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Design and specificity of hammerhead ribozymes against calretinin mRNA

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ABSTRACT

We obtained a partial sequence of mouse calretinin mRNA from cDNA clones, and designed hammerhead ribozymes to cleave positions within it. With a view to optimising hammerhead ribozymes for eliminating the mRNA in vivo, we varied the length and sequence of the three duplex 'arms'and measured the cleavage of long RNA substrates *in vitro* at 37°C (as well as 50°C). Precise cleavage occurred, but it could only go to completion with a large excess of ribozyme. The evidence suggests that the rate-limiting step with a large target is not the cleavage, but the formation of the active ribozyme:substrate complex. The efficiency varied unpredictably according to the target site, the length of the substrate RNA, and the length of the ribozyme; secondary structure in vitro may be responsible. We particularly investigated the degree of sequence-specificity. Some mismatches could be tolerated, but shortening of the total basepairing with the substrate to less than 14 bp drastically reduced activity, implying that interaction with weakly-matched RNAs is unlikely to be a serious problem *in vivo*. These results suggest that specific and complete cleavage of a mRNA in vivo should be possible, given high-level expression of a ribozyme against a favourable target site.

INTRODUCTION

Hammerhead ribozymes are catalytic RNAs with sequence-specific endoribonuclease activity. They are defined by shared elements of primary and secondary structure, which consist of three double-stranded 'arms' of variable length and sequence, linked by short unpaired stretches of highly conserved sequences. They occur naturally in the single-stranded RNA genomes of certain plant viruses and virusoids, in which the cleavage site is part of the same molecule (1–4). Uhlenbeck (5) showed that the ribozyme could act in *trans*, with the ribozyme and substrate being separate oligoribonucleotides basepaired together; and that one ribozyme molecule could catalyse the cleavage of many molecules of substrate. Haseloff and Gerlach (6) devised a different arrangement whereby the substrate could be any RNA molecule (provided it contained a GUC or similar trinucleotide at the target site), and the ribozyme could be made as a ~40-nucleotide RNA with specific basepairing to the substrate.

They further showed that their ribozymes against chloramphenicol acetyltransferase (CAT) mRNA cleaved efficiently *in vitro*, showing catalytic turnover at 50°C. The target site is usually GUC in natural viral ribozymes, but GUA or NUY are permissible (7,8).

This offered the possibility of using ribozymes to destroy designated mRNAs in living cells, which could be useful both for anti-viral therapy, and for research on gene functions by producing null phenotypes.

Indeed, Haseloff-Gerlach ribozymes transcribed from expression plasmids in COS cells did diminish CAT mRNA, more so than an antisense oligoribonucleotide (9). Other studies in cultured cells have shown more substantial decreases in target RNAs caused by ribozymes. Thus ribozymes against various sites in HIV transcripts caused >90% reduction in synthesis of HIV proteins, and reduced viral replication (10–13). A ribozyme against *fos* mRNA in human cells caused reduced levels of Fos and of mRNAs for enzymes involved in DNA synthesis (14). More than 80% reduction in gene expression has also been achieved by ribozymes against *npt* mRNA in plant protoplasts (15), tumour necrosis factor mRNA in human cells (16), and α-lactalbumin mRNA in mouse cells (17).

However, studies with other ribozymes found little or no effect, even thought the same ribozymes had been shown to cleave their targets *in vitro* (17,18). A ribozyme against 28S rRNA in Xenopus oocytes did cut, but did not inhibit protein synthesis any more than an inactive mutant, so was probably acting merely as antisense (19). A ribozyme against HIV *tat* RNA was much less effective in cells than a corresponding antisense RNA (18). A ribozyme against U7 snRNA was effective in Xenopus oocytes but only if it was transported to the cytoplasm (20).

In all of these studies against long substrate RNAs in cells, substantial excess of ribozyme over target (>100:1) was required to achieve sufficient suppression, and there was no evidence for catalytic turnover. This contrasts with the well-documented ability of ribozymes in vitro to give efficient cleavage and rapid enzymatic turnover, using short or intramolecular substrates.

If ribozymes are to be used *in vivo*, the target will be a whole mRNA, in the presence of total cellular RNA, and the reaction may be limited not by the catalytic rate but by association with

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the correct substrate and competition with mismatched substrates. Therefore, we need to optimise both the efficiency and the specificity of cleavage. Variations in the lengths of the three arms may affect these properties.

Arm II, which is entirely within the ribozyme, is unlikely to affect specificity but, as the achievement of an active conformation could be a rate-limiting step, there may be scope for improvement by optimising the stability of arm II. In fact, arm II can be dispensed with, being replaced by a short flexible linker of 4–5 nucleotides (21). When this linker was made of five deoxythymidine residues, the construct was as active as the original, all-RNA ribozyme.

Arms I and III comprise the basepairing between ribozyme and substrate. They do not need to be long for an efficient catalytic step, as they are only 4–6 bp each in natural viral ribozymes, where cleavage is intramolecular. But longer arms might assist ribozyme efficiency in *trans* by enhancing the rate of association with substrate. On the other hand, arms longer than ~7 bp might not dissociate readily at 37°C, in which case the opportunity of catalytic turnover would be lost. If so, the ribozyme would be functioning as an antisense RNA, although it would still have the advantage of irreversibility.

Concerning specificity, an exact match of 14 bp with the target, comprising the sum of arms I and III, should only occur by chance in one RNA per cell. But if there is substantial activity against RNAs with shorter or imprecise basepairing, the ribozyme might cause cleavage of unrelated mRNAs, and its intended activity might be reduced by competition. The same problems have been shown to apply to antisense RNA (22).

Herschlag (23) has calculated that adequate specificity might be impossible. According to his analysis, there must be an optimal length of arms for specificity; longer arms stabilise binding to partially mismatched RNAs, so that these too are cleaved. He predicted that even an optimal ribozyme would cleave incorrect substrates many-fold more frequently than correct substrates. This might have detrimental effects on a cell, and also competitively inhibit the intended reaction. But these conclusions are very sensitive to the energies assumed for the basepairing interactions, and take no account of the stability of the active complex in three dimensions, so they require experimental testing.

Our eventual aim is to express ribozymes in transgenic mice to eliminate expression of calretinin. Calretinin is a neuronal cytosolic calcium-binding protein of unknown function (24–28). The sequence is strongly conserved between chick, human, and rat (25,29,30). Calretinin is expressed in a limited number of cells, almost all of which are neurons. It does not appear until the second half of gestation (embryonic day 14 in rat brain)(31). This expression pattern suggests that calretinin deficiency is unlikely to be lethal to the embryo. Therefore, calretinin appears to be a suitable gene to test the ability of ribozymes to produce a null phenotype in transgenic mice.

MATERIALS AND METHODS

Cloning of calretinin cDNA

RNA from brains of adult female mice (strain ABxT6) was prepared by the guanidinium isothiocyanate/hot phenol method (32), and then selected on oligo(dT). Double-stranded cDNA was synthesised from it using kits (cDNA Synthesis System Plus and λgt10 cDNA Cloning System; Amersham), following the manufacturer's instructions. In brief, this cDNA was primed with oligo(dT), second-stranded with RNase H plus *E. coli* DNA polymerase I, blunt-ended, fitted with linkers, ligated into the EcoRI site of λgt10, packaged, and plated out on *E.coli* NM514.

The library was screened with a human calretinin cDNA clone kindly provided by Dr. M. Parmentier (29). This was a M13 clone and the probe was a ^{32}P-labelled single-stranded DNA, made essentially as in (33). Plaque hybridisation was by standard techniques.

Construction of ribozyme and substrate genes

Construction, growth and subcloning of clones were done by a variety of standard techniques. Oligonucleotides were made by the Oligonucleotide Synthesis facility of the University of Cambridge, Departments of Biochemistry and Pathology. Sequencing of M13 and plasmid clones was done by the dideoxy technique using Sequenase 2 (U.S. Biochemicals).

All plasmid constructs used for *in vitro* transcription were checked by sequencing. To purify plasmid DNA for this purpose, it was either purified on Qiagen tip-5 (Diagen), or prepared using the Magic Minipreps system (Promega). Then, several micrograms of DNA were denatured in 200 mM NaOH/2 mM EDTA at 37°C for 20–30 mins, neutralised with sodium acetate pH 4.5, ethanol-precipitated, redissolved in 10 mM Tris pH 8.0/0.1 mM EDTA, and used for sequencing as for M13 templates. The primer for sequencing pGEM constructs was a 20-mer complementary to the T7 promoter (Promega).

Oligonucleotide-directed mutagenesis was performed according to (34). M13 template was prepared from *E. coli* strain CJ236 (*dut⁻ung⁻*) which incorporates uracil residues in the viral DNA. (We found the efficiency of transfection of CJ236 was about 1% of that typically obtained with *E. co*li JM101.) Oligonucleotides with the intended mutant sequence were 5′-phosphorylated, then annealed to the uracil-containing template as primer, and extended with Klenow enzyme; the products were transfected into *E. coli* JM101. Bacterial repair should favour the mutant strand over the uracil-containing template strand. Sequencing of clones obtained from various experiments showed mutant frequencies of 6–38%. The inserts were double-stranded *in vitro* with Klenow enzyme, excised with restriction enzmes, and cloned into pGEM4.

Synthesis and testing of ribozymes

Template DNA for in vitro transcription was prepared by a variant of the LiCl/Triton X-100 method (35), followed by ribonuclease A treatment and several phenol/chloroform extractions. Template was linearised by BamHI (for pGEM4 constructs) or HindIII (for pGEM3 constructs). For pSubS (small substrate, in pGEM3), to avoid producing a transcript with polylinker complementary to sequence in the ribozymes, linearisation was with XbaI, and it was normally necessary to gel-purify the linear form before use.

Ribozymes, substrates, and size markers were produced by in vitro transcription. This was done with T7 RNA polymerase and other reagents from Promega, using a protocol derived from that of the manufacturers. Strict precautions against ribonuclease were maintained. The reactions were labelled with α-^{32}P-UTP (Amersham or DuPont/NEN) so that the amount of RNA produced could be quantitated accurately: final specific activity 0.1 μCi/nmole for ribozymes, 50 μCi/nmole for substrates. After 2–3 hours of transcription at 37°, DNase I (RNase-free, Promega) was added to digest the template for 15–25 min, and the reaction was terminated with phenol:chloroform extraction, then a spun Sephadex G-50 minicolumn, and ethanol

precipitation. Typically, synthesis produced a single major band, so gel purification was not needed.

Ribozyme cleavage reactions were done in 20 μl with 50 mM TrisCl (pH 7.5 at reaction temperature), 20 mM $MgCl_2$, 1 mM Na_2EDTA, and 100 fmol of substrate RNA, unless otherwise stated. (EDTA was found necessary to prevent non-specific degradation of the RNA. In the set of experiments which included $MnCl_2$ replacing $MgCl_2$, Tris was replaced with HEPES to avoid oxidation of the Mn^{2+}.) Ribozyme and substrate were not pre-heated nor pre-annealed, as those steps would not be possible *in vivo*. Reactions were initiated by addition of ribozyme, and continued for 90 minutes at either 37° C or 50° C, then terminated by 20 μl of stop buffer (95% formamide, 60 mM Na2EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol FF). The stopped reactions were heated at 80 °C for 2 mins, then electropheresed on a 4% polyacrylamide/8 M urea gel (20 cm×40 cm×0.15 cm) in Tris-borate-EDTA buffer (89 mM Tris base, 8.9 mM *ortho*-boric acid, 10 mM EDTA), at 460−640 V for up to 3 hours. ^{32}P-labelled RNA size markers were provided from *in vitro* transcription of a mixture of known templates. The gel was autoradiographed on Fuji XR X-ray film.

To quantitate ribozyme-induced cleavage, bands corresponding to the uncleaved substrate and the cleavage products were excised from the gel, placed in vials with 3 ml of liquid scintillant (Quicksafe A, Zinsser Analytic; or EcoLume, ICN Labs), and counted in a scintillation counter. The counts from all the slices from a lane were added together and the contributions of each band were calculated relative to this amount. Two corrections were applied to take account of non-specific degradation. (i) To take account of contamination of the cleavage products with non-specific degradation products of larger transcripts, as measured in parallel lanes from reactions without ribozyme. This typically amounted to ~5% of substrate in 20 mM Mg^{2+} at 37°C, or more with higher [Mg^{2+}] or temperature. (ii) To take account of size-dependent loss of bands due to non-specific degradation. A formula was derived to make this correction from the measured ratio of the two cleavage products, on the assumption that they should be equimolar and that degradation was due to random cleavages. The frequency of degradation was typically 10^{-3} per nucleotide at 37°C.

```
  K  I  E  M  A  E  L  A  Q  I  L  P  T  E  E  N  F  L  L  C
GAAAATTGAGATGGCGGAGCTGGCGCAGATCCTGCCGACCGAAGAGAATTTCCTTTTGTG    60

  F  R  Q  H  V  G  S  S  A  E  F  M  E  A  W  R  K  Y  D  T
CTTCAGGCAGCACGTGGGCTCCAGCGCTGAGTTTATGGAGGCTTGGCGGAAGTATGACAC   120

  D  R  S  G  Y  I  E  A  N  E  L  K  G  F  L  S  D  L  L  K
AGACAGAAGTGGCTACATCGAAGCCAATGAGCTCAAGGGATTCCTGTCTGACCTCCTGAA   180
                                              A↑
  K  A  N  R  P  Y  D  E  P  K  L  Q  E  Y  T  Q  T  I  L  R
GAAGGCCAACAGGCCCTATGATGAACCTAAGCTCCAGGAGTACACCCAGACCATACTACG   240

  M  F  D  L  N  G  D  G  K  L  G  L  S  E  M  S  R  L  L  P
GATGTTTGACTTAAATGGAGATGGCAAATTGGGTCTCTCAGAGATGTCTAGACTCTTGCC   300
                                              Xba I
  V  Q  E  N  F  L  L  K  F  Q  G  M  K  L  T  S  E  E  F  N
TGTACAAGAGAACTTCCTGCTGAAATTTCAGGGTATGAAGCTGACCTCAGAAGAGTTCAA   360

  A  I  F  T  F  Y  D  K  D  G  S  G  Y  I  D  E  N  E  L  D
TGCCATCTTCACATTTTATGACAAGGATGGAAGCGGCTATATTGATGAGAATGAACTGGA   420

  A  L  L  K  D  L  Y  E  K  N  K  K  E  M  N  I  Q  Q  L  T
CGCCCTCCTGAAGGATCTGTATGAGAAGAACAAGAAGGAGATGAACATCCAACAGCTCAC   480

  T  Y  R  K  S  V  M  S  L  A  E  A  G  K  L  Y  R  K  D  L
CACCTACAGGAAGAGTGTCATGTCCTTGGCCGAGGCAGGGAAGCTCTACAGAAAGGACCT   540
          B↑          Sty I
  E  I  V  L  C  S  E  P  P  V  *
GGAGATTGTGCTCTGCAGTGAGCCCCCCGTGTAAAGGGGTGAAGGGACAGGGGCTGCTTC   600

TGCGCCTCCCTTGAACCCCGCCCCCGCTGTCCTGACTCTCTTGACACTCCTTCCCAGACC   660

TCCCCACCCCCTGCCACCTGCACACACCAGCCTGTGGATCTGGAAAGGAGAGATGGAGAG   720

AGGGTGGCTGGTAGGGTTCCTTAGGCCTGATAGACAGTTGTGCCTGCGTTGGGTCACGGT   780

TGGTGGGCGGGGCTGCAGGGGAAGCTTCTGTCGTCTCCGCTGCGATGCATGAGTTCCTTC   840
                    Hind III
GCTGTATGATTTAGGCTTCTGAGTCCCACAGAGTGGACTCCTTCCTCTTGCTGCGCCACC   900

CCTCCCCCTTTCTCCCCGGTCCACCGCTGACCCCGGACTTCCAGGTTCTGCCCACCAGCT   960

TGCTTAATGATCTAGCTGTCTTCTCAGAGCTACTGTGGAGGGTGACTGCCCTCTCCTTGT  1020

GTTCTTGACGTGTGTGTTCCTTTTCTCTTTGGCTTCCCCTGTCTTCTTGTTTACCAAAGA  1080

AGAGTTTACAGAGAATAAAGTGGAAATGTTCTGTCAAAAAAAAAAAAAAAA           1131
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Figure 1. Partial sequence of mouse calretinin cDNA, from two independent cDNA clones. The first 72 nucleotides were absent from one clone, and the first 144 nucleotides were only determined on one strand; however, these sequences were clear and they only differ from the rat sequence (30) by two non-coding positions. The encoded aminoacids are identical ⊙ the human sequence (29) except for the five positions circled. (EMBL accession number X73985.)

RESULTS

Cloning and sequencing of mouse calretinin cDNA

As the eventual goal is to express ribozymes aganst calretinin in mice, it was first necessary to obtain sequence from mouse calretinin mRNA. Using a probe from human calretinin cDNA

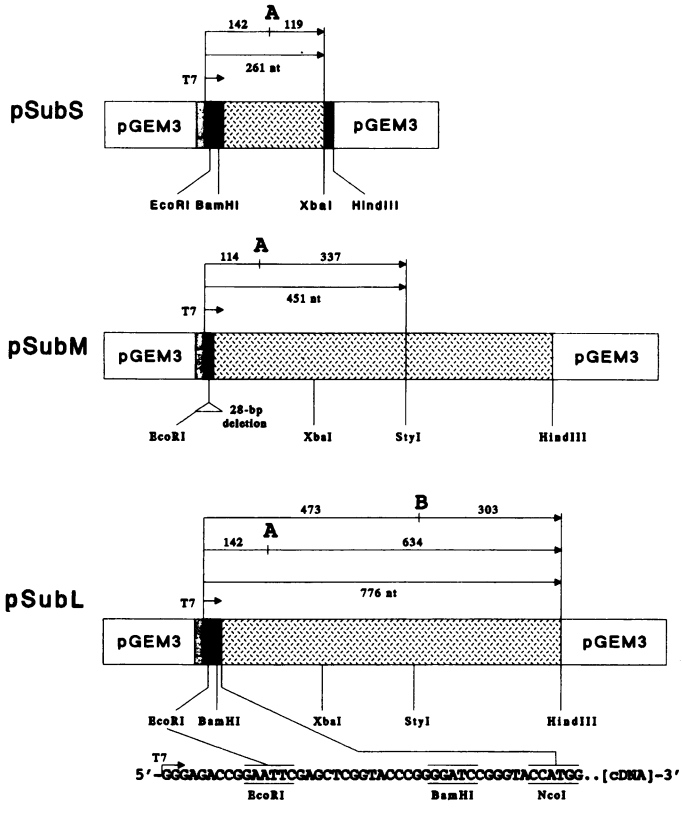

Figure 2. Structures of substrates for ribozymes, showing the plasmids from which the substrates were transcribed, and the expected products of cutting the substrate RNAs at site A or B. To create pSubL, a fragment of mouse calretinin cDNA, beginning at nucleotide 73 of Fig. 1 with an adaptor containing BamHI and NcoI sites (sequence at bottom), was inserted into pGEM3 as shown. The lightly dotted region indicates calretinin cDNA. pSubL produces a 776-nucleotide T7 transcript when linearised with HindIII. pSubM was derived by cutting pSubL with EcoRI and NcoI, filling in with Klenow enzyme, and religating; it can be cut uniquely with StyI to produce the 451-nucleotide T7 transcript. pSubS is similar to pSubL but the insert is shorter, producing a 261-nucleotide T7 transcript when linearised with XbaI.

(29), a 'northern blot' of mouse brain RNA showed a single band of about 2.0 kb (data not shown).

A cDNA library was prepared from mouse brain RNA, cloned into λgt10, and screened using the human calretinin probe. Two positive clones were obtained, which appear to be identical except that one is 72 nucleotides longer at the 5′ end. They yielded a combined sequence of 1131 bases (Fig. 1). Comparison with the human calretinin sequence indicates that this sequence contains part of the coding sequence and the 559-nucleotide 3′ untranslated region of mouse calretinin mRNA, ending in a poly(A) tract. About 82 codons are missing from the 5′ end of the mouse cDNA clones.

The cloned coding sequence specifies 190 aminoacids, which are 97% identical to the C-terminal 190 aminoacids of human calretinin: there are only five aminoacid differences, all in EF-hand domains V and VI, with no differences at all in domains III and IV. In contrast, the nucleotide identity in the coding region is only 90%. Compared with the recent sequence of rat calretinin cDNA (30), the nucleotide identity in the coding region is 96%, and there is only one aminoacid difference, in the last residue.

Construction and testing of ribozymes against two sites in calretinin mRNA

To provide an in-vitro substrate for ribozymes, segments of the mouse calretinin cDNA clone were subcloned into pGEM3 under the control of the T7 promoter and transcribed in vitro (Fig. 2). These produced substrates of 776, 451, and 261 nucleotides.

Ribozymes were designed against two sites in the mouse calretinin mRNA, named A and B (Fig. 1). The initial ribozymes were encoded by synthetic oligonucleotides which were subcloned into pGEM4 under the control of the T7 promoter, and transcribed in vitro (Fig. 3). One was named RzA8 (against site A, with messenger-complementary arms of 8 and 10 nucleotides). The other was named RzB9 (against site B, with arms of length 9 nucleotides each). RzB9 was designed to have a particularly stable loop II (sequence UUCG)(36–38) and a particularly low stability for any secondary structure in the remainder of the sequence. Using the FOLD program (39) to assess secondary structure in the absence of substrate, the least stably folded of several possible sequences was chosen.

Ribozyme RzA8 was tested against the in-vitro substrates, and produced cleavage products of the expected sizes (Fig. 4a,c). The cleavage products showed no heterogeneity, and the fact that the long and short substrates gave the same 142-nucleotide product proved that cleavage was occurring at site A as intended. RzB9 also produced the expected cleavage products (Fig. 4d). No such cleavages were seen during incubation without ribozyme, nor with 'anti-ribozyme' RNA produced by transcribing the ribozyme gene in opposite orientation (data not shown).

Effect of temperature: At 37°C, a substantial excess of ribozyme was required to obtain partial cleavage of the target RNA: 60% cleavage was obtained with 50-fold excess ribozyme. Most ribozyme:substrate combinations were about twice as active at 50°C as at 37°C (Figs. 4b, 5); nevertheless, complete cleavage required a >10-fold excess of ribozyme, even for the most favourable combination (RzA8 against the short substrate). Thus, catalytic turnover was not observed.

RzA8 could cleave a majority of the target RNA, but RzB9 was much less efficient (Figs. 4, 5). Therefore, all subsequent experiments were done with RzA8 and derivatives thereof, directed against site A. The effects of different reaction conditions

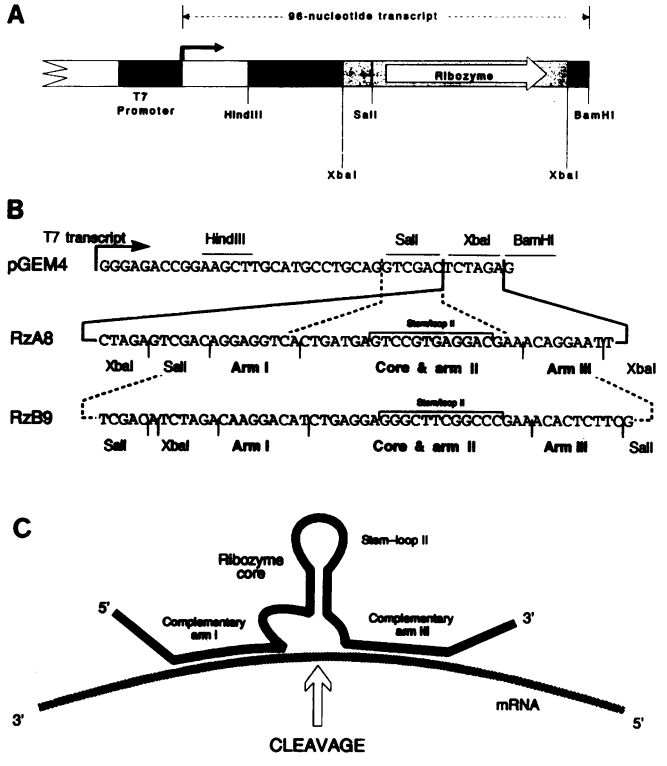

Figure 3. Sequences and predicted structures of ribozymes RzA8 and RzB9, as transcribed by T7 RNA polymerase from pGEM4 clones linearised with BamHI. (Top) structure of insert in pGEM4; (middle) sequences of the transcripts; (bottom) structure of ribozyme in active complex with target site, identifying arms I–III.

were explored using a quantitative assay, in which the percentage cleavage was measured by counting the radioactivity in gel slices.

Effect of cations: The cleavage reaction required Mg^{2+} (data not shown), and was optimal between 20 and 40 mM Mg^{2+}, in agreement with (5).

It has been reported that the reaction was several times more efficient with Mn^{2+} replacing Mg^{2+} (5,40). In contrast, we found that replacing Mg^{2+} with 20 mM Mn^{2+} produced only a very slight increase in cleavage (data not shown).

The effect of varying salt concentration was also tested. Using RzA8 on the short substrate at 37°, varying the Na^{+} concentration between 0 and 100 mM had no significant effect on the cleavage.

Effect of length of substrate: It was notable that RzA8 cleaved the long substrate much less than the short substrate at 37° (Fig. 5), although there was little difference at 50°. The most likely explanation is secondary structure in the substrate or unintended pairing between the ribozyme and non-target regions of the substrate. Outside the target region, the next closest complementarity to the RzA8 arms in the long substrate is a 7-bp match at nucleotides 657–663. We therefore prepared an intermediate-length substrate (451 nucleotides long; SubM in Fig. 2) which lacks this sequence. However, this showed even less cleavage than the others (Fig. 5). It thus appears that the susceptibility of the target site varies unpredictably with the length of the substrate.

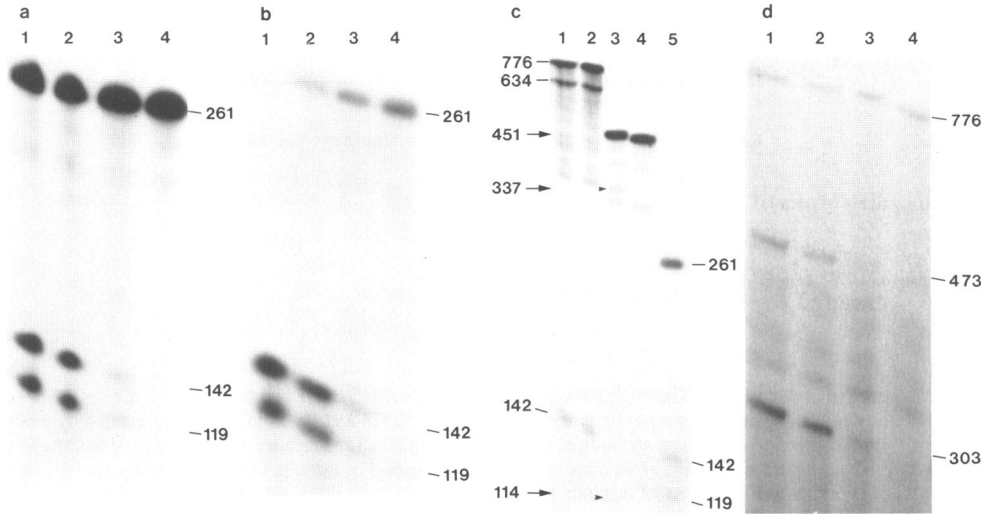

Figure 4. Cleavage of RNA substrates by ribozymes. **(a)** Cleavage of the 261-nt substrate by RzA8 at 37°, forming 119-nt and 142-nt fragments as predicted in Fig. 2. Ribozyme:substrate ratios were (lane 1) 50:1, (lane 2) 10:1, (lane 3) 1:1, (lane 4) 1:10. **(b)** Cleavage of the 261-nt substrate by RzA8 at 50°, showing higher efficiency. Ribozyme:substrate ratios as in (a). **(c)** Cleavage of three substrates with different lengths by RzA8 at 37°. (Lanes 1 and 2), 776-nt substrate cleaved to 634 nt plus 142 nt. (Lane 3), 451-nt substrate cleaved to 337 nt plus 114 nt. (Lane 4), same without ribozyme. (Lane 5), 261-nt substrate cleaved to 142 nt plus 119 nt. Lengths of cleavage products are as predicted in Fig. 2; the comigration of the 142-nt fragment from both large and small substrates confirms that cleavage is at site A. **(d)** Cleavage of the 776-nt substrate by RzB9 at 50°. The predicted fragments of 473 and 303 nt are visible. Ribozyme:substrate ratios were (lane 1) 50:1, (lane 2) 10:1, (lane 3) 1:1, (lane 4) 1:10. (In this experiment, there was more non-specific degradation of RNA than usual.)

Changes to arm II

The sequence UUCG forms a particularly stable loop structure for closing RNA hairpins (36−38). Therefore, if arm II were closed with this sequence, it might favour the formation of the active ribozyme structure and enhance cleavage efficiency. To achieve this, the RzA8 sequence in M13-mp18 was modified by oligonucleotide-directed mutagenesis, and transferred to pGEM4 for expression; this ribozyme was named RzM8 (Fig. 6).

When tested against either the long or the short substrate, at 37° in either 10-fold or 50-fold excess over substrate, RzM8 produced only 0.67 to 0.85 times as much cleavage as RzA8.

Shortened arms I and III

It was important to test ribozymes with reduced basepairing to the target, to answer two questions. On the one hand, would reduced basepairing permit catalytic turnover and thus increase the efficiency of cleavage? On the other hand, would activity be limited by a requirement for a minimum length of basepairing? The minimum basepairing requirement will determine the specificity of ribozyme activity in mixed cellular RNA, where the longest exact complementarity expected to occur by chance would be ~14 nucleotides.

To reduce the lengths of the arms, the RzA8 sequence was subjected to oligonucleotide-directed mutagenesis in M13-mp18 (Fig. 6). First, the outer part of arm III (originally 8 bp) was mutagenised, creating ribozymes RzD8 to RzI8 (Table 1). All these were somewhat less active than RzA8, but activity was not eliminated even when the arm was reduced from 8 bp to 4 bp (RzH8). The activity correlates well with the predicted stability of the basepaired arm (Table 1). This is evident with two ribozymes that had a shortened arm separated by a mismatch from a further 2-bp complementarity (RzF8 and RzH8). The detached 2-bp match makes no net contribution to binding energy nor, according to Fig. 7, to the activity of the ribozyme. However,

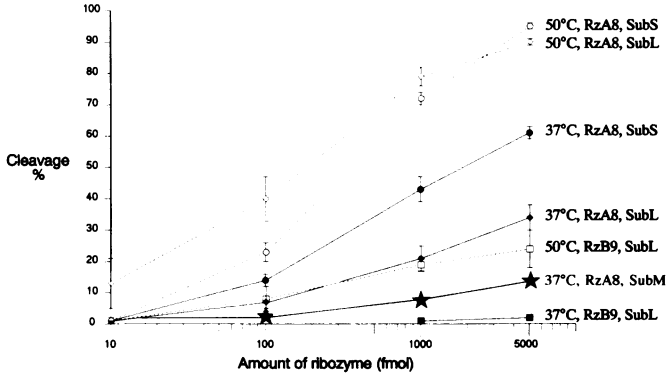

Figure 5. Effects of temperature and substrate length on ribozyme activity. The chart shows the cleavage of 100 fmol of 261-nt substrate (SubS) or 776-nt substrate (SubL), at 37° (filled symbols) and at 50° (open symbols), and of 451-nt substrate (SubM) at 37° (stars). See Fig. 2 for substrate structures. These are averages of 3−5 experiments (± s.e.m.).

RzI8 gave an interesting result. This ribozyme has a single mismatch only 3 nucleotides from the cleavage site, but it cut as well as other ribozymes with the same degree of overall complementarity in this arm.

The least active of the arm-III mutants (RzH8, with arm III rduced to 4 bp) was used as starting-point for mutagenesis of arm I (starting length 10 bp;Fig. 6). All these mutants had arm I reduced to 7 bp or less, so the total basepairing to target was reduced to 11bp or less, and all these showed extremely low or zero activity (Fig. 7).

Lengthened arms I and III

Another ribozyme was made with much longer arms: RzA14, identical to RzA8 except that arms I and III were extended to

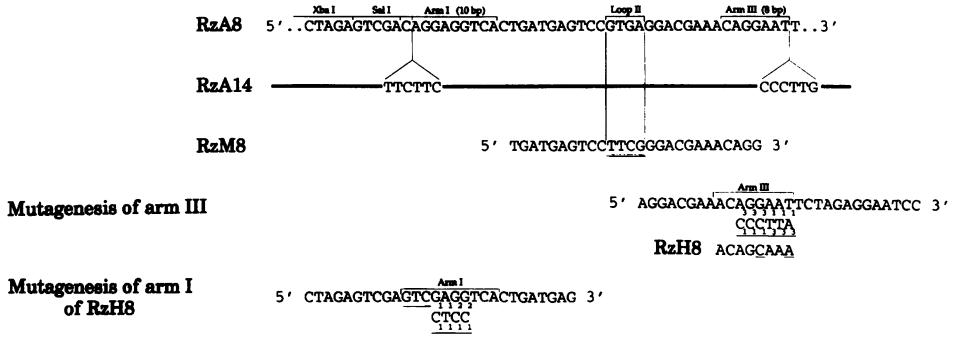

Figure. 6. Derivation of modified ribozymes from RzA8. The modified genes were created in M13-mp18 and then transferred to pGEM4 and checked by sequencing. RzA14 was constructed independently to contain arms that are 6 bp longer on each side. RzM8 and RzD8 to RzI8 were created by mutagenesis of the RzA8 gene using the oligonucleotides shown. Where alternative positions are shown, the oligonucleotide had redundancy, and the ratio of wild-type to variant nucleotide varied from 3:1 to 1:1 along the sequence so as to produce a range of mutants in which the outer part of the arm was preferentially altered. One of these mutants, RzH8, was used for further mutagenesis of the other arm by the same method; in this case the ratio of wild-type to variant nucleotide varied from 2:1 through 1:1 to 0:1 along the arm, and the products were RzJ8, RzK8, and RzL8. Sequences of individual mutants are listed in Table 1.

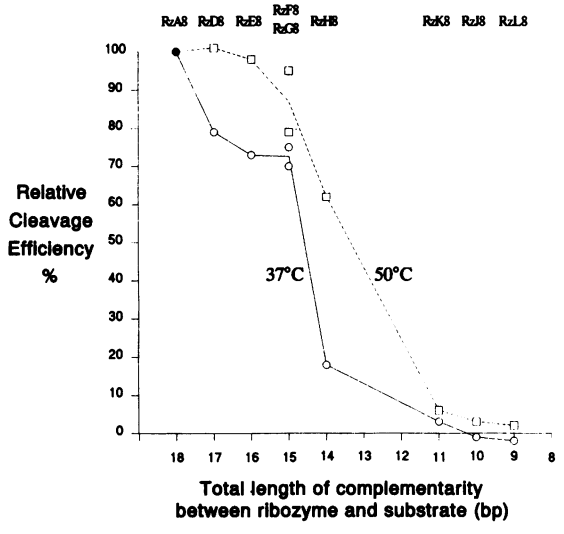

Figure. 7. Effect of total complementary arm length on cleavage efficiency. Data are taken from Table 1. (Arm lengths for RzF8 and RzH8 exclude two A:U basepairs that lie betwen mismatches, as these make no net contribution to stability.) The 50° points for RzJ8, RzK8, and RzL8 are overestimates, as a higher ribozyme:substrate ratio was used in these experiments.

16 and 14 bp respectively (Fig. 6). Surprisingly, this was much less effective than RzA8 (Table 1).

DISCUSSION

Catalytic properties of ribozymes

One of our ribozymes, RzA8, is capable of cleaving the majority of its target RNA, but the rate of reaction is much slower than reported for ribozymes against small or intramolecular substrates, and there is no evidence for catalytic turnover in the system.

The maximum cleavage obtained against our substrate RNA at 37° was 61% in 90 mins with a ribozyme:substrate ratio of 50:1. This is in striking contrast to cleavage reported for an oligonucleotide (41): 90% after 40 mins at 25° with a ribozyme:substrate ratio of 1:25. As our ribozyme core sequence is the same as in ribozymes which can catalyse rapid cleavage on an intramolecular substrate (satellite tobacco ringspot virus) or oligonucleotide substrate (42), the limitation on rate is probably due to the use of a large RNA as substrate. This is consistent with the results of Heidenreich and Eckstein (43). In a direct comparison, they found that a ribozyme cleaved a 19-mer oligoribonucleotide substrate 1000 times more efficiently than a 985-nucleotide substrate containing the same target sequence.

This suggests that catalysis is not rate-limiting against a large substrate. This is supported by the lack of effect of Mn^{2+}. Against an oligonucleotide substrate, use of Mn^{2+} in place of Mg^{2+} increased the rate of reaction by an order of magnitude (5,40); this was an effect on k_{cat} (44). We found that Mn^{2+} had no effect, as did another study using large substrates (45). Thus the rate-limiting step against a large substrate is not catalysis.

Studies of ribozymes against oligonucleotide substrates show reduced k_{cat} when arms I and III are each longer than 6 bp, suggesting that dissociation of products from ribozyme may be rate-limiting in these cases (41,46,47). But this cannot be the rate-limiting step in our experiments, as the ribozyme was used in great excess.

The rate-limiting step is likely to be either finding the target, or refolding the substrate and ribozyme to form the active hammerhead complex. This accords with the marked disparities in efficiency shown between substrates of different lengths and between two different sites in the substrate.

Alteration of arm II

Mutation of the loop of arm II to a particularly stable structure, UUCG (36–38), had little effect on ribozyme activity. This could be because the formation of arm II is not implicated in the rate-limiting step. However, it has subsequently been shown that the original loop of arm II (GUGA), which was taken from satellite tobacco ringspot virus, itself belongs to another family of stable loop structures, GNRA (48). Thus it may already be optimal for hammerhead formation.

Shorter arms I and III

The main issue that we wished to address was that of the optimum lengths of basepairing to substrate, for activity and for specificity. If ribozymes are to be useful in vivo, the complementarity required for recognition must be sufficiently large to avoid a lot of cleavage of mismatched RNAs. Against short substrates, the

Table 1. Structures and activities of ribozymes with different extents of basepairing to the substrate at site A.

Ribozyme construct	Arm I sequence	Arm III sequence	Total binding energy (kcal/mol)	Effective arm lengths (bp)	Efficiency at 37°	Efficiency at 50°
RzA8	CAGGAGGUCA	ACAGGAAU	−14.3	10 + 8	100%	100%
RzD8	CAGGAGGUCA	ACAGGAAA	−13.4	10 + 7	78.5 (±2.2)%	101 (±3.8)%
RzE8	CAGGAGGUCA	ACAGGAUA	−12.5	10 + 6	72.6 (±5.5)%	97.5 (±2.8)%
RzF8	CAGGAGGUCA	ACAGGUAU	−10.3	10 + 5	75.0 (±2.2)%	95.0 (±0.9)%
RzG8	CAGGAGGUCA	ACAGGUAA	−10.2	10 + 5	69.6 (±5.5)%	78.5 (±2.2)%
RzI8	CAGGAGGUCA	ACCGGAAU	−10.0	10 + 5(+2)	68.0 (±2.9)%	80.4 (±4.3)%
RzH8	CAGGAGGUCA	ACAGCAAA	−7.4	10 + 4	18.0 (±2.6)%	62.0 (±3.3)%
RzK8	GUCGAGGUCA	ACAGCAAA	−1.0	7 + 4	3.3 (±2.3)%	5.9 (±0.3)%
RzJ8	GUCCAGGUCA	ACAGCAAA	+1.3	6 + 4	−0.8 (±0.6)%	2.6 (±1.0)%
RzL8	GUCCUGGUCA	ACAGCAAA	+3.0	5 + 4	−1.6 (±0.0)%	2.5 (±1.0)%
RzA14	CUUCUUC-AGGAGGUCA	ACAGGAA-UCCCUUG	−36.6	16 +14	26.0 (±3.0)%	29.0 (±4.1)%

In the sequences of arms I and III, underlining marks mismatched residues. Binding energies were calculated using the program FOLD (39). Relative cleavage efficiencies were measured against 261-nt substrate in standard buffer; at 37°, with ribozyme:substrate ratio of 50:1; at 50°, with ratio of 10:1, except for those marked * which had a ratio of 20:1. The figures are the average (±s.e.m.) of 4−7 experiments for RzD8 to RzI8, and 2−3 experiments for RzJ8 to RzL8. RzA14 efficiencies were measured in separate experiments from RzA8.

ribozyme is most active with 10−12 bp total complementarity in arms I and III, to maintain rapid dissociation of products from ribozyme (41,46,47). Cleavage of RNAs with only a 10-bp match might lead to considerable cleavage in *trans* of normal cellular RNAs. Symons (4) suggested that this might account for some of the pathogenic effects of the viruses and similar molecules that contain catalytic RNA. Herschlag (23) calculated that this might be an unavoidable byproduct of ribozyme activity. If so, low-specificity interactions might damage the cell and also competitively inhibit the desired reaction. However, if basepairing with the target is rate-limiting for cleavage of large and complex RNAs, the optimal length for activity might be considerably greater than 12 bp.

We started with a ribozyme with a total of 18 bp basepairing to target in arms I and III, and found that minor mutation or shortening of the arms was well tolerated, as long as the total length of basepairing was not less than 14 bp. When the total length was reduced to 11 bp or less, very little activity remained. Our results agree with another study (43), which found that activity against a 1-kb substrate was dramatically reduced by shortening arms I and III in total from 14 bp to 10 bp. This is encouraging for the utility of ribozymes in eukaryotic cells, where a chance match of 14 bp would be expected in only 1 mRNA species. Moreover, given the variation in susceptibility of different sites found in our and previous studies, the real incidence of accidental cleavages would probably be less.

We have also done a preliminary experiment to determine whether complex cellular RNA would competitively inhibit cleavage (unpublished results). RzA8 was incubated with the 261-nucleotide substrate in the presence of a 200-fold molar excess of cellular poly(A)$^+$ RNA plus a 100-fold molar excess of poly(A)$^-$ RNA, and cleavage occurred at 60% of the control level. Thus, while mild inhibition occurs, worries about massive competition appear to be unfounded.

It seems likely that longer arms are better for longer substrates because they increase the probability of successfully nucleating the correct ribozyme:substrate complex, in competition with non-specific hybrids and internal structure in the substrate. In this context it is interesting to note the results with a 38-nucleotide substrate (19), which was cleaved efficiently by a ribozyme with 16 bp basepairing, and very poorly with only 12 bp basepairing. Thus a substrate does not have to be very long before the advantages of long arms I and III become evident - presumably as a function of self-folding of the substrate.

Longer arms I and III

As catalytic turnover was not observed in our system, one would not predict any upper limit on the lengths of arms I and III. It is therefore odd that RzA14 (with total basepairing 30 bp) gave severalfold lower activity than RzA8. This may be an idiosyncrasy of this particular ribozyme, which has more stable self-folding than RzA8 (−0.27 kcal/mole/nucleotide for RzA14, −0.19 kcal/mole/ nucleotide for RzA8, according to the FOLD program). A recent study (13) showed that a ribozyme with arms I and III totalling 27 bp was very active against a large RNA substrate, independent of substrate size: 73% cleavage of a 380-nucleotide target in 5 mins with equimolar ribozyme.

Therefore, it appears that RNA recognition is still the rate-limiting step, and that in the absence of catalytic turnover, the most efficient ribozyme may be one that is attached to a substantial

length of antisense RNA. The ribozyme may still be more effective than antisense RNA alone in inhibiting gene expression because of its irreversible effect. But ribozyme constructs will have to be checked individually to see if they can readily form the active complex with the target, in competition with self-folding.

Use of tandem ribozymes may also be advantageous (12). In principle, tandem ribozymes might be synergistic because of the presence of multiple substrate-complementary regions within the polyribozyme molecule. However, we have tried a double ribozyme with the RzB9 and RzA8 sequences in tandem, and this was no more effective than RzA8 alone (unpublished data). Therefore any advantage of tandem ribozymes will also need to be established in individual cases.

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