC (3 μg) was cotransfected with 2.5-20-fold molar excess of Rz13st and anti-PCst constructs and analyzed as described in Fig. 7. A, autoradiograph of cotransfections with P and C proteins indicated. The asterisk indicates a cellular or viral protein which was immunoprecipitated by the polyclonal anti-P or anti-C sera as described in Fig. 7. B, relative level of P compared with pcPC alone (−) or pcPC cotransfected with negative control, pcRe2.

Intracellular expression of ribozymes via eukaryotic vectors results in changes to the basic ribozyme structure due to incorporation of the vector-derived sequences (or NES). Previous studies have demonstrated that additional sequences can increase stability of the ribozymes against cellular nucleases (44, 45). On the other hand, these sequences could also reduce the ability of a ribozyme to interact productively with the substrate (17-20). Since we introduced ribozymes into cells using a T7 expression system, we also examined the influence of these extraneous vector-derived sequences on ribozyme activity. Cell-free assays demonstrated that ribozyme efficiency increased as the length and the complexity of the nonhybridizing vector-derived sequences (NES) were reduced (Table III). In contrast, the constructs linearized at BamHI, which contained minimal 3′-NES (1 nt 3′-NES; Fig. 3A), were essentially inert intracellularly. These results imply that NES may be necessary to protect the ribozymes from intracellular nucleases. However, as the extended 5′-NES (35 nt of Rz1E) reduced ribozyme activity, judicious use of extraneous sequences will be necessary for generating effective ribozymes.

The most effective intracellular ribozyme (Rz13st) contained the sRz and Tc cassettes, which upon self-cleavage generated a ribozyme (Rz13s) with 17 nt each of 5′- and 3′-NES. Both 17 nt termini were predicted by RNAFOLD to form stem-loop structures (Fig. 3). Although the secondary structure of the RNA has not been determined experimentally, it was shown that its stability was consistent with the RNAFOLD analysis. The half-life of Rz13s in the presence of RNase T2 was about 8-fold longer than Rz13B (without structure) and about 3-fold longer than Rz13E (with weaker stem-loop). These results are also consistent with previous studies showing that stem-loop structures can increase RNA half-life (44, 46). The presence of preformed secondary structures in Rz13s was further suggested by the 100-fold increase in cleavage activity following heat denaturation prior to annealing (Table IV). Thus, the increase in intracellular activity of Rz13s (and anti-PCs) is most likely related to its increased stability due the presence of the predicted stem-loop structures at their termini. In addition, self-cleavage by the cis-acting ribozyme (sRz) in these constructs results in a 3′-termini containing a 2′,3′-cyclic phosphate (22), which could further enhance RNA stability by protecting the transcripts from 3′-exonuclease digestion. The increased activity with Rz13s and anti-PCs could be due to increased efficiency of the R-S interaction. It is plausible that the stem-loops could...
also sequester the terminal NES into defined structures which would subsequently limit their nonproductive interactions with other areas of the substrate. Although our studies indicated that ribozymes behaved intracellularly, as expected, we have no direct evidence that they indeed cleaved the substrate.

Both the ribozyme (Rz13s) and antisense RNAs (anti-PCs) were able to successfully inhibit p gene expression in transiently transfected cells. However, based on the predicted secondary structures combined with the cell-free and intracellular assays, ribozyme activity appears to be limited by the rate of ribozyme-substrate interaction, rather than turnover efficiency. This brings up the question whether the ribozymes provide an advantage over antisense RNAs. At higher intracellular R:S ratios (10:1 and 20:1), there is no obvious advantage over antisense RNAs. At higher intracellular R:S ratios (2.5:1 and 5:1) Rz13s was slightly more effective than anti-PCs. This indicates that addition of catalytic sequencesto antisense RNA imparts an advantage where recycling of ribozyme is possible. However, even in the absence of efficient turnover, the irreversible nature of the trans-cleavage reaction would make ribozyme more desirable than antisense RNA alone. Unquestionably, all these presumptions have to be experimentally tested.

In conclusion, our results suggest that appropriate sized ribozymes (or antisense RNAs) with nucleo-resistant structures at their 3'- and 5'-termini can serve as effective modulators of gene expression.

Acknowledgments—We thank Dr. Bernard Moss, National Institutes of Health, for providing the vaccinia virus, vTF7–3. We also appreciate the help of Dr. Arnold Hampel during the course of this work.

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