Catalytic Properties of Hairpin Ribozymes Derived from Chicory Yellow Mottle Virus and Arabis Mosaic Virus Satellite RNAs†

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ABSTRACT: Regions of the negative strands of the satellite RNAs of chicory yellow mottle virus (sCYMV1) and arabis mosaic virus (sArMV) have similarity in sequence and predicted secondary structure compared to the tobacco ringspot virus satellite RNA (sTRSV) hairpin ribozyme, suggesting that they may also be catalytic RNAs of a similar type. Our experiments show that the hairpin ribozyme-like sequences derived from sCYMV1 and sArMV have high phosphodiesterase activity. The k_{cat} values determined are similar to that of the highly active native sTRSV hairpin ribozyme under the same conditions, although the K_m values are much higher. The K_m of the sArMV ribozyme was reduced 3-fold, with no change in k_{cat}, by extending substrate hybridization in helix 2. Additionally, the three hairpin ribozymes prefer different GUX sequences on the immediate 3′-side of the cleavage site. The sTRSV hairpin ribozyme cleaves GUX substrates with catalytic efficiencies in the relative order GUC ≫ GUU > GUG = GUA. The sCYMV1 ribozyme cleaves GUA > GUC, GUG, GUU. The sArMV ribozyme prefers GUA > GUG > GUU > GUC. The functional domain, regulating substrate selection at this position, must reside in the nucleotides that vary between the ribozyme−substrate complexes. The sTRSV ribozyme is most efficient at cleaving GUC sequences, while the sCYMV1 and sArMV ribozymes are most efficient for cleaving GUA-containing sequences.

Ribozymes catalyze phosphodiester bond cleavage of RNA and are emerging as a useful tool for controlling the cellular RNA levels of specific genes. Several ribozyme structural families have been identified, including group I introns, RNase P, the hepatitis δ virus ribozyme, hammerhead ribozymes, and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA (sTRSV)[1] (Sullivan, 1994). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication (Symons, 1989, 1992). Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans cleavage of mRNAs or viral RNA genomes for gene therapy (Sullivan, 1994). A hairpin ribozyme has been developed which successfully cleaves HIV-1 genomic and mRNAs *in vitro* and in cells (Ojwang et al., 1992; Yu et al., 1993) and is being prepared for clinical testing in human AIDS patients (Wong-Staal, 1994). Given the therapeutic potential of the hairpin ribozyme as well as the general importance of a designed "molecular knife", it is desirable to understand the structure−function relationships within this ribozyme. Practical applications from such basic knowledge could be improvement of catalytic activity and broadening substrate selectivity.

The sTRSV hairpin ribozyme has a well-defined secondary structure (Tritz & Hampel, 1989; Hampel et al., 1990, 1993). Mutagenesis data have identified and confirmed the presence of four primary helices and five loops in the RNA structure (Hampel et al., 1990; Anderson et al., 1994; Berzal-Herranz et al., 1993). The ribozyme can be adapted to cleave non-native substrates by modifying the bases in helices 1 and 2 to complement the mRNA of choice. The three-dimensional structure of the ribozyme is not yet known, although mutagenesis studies suggest a hinge between helices 2 and 3 that allows tertiary interactions between loops 1 and 5 with loops 2 and 4. This has been supported by cross-linking studies (Monforte, 1993) and by tethering of the 3′-end of the ribozyme to the 5′-end of the substrate to show that a bent structure exists (Komatsu et al., 1994).

In the past, two approaches were used to characterize the necessary nucleotides for activity: individual mutagenesis of 1−3 single bases of the sTRSV ribozyme (Anderson et al., 1994) and *in vitro* selection of variants capable of both cleavage and ligation after randomization of ribozyme domains (Berzal-Herranz et al., 1993). Both of these methods use the sTRSV hairpin ribozyme as a template and share the limitation that a relatively small area of the ribozyme is varied in each experiment. Alternative sequences with improved properties may exist when larger or multiple interacting domains are mutated simultaneously, but this is difficult to achieve *in vitro*. However, natural selection has evolved additional putative hairpin-like ribozymes in the satellite RNAs of chicory yellow mottle virus (sCYMV1) and arabis mosaic virus (sArMV). The satellite RNA sources of these sequences differ somewhat in size, 457 nt for sCYMV1 (Rubino et al., 1990) and 300 nt for sArMV (Kaper et al., 1988) compared to 359 nt for sTRSV (Buzayan et al., 1986; Fritsch et al., 1993) and are associated with nepoviruses that are not serologically related to sTRSV

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[1] Abbreviations: DTT, dithiothreitol; PEG, polyethylene glycol; sTRSV, satellite RNA of tobacco ringspot virus; sCYMV1, satellite RNA 1 of chicory yellow mottle virus; sArMV, satellite RNA of arabis mosaic virus.

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Table 1: Substrate Sequences Used[a]

sequence (1 8 14 (nt number))	origin
UGACA*GUCCUGUUU	sTRSV
UGACA*GUACUGUUU	sTRSV-C8A
UGACA*GUGCUGUUU	sTRSV-C8G
UGACA*GUUCUGUUU	sTRSV-C8U
GCGCA*GUACUGUUU	sCYMV1
GCGCA*GUCCUGUUU	sCYMV1-A8C
GCGCA*GUGCUGUUU	sCYMV1-A8G
GCGCA*GUUCUGUUU	sCYMV1-A8U
ACGCA*GUACUGUUU	sArMV
CCGCA*GUACUGUUU	sArMV-A1C
CCGCA*GUCCUGUUU	sArMV-A1C-A8C
CCGCA*GUGCUGUUU	sArMV-A1C-A8G
CCGCA*GUUCUGUUU	sArMV-A1C-A8U

[a] Underlined nucleotides are mutations in the original sequence. The asterisk (*) is the site of cleavage.

(Kaper et al., 1988), indicating probable evolutionary divergence.

Two characteristics desired in an alternative hairpin ribozyme structure are improved catalysis and more flexibility in choice of substrate sequence. Optimum catalysis by the sTRSV ribozyme is achieved with a BN*GUC sequence in the substrate, in which B is C, G, or U and N is any nucleotide (Hampel et al., 1990; Anderson et al., 1994). Substrate cleavage occurs at the asterisk between the N and the G nucleotides. This sequence requirement limits potential cellular hairpin ribozyme targets to RNAs having nonvariable BN*GUC sequences accessible to ribozyme binding, i.e., lacking strong helical RNA structure and bound proteins in the target region. Increasing the number of potential targets improves the probability of finding a suitable site. The native sequences of the putative substrates of both the sCYMV1 and the sArMV systems have a GUA at the presumed cleavage site. This may indicate that either evolution has favored suboptimal cleavage or that compensatory base changes have evolved that allow more flexibility in substrate selection.

The present study explores the possibility of cleavage in a trans reaction of putative hairpin-like ribozymes from sCYMV1 and sArMV, their catalytic properties, and the ability of these ribozymes to cleave variants of the GUC substrate sequence compared to that of the sTRSV hairpin ribozyme. The ribozymes derived from sCYMV1 and sARMV are both highly active in cleavage of their native substrate sequences. The k_{cat} values are similar to that of the sTRSV-derived hairpin ribozyme, however; the K_m values are much larger. In the native form the sARMV ribozyme has a predicted 3 bp helix 2. When this is extended to 4 bp, as is found in the sTRSV and sCYMV1 ribozymes, the k_{cat} value is unchanged; however, the K_m value is reduced 3×, giving a 3× increase in catalytic efficiency. The three hairpin ribozymes prefer different GUX sequences on the immediate 3′-side of the cleavage site. The sTRSV hairpin ribozyme cleaves GUC substrates best, while the sCYMV1 ribozyme and sARMV ribozyme both prefer GUA. The functional domain of the ribozyme responsible for selection of substrate sequence at this position must be either dependent upon or within the nucleotides that differ between the various ribozyme−substrate complexes.

MATERIALS AND METHODS

Sequences of Ribozymes and Substrates. The ribozyme sequences chosen for study were selected regions from the sequence of the native negative strands of the satellite RNAs of tobacco ringspot virus (sTRSV) (nt 224−175) (Buzayan et al., 1986), chicory yellow mottle virus (sCYMV1) (nt 306−255) (Rubino et al., 1990), and arabis mosaic virus (sArMV) (nt 157−106) (Kaper et al., 1988). The ribozyme sequences were as follows:

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sTRSV ribozyme
AAACAGAGAAGUCAACCAGAGAAACACACGUUGUGGUAUAUUACCUGGUA
1                   20                  40          50
sCYMV1 ribozyme
CAACAGCGAAGCGCGCCAGGGAAACACACCAUGUGUGGUAUAUUAUCUGGCA
1                   20                  40          52
sArMV ribozyme
CAACAGCGAAGCGGAACGGCGAAACACACCUUGUGUGGUAUAUUACCCGUUG
1                   20                  40          52
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For comparison in this study, numbering of these sequences has been adopted to start from the 5′-terminus with nt 1.

The substrate sequences used are given in Table 1. The native substrate sequences correspond to nucleotides 53−40 of sTRSV, 52−39 of sCYMV1, and 55−42 of sArMV, respectively, from the original sequences. Again, for this study, the numbering has been adopted such that nt 1 is the 5′-terminus of these sequences. Mutations were made in these sequences (Table 1).

Transcription Reactions. RNA was transcribed from oligodeoxynucleotide partial duplexes according to a modification of the method of Milligan et al. (1987) (Hampel et al. 1993). To improve transcription, GGG and GCG sequences were added to the 5′-ends of ribozyme and substrate RNA sequences, respectively. Oligodeoxynucleotide sequences were synthesized with T7 promoter sequences at the 3′-end on an Applied Biosystems Inc. 392 RNA/DNA synthesizer (Foster City, CA) according to standard techniques. Oligodeoxynucleotides were purified by HPLC and annealed to a T7 complement to form the transcription template. DNA template (0.5−1 μg) was combined with 4% PEG, 0.1% Triton X-100, 1 mM spermidine, 5 mM DTT, 40 mM Tris, 6 mM $MgCl_2$, 40 mM Tris (pH 8.0), each nucleoside triphosphate (1 mM), and 20−100 units of T7 RNA polymerase (Ambion or United States Biochemicals Corp.) in a 50 μL reaction volume. α-^{32}P-CTP (ICN) was used to label the transcripts. After 3 h at 37 °C, 2 units of DNase (Ambion) was added to remove the DNA template. Transcribed RNA was purified on 10% or 15% polyacrylamide/7 M urea electrophoresis gels for ribozymes or substrates, respectively, extracted from the gel matrix, and quantitated based on radioactive content.

Cleavage Reactions and Kinetic Studies. Transcribed RNA was redissolved in water at initial concentrations ranging from 0.5 to 8 μM RNA. Kinetic reactions were performed in volumes ranging from 4 to 8 μL with substrate

RNA concentrations ranging from 60 to 4000 nM, depending on the K_m of the ribozyme−substrate complex. An appropriate range of substrate concentrations was used for each K_m and k_{cat} determination. Ribozyme concentrations were kept constant and as low as possible to allow multiple reaction turnover. Concentrations of ribozyme for the various determinations ranged from 1 to 7 nM.

The more active ribozyme−substrate combinations required as little as 30 min for reaction, although ribozyme−substrate combinations with lower catalytic activity were incubated for up to 6 h to allow product detection. To remain near the linear range, no more than 15% substrate cleavage was permitted in a given experiment. Cleavage was done in buffer containing 12 mM $MgCl_2$, 2 mM spermidine, and 40 mM Tris, pH 7.5, at 37 °C (Hampel & Tritz, 1989). Reactions were terminated by addition of electrophoresis buffer containing 98% formamide and 10 mM EDTA. Separation of substrate and products was achieved by electrophoresis over a 15% polyacrylamide/7 M urea gel in 1× TBE. The reaction result was analyzed by autoradiography. Reactants and products were excised from the gel and quantitated by scintillation counting in BioSafe II scintillation fluid (Research Products Intl. Co.).

Reaction velocities were calculated from product concentrations and incubation times. The kinetics of the ribozyme reactions were analyzed according to the Michaelis−Menten equation using a curve-fitting program (Tablecurve 2D v3 for win32, Jandel Scientific Software Co., San Rafael, CA) which derived K_m and k_{cat} values from reaction velocities and substrate concentrations. The r^2 determination of goodness of fit exceeded 0.90 in all cases. Data given are the mean of all trials. Reproducibility for repetitive experiments is within 25%.

RESULTS

Sequences and Proposed Structures of sTRSV, sCYMV1, and sArMV Hairpin Ribozymes. Sequences from the native negative strands of the satellite RNAs of chicory yellow mottle virus (sCYMV1) (Rubino et al., 1990) and arabis mosaic virus (sArMV) (Kaper et al., 1988) were chosen to be tested for phosphodiesterase activity on the basis of both sequence and predicted structural homology to the hairpin ribozyme derived from the negative strand of the satellite RNA of tobacco ringspot virus (sTRSV) (Hampel et al., 1990). The structure of the sTRSV hairpin ribozyme and substrate was previously determined experimentally by mutating individual bases believed to participate in helices to inactivate the ribozyme and then mutating candidate complementary bases to reform the putative helix structure and restore activity (Hampel et al., 1990; Anderson et al., 1994). No such structural data has yet been obtained for the sCYMV1 and sArMV sequences. Thus, ribozyme and substrate sequences were selected by sequence and predicted structural homology with the known sTRSV hairpin ribozyme−substrate two-dimensional structure. The sequences emphasize these similarities and suggest strong structural similarities between the sTRSV hairpin ribozyme−substrate complex with sCYMV1 and sArMV sequences (Figure 1).

The sequence of sCYMV1 derived ribozyme differs from that of the sTRSV hairpin ribozyme by 4 nt in the substrate and 13 nt in the ribozyme for an overall base composition difference of 27%. The sArMV ribozyme sequence differs from the sTRSV hairpin ribozyme by 4 nt and 16 nt in substrate and ribozyme sequences, respectively, for an overall difference of 31%. The sCYMV1 and sArMV sequences differ from each other in only 1 nt in the substrate and 11 nt in the ribozyme for an overall difference of 23%. Both the sCYMV1 and sArMV ribozyme can be readily drawn in the standard hairpin motif with the known four helical domains and known five loop domains of the sTRSV hairpin ribozyme. Helix 2, however, for the native substrate sequence has 3 bp in the sArMV sequence as compared to 4 bp for the sTRSV and sCYMV1 sequences.

Hairpin sArMV and sCYMV1 Sequences are Catalytically Active. The hairpin-like ribozymes from sCYMV1 and sArMV have catalytic activity in a trans cleavage reaction (Figure 2). Under conditions of this assay, the sCYMV ribozyme sequence cleaved its corresponding substrate (lane 4) to 88% completion, the sArMV ribozyme sequence cleaved its substrate to 60% completion (lanes 5 and 6), while the control sTRSV hairpin ribozyme cleaved to near completion, 85%, its corresponding substrate (lane 3) under these conditions. No substrate degradation was observed in the absence of ribozyme (sTRSV, lane 1; data for sCYMV1 and sArMV substrate not shown). These results clearly show the sequences derived from sCYMV1 and sArMV are capable of phosphodiester cleavage of a corresponding target RNA substrate.

The sArMV ribozyme has a G opposite an A in the first 5′-base of the substrate in the proposed helix 2 of the hairpin model. The A base in the substrate was mutated to a C in this position to give the substrate sArMV-A1C. If the models are correct, this would allow formation of a C·G base pair and thus a 4 bp helix 2. The sArMV-A1C substrate sequence served as a substrate for the sArMV ribozyme (Figure 2, lane 8) with cleavage to 90% completion. This data clearly shows that for the sArMV system, a proposed conventional 4 bp helix 2 is catalytically active.

To further investigate the nature of mismatches in this position, we tested a mismatch in the chicory system. When the original sArMV substrate was tested with the sCYMV1 ribozyme, cleavage also occurred with this ribozyme (Figure 2, lane 7) to 70% completion under these conditions. This combination would give a proposed A·C mismatch in this first position of helix 2 for the chicory system resulting in a 3 bp helix 2.

Kinetic Analysis of sCYMV1 and sArMV Sequences. The catalytic parameters K_m and k_{cat} were determined for the sCYMV1 and sArMV ribozymes and various substrates under conditions of substrate excess with multiple turnover events for the ribozyme (Table 2). Thus these kinetic values represent true Michaelis−Menten catalytic parameters. Extensive ribozyme turnover was observed for both the sCYMV1 and the sArMV sequences, and the determined k_{cat} values were similar to the k_{cat} determined for sTRSV in side-by-side experiments. However, K_m values for the sCYMV1 and sArMV ribozymes, 400 nM (Table 2, line 5) and 2600 nM (Table 2, line 14), respectively, were higher than the value of 96 nM for sTRSV by a factor of four for the sCYMV1 ribozyme and by a factor of more than 20 for the sArMV ribozyme.

A likely contributing factor to the extremely high K_m for the sArMV ribozyme was due to the possible A·G mismatch in helix 2 at position one of the substrate to give a 3 bp

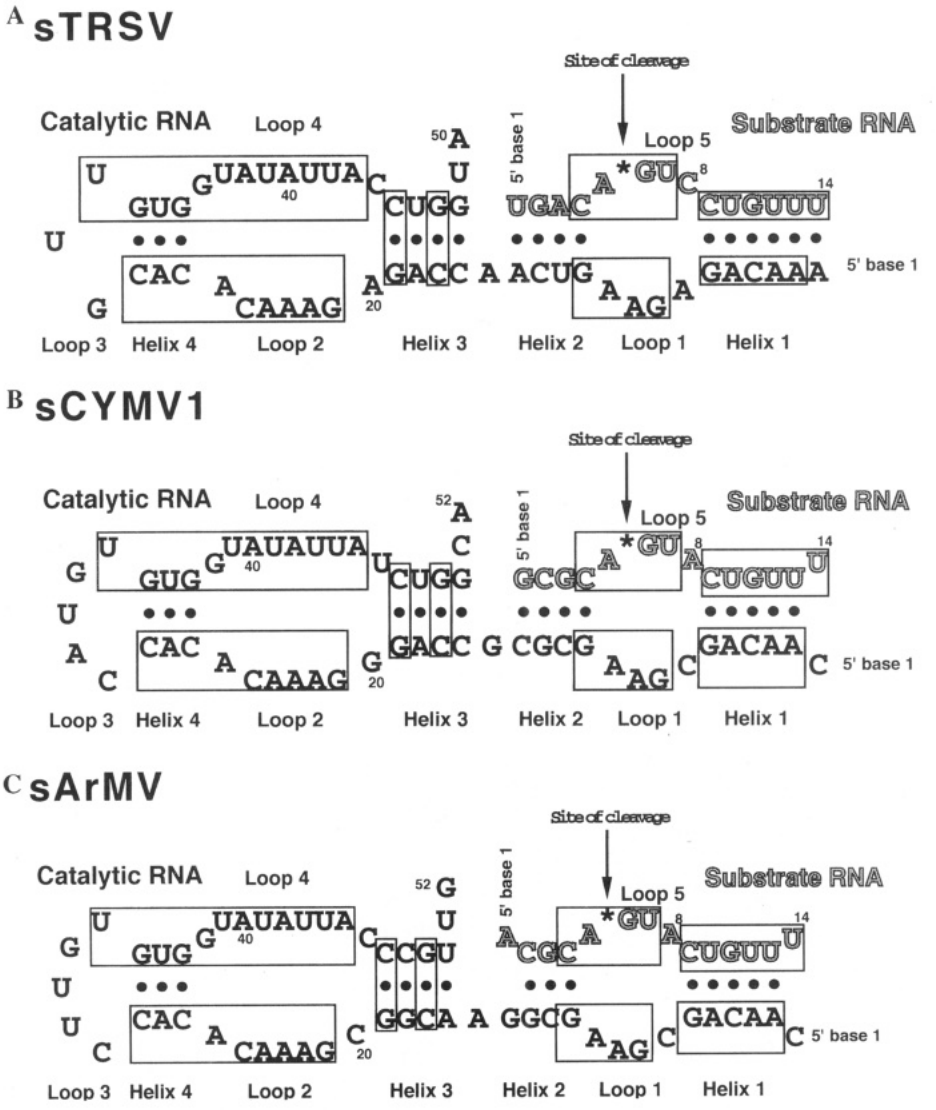

FIGURE 1: Schematic representation of the hairpin ribozyme−substrate complex. (A) sTRSV origin; (B) sCYMV1 origin; (C) sArMV origin. Boxed regions are conserved in all three RNAs. Ribozyme and substrate RNAs are numbered sequentially from 5′ to 3′.

helix 2. To test this in more detail the first base in the sArMV substrate was mutated from A1C to allow a C·G base pair in this position and thus give a proposed 4 bp helix 2. This substrate sequence, sArMV-A1C, lowered K_m by a factor of 3 and had no effect on k_{cat} (Table 2, line 10). This gave an overall 3× increase in catalytic efficiency. In a converse analysis, the K_m for the sCYMV1 ribozyme was increased by a factor of over 3 when the A·C mismatch was introduced into its substrate (this is the sArMV substrate) to give a 3 bp helix 2 (Table 2, line 9). This change from a 4 bp to a 3 bp helix 2 gave overall reduction in catalytic efficiency of 6×. Taken together, these results show optimal activity is obtained with a 4 bp helix 2 and the proposed helix 2 structure in the sCYMV1 and sArMV ribozyme−substrate complex is likely correct.

Mutational Analysis of the GUX Sequence at the Target Site. The GUX sequence is the sequence immediately following the cleavage site of the substrate. The sTRSV system has GUC in this position while the sArMV and sCYMV1 native sequences have a change in the base at position 8 to give GUA. A previous study mutated selected individual bases of the sTRSV substrate to match those of sArMV and examined the effects on phosphodiesterase activity (Anderson et al., 1994). Mutation of sTRSV substrate base 8 from C to A significantly diminished the extent of cleavage. Successful cleavage of native A8 substrates by corresponding native sCYMV1 and sArMV ribozymes suggested that these ribozymes may tolerate sequences other than GUC at this position. Therefore, a panel of substrates (Table 1) was constructed for each of the sTRSV, sCYMV1, and sArMV ribozymes to examine the effects of varying nucleotide 8 in the substrate. These are GUX variants. To make the comparison useful, the sArMV substrate used had a 4 bp helix 2 to make it equivalent to the sTRSV and sCYMV1 systems.

At an equimolar ratio of ribozyme to substrate (125 nM each) and for 1 h, cleavage occurred with all variants in position 8 (Figure 3). Under the experimental conditions of Figure 2, which include a 1:1 ribozyme:substrate ratio, sTRSV ribozyme cleaved sTRSV substrate C8A to 28% completion, C8 (93%), C8G (18%), and C8U (87%). The sCYMV1 ribozyme cleaved sCYMV1 substrate A8 to 92% completion, A8C (92%), C8G (73%), and C8U (92%). sArMV ribozyme cleaved sArMV-A1C-A8 to 93% completion, A1C-A8C (80%), A1C-A8G (80%), and A1C-A8U (90%). The sArMV ribozyme also cleaved A8-mutated substrates mismatched at nucleotide 1 with the same relative nucleotide preferences, but to a lesser extent (data not

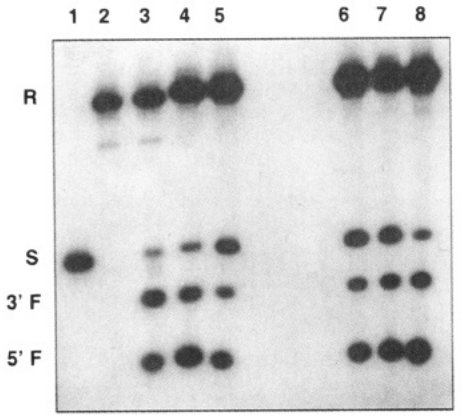

FIGURE 2: Substrate cleavage by ribozymes derived from sTRSV, sCYMV1 and sArMV sequences. Sequences of the ribozymes are in Figure 1, and the substrates are in Table 1. Cleavage of respective substrates by sTRSV (lane 3), sCYMV1 (lane 4), and sArMV (lane 5) ribozymes. Lanes 1 and 2 show that sTRSV substrate and ribozyme do not degrade individually under the incubation conditions. Also shown is cleavage of native sArMV substrate by sArMV ribozyme (lane 6) and sCYMV1 ribozyme (lane 7), which both mismatch the substrate at the first nucleotide to give a 3 bp helix 2. Lane 8 shows sArMV ribozyme cleavage of substrate sArMV-A1C, in which substrate base A1 has been mutated to a C to give a 4 bp helix 2. Ribozyme and substrate, 125 nM each, were incubated for 1 h at 37 °C in cleavage buffer and processed as described in Materials and Methods. (R) ribozyme; (S) substrate; (3′F) 3′-cleavage fragment; (5′F) 5′-cleavage fragment.

shown). This data clearly shows GUX sequences can be cleaved by the sArMV and sCYMV1 ribozymes.

True Michaelis−Menten kinetic parameters, K_m, k_{cat}, and k_{cat}/K_m (catalytic efficiency), for ribozyme cleavage of these GUX substrate mutants were determined (Table 2). For the sTRSV hairpin ribozyme, the native GUC sequence is clearly preferred, with GUC having a catalytic efficiency (k_{cat}/K_m) at least 30× greater than that of the GUU, GUA, and GUG sequences. For the sCYMV1 sequence the native GUA in the substrate is clearly preferred, with a catalytic efficiency 9× greater than the corresponding GUC sequence. The sCYMV1 substrates with GUG and GUU had such high K_m values that it was not possible to determine them. Thus these substrates cleave at a low efficiency. For the sArMV system, with a 4 bp helix 2, the native GUA is also the preferred sequence in the substrate. However the GUG sequence has a catalytic efficiency of nearly half that of the native GUA, indicating a new class of targetable substrates with GUG at the cleavage site may be possible. The GUU has a catalytic efficency of about one-third that of the preferred GUA in the substrate, and the GUC sequence is very poor for the sArMV system.

These data show that mutations in position 8 of the sTRSV substrate from C to G, U, or A produce reduction in catalytic efficiency which confirms earlier reports that the native GUC sequence is preferred (Hampel et al., 1990; Anderson et al., 1994). The preference order at substrate position 8 based on the catalytic efficiencies are C > U > G = A for sTRSV. This is the first complete determination of the kinetic values for all GUX variants of sTRSV based hairpin ribozyme. The values in Table 2, determined in this study, show a reproducible decrease in catalytic efficiency (k_{cat}/K_m) of more than 100-fold for the GUA and GUG variants. A reproducible decrease of 30× was seen for the GUU variant.

For the sCYMV1 system, the native GUA is the preferred sequence with order of preference in position 8 being A > C, G, U. For sArMV with a 4 bp helix 2, the order of preference in this position is A > G > U > C, again the preferred sequence after the site of cleavage is the native GUA. Most of the decrease in catalytic efficiency for the sCYMV1 and sArMV substrates is associated with an increase in K_m.

The GUA-containing substrate sequences for the chicory system had a catalytic efficiency 20× greater than that of the corresponding GUA sequence for the sTRSV system. The arabis system with GUA and a 4 bp helix 2 had a catalytic efficiency 15× greater than that of the GUA in the sTRSV system. This clearly shows that all three systems prefer their native sequences at this position, and greatly improved cleavage of GUA-containing substrates is seen in both the chicory and arabis systems over the sTRSV system. The arabis system also cleaves GUG-containing sequences at catalytic efficiencies 7× greater than cleavage of GUG-containing sequences by the sTRSV system. These results may allow broadened substrate applications for mRNA targeting.

DISCUSSION

Sequence and probable structural similarities of sCYMV1 and sArMV hairpin-like ribozymes with the sTRSV hairpin ribozyme indicate that they are likely members of the hairpin ribozyme family. Such a correlation for sArMV has been previously suggested (Haseloff & Gerlach, 1989). While the sequence homology is diverse, the structural similarities within the proposed models are striking. Major sequence differences are mostly in regions of nonrequired sequence for sTRSV hairpin ribozyme, with the most significant differences being in loop 3, which is known not to have required nucleotides (Anderson et al. 1994).

This study clearly shows, for the first time, that sequences derived from the satellite RNAs of sCYMV1 and sArMV have trans ribozyme activity. Both are capable of trans cleavage reactions. Although the native sTRSV ribozyme has the best catalytic parameters of the family, the sCYMV1 and sArMV ribozymes are also very active. Minor sequence alterations can improve ribozyme activity for the sArMV ribozyme. The catalytic efficiency of the sArMV ribozyme was increased 3× by changing the 5′-terminal base of the substrate from A to C. According to our model, this would change the length of helix 2 from 3 bp to 4 bp, and thus the data support the model. For the sCYMV1 ribozyme, when the proposed helix 2 was shortened from 4 bp to 3 bp, the catalytic efficiency was reduced 6×, showing the preference for a 4 bp helix 2 in the hairpin family of structures, again supporting this portion of the model.

For certain GUX sequences, the sCYMV1 and sArMV ribozymes have greater catalytic activity than the sTRSV ribozyme. The native sequences found in the sCYMV1 and sArMV substrates, having GUA after the cleavage site, are cleaved at very high catalytic efficiencies. This very clearly shows that a sCYMV1 or sArMV designed ribozyme may be superior to a sTRSV-based hairpin ribozyme for cleaving target a RNA sequence containing a GUA after the cleavage site. We have likely expanded the repertoire of cleavable target RNAs by the hairpin ribozyme family from GUC-containing sequences to include GUA-containing sequences.

The arabis system also cleaves GUG-containing sequences at significant catalytic efficiencies which are 7× greater than

Table 2: Kinetic Parameters and Comparison of the Efficiency of GUX Cleavage by sTRSV, sCYMV1, and sArMV Ribozymes

ribozyme	substrate	GUX	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m 10^4 M^{-1} min^{-1}
sTRSV	sTRSV-C8A	GUA	0.038	2500	2
	sTRSV	**GUC**	0.36	96	**360**
	sTRSV-C8G	GUG	0.01	540	2
	sTRSV-C8U	GUU	0.045	390	12
sCYMV1	sCYMV1	**GUA**	0.32	400	**80**
	sCYMV1-A8C	GUC	0.14	1500	9
	sCYMV1-A8G	GUG	ND[a]	>6500	
	sCYMV1-A8U	GUU	ND	>10000	
	sArMV	GUA	0.19	1400	14
sArMV	sArMV-A1C	**GUA**	0.26	880	**30**
	sArMV-A1C-A8C	GUC	0.22	5400	4
	sArMV-A1C-A8G	GUG	0.26	1700	15
	sArMV-A1C-A8U	GUU	0.40	3000	10
	sArMV	GUA	0.29	2600	11

[a] ND. These were not determined. V_{max} could not be reached.

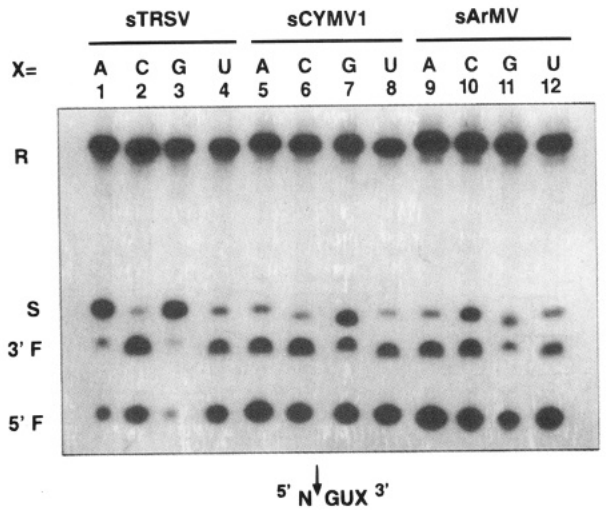

FIGURE 3: Comparison of sTRSV, sCYMV1 and sArMV ribozyme cleavage of substrates modified in GUX at the target site. The X base is at position 8 in the substrate. The sTRSV ribozyme cleavage of sTRSV-C8A, lane 1; native sTRSV, lane 2; sTRSV-C8G, lane 3, and sTRSV-C8U, lane 4. The sCYMV1 ribozyme cleavage of sCYMV1 native substrate, lane 5; sCYMV1-A8C, lane 6; sCYMV1-A8G, lane 7; and sCYMV1-A8U, lane 8. The sArMV ribozyme cleavage of sArMV substrate sArMV-A1C, lane 9; sArMV-A1C-A8C, lane 10; sArMV-A1C-A8G, lane 11; and sArMV-A1C-A8U, lane 12. Experiments were performed as in Figure 2.

cleavage of GUG-containing sequences by the sTRSV system. The hammerhead ribozyme does not cleave GUG sequences well (Perriman et al., 1992), and therefore in the future, an sArMV-based ribozyme may be developed for designing ribozymes to cleave GUG-containing sequences in target RNA sequences as well.

Maintenance of these configurations and judicious substitution of sequence from the sCYMV1 or sArMV ribozymes with sTRSV sequence may preserve altered sequence specificity while improving the catalytic efficiency. Even without catalytic improvement, the sCYMV1 and sArMV ribozymes may be useful in targeting cellular RNAs. Reductions in RNA within cells has been produced by ribozymes with catalytic efficiencies as low as 7×10^4 M^{-1} min^{-1} (DeYoung et al., 1994), although high levels of stable ribozyme expression are required for this result.

Previous studies suggested that sCYMV1 and sArMV ribozyme cleavage of native substrate would be poor on the basis of the activity of the sTRSV ribozyme when base 8 in the substrate is mutated from C to A (Fujitani et al., 1993; Anderson et al., 1994; Figure 3, Table 2). Alteration of bases A20 and A7 in the ribozyme to match sArMV ribozyme sequence failed to restore activity to wild-type levels, again suggesting that the sArMV ribozyme−substrate complex would have low activity (Anderson et al., 1994). The high level of catalytic activity for the sCYMV1 and sArMV ribozymes demonstrates that the sTRSV ribozyme may not be an ideal paradigm of hairpin ribozyme substrate selection. Similarly, a paradigm based on sArMV substrate selection characteristics would not predict the high catalytic efficiency of the sTRSV ribozyme−substrate complex. Formulation of a more general hairpin ribozyme model can be achieved by careful comparison of these three representatives of the family. Sequence elements regulating substrate selection should be found in the 27%−30% of the sCYMV1 and sArMV ribozyme complexes that differ from the sTRSV complex. The conserved areas shown as boxed regions in Figure 1 do not determine substrate selection at postion 8. A more general ribozyme model may also increase understanding of the function of U6 snRNA from *Schizosaccharomyces pombe*, which differs from the sTRSV ribozyme−substrate complex by 33% and cleaves substrate when the difference is reduced to 18% (Tani et al., 1992).

Although sArMV and sCYMV1 ribozyme activities evolved by natural selection to cleave GUA substrate, the given sequences were not necessarily optimized for trans cleavage. Adding an additional base pair to the proposed helix 2 improved the catalytic efficiency of the sArMV system 3×. This change is probably preferred for trans cleavage, because the native sequence cleaves in cis within the context of the satellite RNA. Cis-acting systems cleave much faster than the same sequences in trans (Altschuler et al. 1992). Hence, additional hybridizing bases might not be needed to more effectively cleave replicated copies in the native context or might participate in an unfavorable satellite RNA structure which would be selected against.

A range of kinetic values have been reported in the literature for the sTRSV hairpin ribozyme structure determined by Hampel and Tritz (1989). The k_{cat} values of 2.1 min^{-1} (Hampel & Tritz, 1989), 1.3−2.2 min^{-1} (Joseph et al., 1993), and 0.21 min^{-1} (Sekiguchi et al., 1991) have been published. A value for k_{cat} of 0.16 min^{-1} for a substrate that is three bases longer and using a ribozyme which was missing the essential 3′-terminal base was also published

(Feldstein et al., 1990). The k_{cat} value for this study, 0.36 min^{-1}, is within this range and was achieved in four separate experiments (Table 2). Published values for the K_m are 30 nM (Hampel & Tritz, 1989), 42 nM (Joseph et al., 1992), and 22 nM (Sekiguchi et al., 1991). A value of 290 nM, again for a longer substrate sequence and the partly inactive ribozyme, was also reported (Feldstein et al., 1990). The value of 96 nM reported here falls within this distribution. Large variations in ribozyme kinetic values have been reported elsewhere for other systems (Herschlag & Cech, 1990; Tuschl & Eckstein, 1993). Ribozyme activity is highly sensitive to changes in temperature, pH, and media composition (Hampel & Tritz, 1989), which may explain some of the differences.

The data in Table 2 show a range of K_m values for the various substrates, even in cases where the only change is in base 8 which is not explicitly identified as binding to the ribozyme in these two dimensional models. This two-dimensional model, does not address interactions affected by changes at this position at higher levels of structure. We have no direct information on what these might be; however, the likelihood of a hinge between helices 2 and 3, along with the large number of required bases in loops 2 and 4 (Anderson et al., 1994; Komatsu et al., 1994), clearly suggests the ribozyme may be folded back on itself to allow other portions of the ribozyme to interact with this region of the substrate during catalysis. If such a higher order folding were required to occur prior to catalysis and after formation of helices 1 and 2, it may be rate limiting for the catalytic reaction, consequently altering K_m.

Although understanding the reasons for the kinetic differences between the various members of the hairpin ribozyme family and their mutant substrates will require additional structural information, sequence comparisons of hairpin ribozyme family members should lead to discovery of the functional domains involved in substrate selection. This, in turn, should permit improved ribozyme engineering and more effective ribozyme therapy for the treatment of genetic and acquired diseases.

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