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Selective cleavage of closely-related mRNAs by synthetic ribozymes

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ABSTRACT

In *Phaseolus vulgaris* L. (French bean) glutamine synthetase (GS) is encoded by four closely-related genes termed *gln-α*, *gln-β*, *gln-γ* and *gln-δ*. We have constructed and characterised *in vitro* a number of hammerhead ribozymes designed to cleave individual RNAs encoded by these genes. The three ribozymes, termed J1, J2 and J3, were targeted to cleave RNA at the start of the γ and β, and the middle of the γ, GS open reading frames respectively. All three ribozymes successfully discriminated between the four (α, β, γ and δ) highly homologous sequences, even though the targeted sites of cleavage shared up to 18 out of 22 identical bases with other gene family members. The ribozyme-mediated cleavage reactions were Mg^{2+} dependent and enhanced at higher temperatures, although the J1 ribozyme retained considerable activity at physiological temperatures. Both J1 and J2 demonstrated a time-dependent cleavage of their targeted GS RNAs, although these two ribozymes differed markedly in their ability to cleave multiple substrate molecules. The rate of cleavage by J1 was found to be reduced in the presence of related GS RNAs and by total leaf poly(A) RNAs. The implications of these results for ribozyme activity *in vivo* are discussed.

INTRODUCTION

Ribozymes are RNA molecules that possess endoribonuclease activity (1). Most naturally occurring ribozyme activities are intramolecular and have roles involved with RNA processing, such as intron removal by self-splicing and cleavage of concatameric transcripts from replicating RNA viruses (1,2,3,4). Symons and coworkers, working on plant virus replication, identified a common secondary structure conserved between a large class of the self-cleaving RNAs of a number of infectious plant pathogenic RNAs. This structure consisted of three double-stranded RNA loops and 17 conserved nucleotides in the shape of a 'hammerhead' configuration (5). They then showed that a small 55-base RNA segment, derived from a plant RNA virus, was able to undergo efficient self-cleavage *in vitro* (6). Haseloff and Gerlach (7) then resolved this self-cleaving domain into two separate catalytic and substrate RNAs, so that most of the conserved residues were retained by the catalytic RNA. Thus, the substrate RNA had very few sequence requirements, other than an ability to base-pair to the ribozyme strand, and to contain a GUX motif (where X can be C, A or U) at the site of cleavage. Taking advantage of this, the authors further demonstrated that they could design ribozymes containing a conserved 22 base catalytic domain flanked by arms of about 8 bases complementary to the sequence flanking a GUC motif in the substrate RNA. Such designer hammerhead ribozymes were shown to act *in trans*, like RNA restriction endonucleases, cleaving a natural RNA *in vitro* in a highly sequence-specific manner (7). These ribozymes are now being investigated as potential antiviral agents in both animals and plants (2,3,8).

Another field in which ribozymes are potentially useful is in the analysis of gene function. As discussed above, ribozymes exhibit not only a catalytic endoribonuclease activity, but they also demonstrate a high degree of sequence-specificity. Thus if they were expressed *in vivo* they may inactivate their complementary RNA and hence interfere with the expression of a specific gene. We wish to exploit these features in our investigation of the roles of the glutamine synthetase (GS) multigene family of *Phaseolus vulgaris* L. (French bean). This family consists of four differentially expressed genes (*gln-α*, *gln-β*, *gln-γ* and *gln-δ*), encoding three cytosolic polypeptides (α, β and γ), and a precursor to the plastid-located polypeptide (δ). The GS genes are between 70% and 86% identical in the coding regions (9). We would like to employ an approach that could distinguish between the gene products of the closely related GS gene family members and hence investigate the individual role of each GS gene *in planta*. This paper describes the design, construction and *in vitro* characterisation of three 'hammerhead' ribozymes which can successfully distinguish between their target, and other closely related, GS RNAs.

MATERIALS AND METHODS

Template DNAs

Restriction enzymes used for construction and linearisation of template DNAs were obtained from BRL or Amersham. The

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methods used are described in Sambrook et al. (10) unless otherwise stated. Plasmids pcGS-α1T, pcGS-β1T and pcGS-δ1T contain the essentially full-length α, β and δ cDNAs cloned into pGEM (Promega) *in vitro* transcription vectors (11). Plasmid pcGS-γ1 contains the essentially full-length γ cDNA in pUC19 but including a 73 bp cloning artefact at the 5′ end (11). This artifact was removed and an *Eco*RV restriction site introduced 65 bp upstream of the initiating ATG codon, using an Amersham *in vitro* mutagenesis kit, to create pcGS-γ10. The entire γ GS cDNA was then cloned as a 1.5 kbp blunt-ended *Taq*I fragment from pcGS-γ10, into *Sma*I linearised pGEM-4Z (Promega), to give pcGS-γ10T. J1, J2 and J3 ribozyme sequences were each synthesized as two complementary 54-mer and 46-mer oligodeoxynucleotides using automated phosphoramidite chemistry. The oligonucleotides were kinased and the complementary pairs hybridised together to give *Bam*HI and *Sac*I compatible ends and then cloned into *Bam*HI-*Sac*I linearised pGEM-4Z, to give plasmids pJ1, pJ2 and pJ3 respectively. All constructs were verified by restriction mapping and dideoxy sequencing, the latter using the Sequenase system (United States Biochemicals).

Synthesis of ribozyme and GS RNAs

Transcription templates were linearised with either 5′ overhang or blunt-end cleaving restriction enzymes as follows. The ribozyme plasmids pJ1, pJ2 and pJ3 were linearised at a *Pvu*I site at the end of the ribozyme sequence. Plasmid pcGS-α1T was linearised near the 3′ end of the cDNA with *Xmn*I. Plasmids pcGS-β1T, pcGS-γ10T and pcGS-δ1T were linearised in the polylinkers at the 3′ ends of the cDNAs with *Sma*I, *Sal*I and *Xba*I respectively. These four linearised plasmids were used to synthesize the essentially full-length α, β, γ and pre-δ RNAs respectively. To produce shortened β and γ GS RNAs, pcGS-β1T and pcGS-γ10T were linearised with *Bam*HI prior to transcription. All linearised DNA was transcribed *in vitro* using T7 polymerase except DNA from plasmid pcGS-γ10T where SP6 polymerase was used. A standard 100 μl transcription reaction contained 2.5 μg template DNA, 80 units of RNasin (Promega), 10 mM DTT, 1 mM of each rNTP (Boehringer Mannheim), 10 μg bovine serum albumin, 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl and 50–100 units of RNA polymerase. To radiolabel GS RNA, 1 μCi [α^{32}P] UTP (Amersham) was included in the reaction. To simply quantitate the amount of either ribozyme or GS RNA synthesized in a transcription reaction, a trace of approximately 0.05 μCi [α^{32}P] UTP was included to determine the efficiency of incorporation. Reactions were incubated at 37°C for 60 minutes, followed by another 15 minute period after the addition of 3 units of RQ1 RNase free DNase (Promega), to remove template DNA. The reaction was terminated by phenol-chloroform extraction, and the RNA recovered by ethanol precipitation. All RNAs were run on denaturing formamide gels (12) to verify their size and the quantitation.

Ribozyme cleavage assays

GS and ribozyme RNAs were initially heat denatured at 80°C for 3 minutes, in a buffer containing 1 mM NaEDTA pH 8.0, followed by quick cooling on ice (however, little difference was observed in the rates of cleavage whether or not the GS and ribozyme RNAs were heat-treated [data not shown]). The standard cleavage reaction was conducted in 6 μl at 50°C in the presence of 50 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, and initiated with the addition of ribozyme RNA. For time courses larger reactions were set up and aliquots of 6 μl were removed at various time intervals. The initial concentration of substrate RNA (S_0)was generally about 50 nM and was in 2-fold excess over the concentration of ribozyme except in the experiments in Figure 2 (where the ribozyme was in excess), Figure 3 (where the substrate:ribozyme ratio was 1:1) and Figure 5 (where the ribozyme concentration was varied as indicated). For the experiment in Figure 6 the competing DNA was ethanol precipitated and resuspended in cleavage buffer followed by subsequent addition of substrate RNA. The reaction was then initiated by adding ribozyme. In all cases the reactions were terminated by the addition of 2 μl of gel loading buffer (95% formamide, 10 mM NaEDTA, 0.02% xylene cyanol and 0.02% bromophenol blue), and then placed on ice. Samples were then heat-denatured at 80°C for 2 minutes prior to running on 6% polyacrylamide, 7M urea gels. The gels were then dried and subjected to autoradiography.

Quantitation of cleavage reactions

Autoradiographs were scanned using a Molecular Dynamics Computing Densitometer with Image-Quant software (release version 3.0).

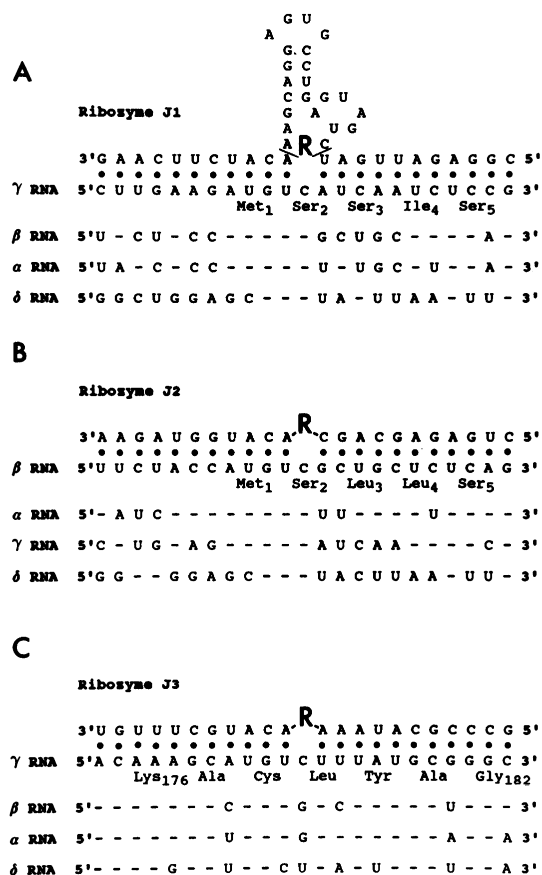

Figure 1. Nucleotide sequences of the synthetic hammerhead ribozymes and their targets in the GS RNAs. The sequences of the other three GS RNAs at the target sites are shown for comparison. The 22 bases of the catalytic domain of the ribozyme shown in part A are replaced by R in parts B and C. **A:** Ribozyme J1 which targets the second codon in the *gln-γ* RNA. **B:** Ribozyme J2 which targets the same position in the *gln-β* RNA. **C:** Ribozyme J3 which targets codon 179 in the *gln-γ* RNA.

RESULTS

Description of the synthetic ribozymes and their GS RNA substrates

Figure 1 presents information about the ribozymes and their designated sites of cleavage within the GS mRNA sequences discussed in this paper. Three hammerhead ribozyme RNA molecules (J1, J2 and J3) were 'designed' according to the guidelines outlined by Haseloff and Gerlach (7). Firstly each ribozyme contains the 22 bases of the highly conserved cleavage domain (Figure 1A, designated as R in Figures 1B and 1C). Secondly the cleavage domain is flanked by arms of 11 bases complementary to their target RNAs, thus enabling accurate positioning of the ribozyme relative to the cleavage site in the substrate RNA. Thirdly the ribozymes were designed to cleave after a GUC motif in the target RNAs. For ribozymes J1 (Figure 1A) and J2 (Figure 1B) this motif occurs in the second codon of the γ and β GS mRNAs respectively whereas the third ribozyme, J3 (Figure 1C), was designed to cleave in the middle of the γ GS RNA in codon 179. (Figure 1C). The sequences of the related GS RNAs over these regions are also shown. Note that the three transcribed ribozymes also contain identical 54 bases of vector sequences at their 5′ ends (not shown). These sequences are not complementary and show no significant homology to any vector or mRNA sequences in the transcribed GS RNAs.

The ribozymes exhibit an absolute specificity of cleavage for their target GS RNAs

To examine whether the J1, J2 and J3 ribozymes demonstrated endoribonuclease cleavage in a sequence specific manner, tRNA (as a control), J1, J2 and J3 ribozymes were each mixed with each of the four full-length radiolabelled GS RNA and incubated under standard cleavage conditions (50 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, at 50°C) for 60 minutes. The reaction products were separated electrophoretically, enabling the resolution of the cleavage products that were smaller than 800 bases (see Figure 2). The J1, J2 and J3 RNAs, but not the tRNA control, demonstrated endoribonuclease activity, with the release of GS RNA cleavage products (Figure 2, open arrow heads). However, J1, J2 and J3 cleaved only the γ, β and γ GS RNAs respectively, releasing fragments of sizes that were consistent with the predicted sites of cleavage (see Figure 2A). These results demonstrated that each ribozyme could cleave only the GS RNA that it was designed against (and only in the targeted position) and not closely-related GS RNAs. This conclusion was supported for the J1 and J2 ribozymes by using shortened γ (γ_s) and β (β_s) GS RNA substrates (see Figure 3A, and materials and methods), which enable the direct visualisation of the substrate and both product RNA fragments from the cleavage reaction (see Figure 3B, closed and open arrow heads respectively). No cleavage of the substrate and no products were observed when J1 was incubated with the β_s RNA or when J2 was incubated with the γ_s RNA (with the substrate in two-fold excess) (data not shown). However when the ribozymes were incubated with the RNA to which they were designed (J1 with γ_s or J2 with β_s) reductions in the amount of substrate and the production of the two cleavage products were observed (Figure 3B). From the intensities of the signals on the autoradiographs it is estimated that the two ribozymes showed less than 0.1% activity with the related but non-homologous substrate.

Endoribonuclease assays were conducted at four different temperatures, 20°C, 37°C, 50°C and 65°C, to investigate the differences in ribozyme activities at physiological, and higher, temperatures. It was observed that both J1 and J2 demonstrated ribozyme activity at all temperatures assayed (Figure 3B). However, it was only at higher temperatures that both J1 and J2 ribozymes effected complete cleavage of their substrates during the time of the assays. At lower temperatures substrate cleavage was less efficient, but in the case of J1, even at 20°C its activity was clearly discernible. Using scanning densitometry it was estimated that at 20°C the activity of the J1 ribozyme was about 21% of the activity at 50°C. Ribozyme cleavage assays were also performed in the absence of 20 mM $MgCl_2$ (Figure 3B). No cleavage activity by either J1 or J2 was observed, even

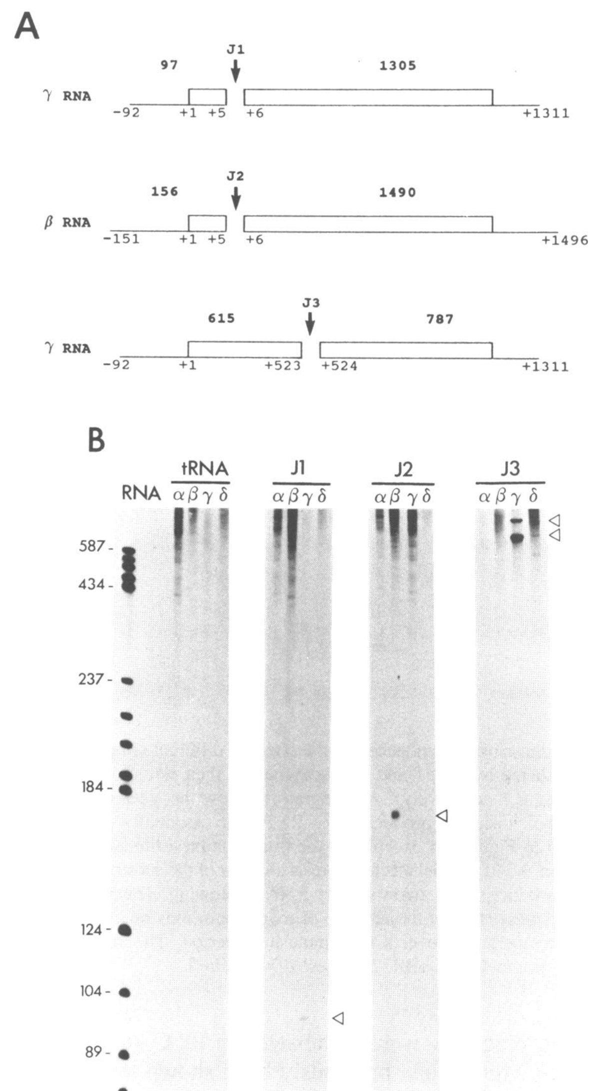

Figure 2. Specificity of ribozyme-mediated cleavage of different GS RNAs. **A:** Diagram to show the position where the ribozymes (J1, J2 and J3) are expected to cleave their homologous targets (*gln-γ*, gln-*β* and *gln-γ* RNAs respectively). Boxes and lines represent the coding regions and the non-coding regions respectively of the *in vitro* synthesized RNAs. The figures below the lines represent the number of bases from the translation initiation codon. Figures above the lines represent the size of the expected cleavage fragments. **B:** Gel electrophoresis analysis of the cleavage of the α, β, γ and δ RNAs by either tRNA or the ribozymes J1, J2 and J3. The position of the cleavage products as shown in part A are marked by the open arrow heads. The size markers are end-labelled fragments of pBR322 digested with *Hae*III. The reactions were carried out for 60 minutes at 50°C.

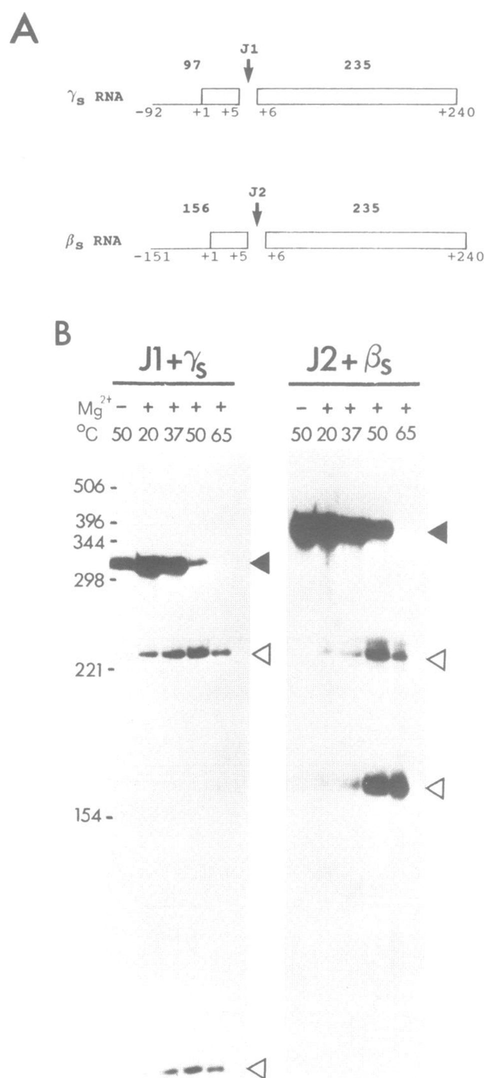

Figure 3. Magnesium-dependence and the effect of different temperatures on the cleavage mediated by the J1 and J2 ribozymes on their homologous shortened RNAs (γ_s and β_s respectively). **A:** Diagram to show the sizes of the substrate RNAs and their cleavage products. The RNAs are essentially those described in the legend to Figure 2A except that the transcription terminated at a *BamHI* site at position +240. **B:** Gel electrophoresis analysis of the substrate (filled arrow heads) and product (open arrow heads) RNAs following cleavage with the J1 and J2 ribozymes either in the absense of magnesium ions or in their presence at different temperatures over a 60 minute time period. The size markers are end-labelled fragments of pBR322 digested with *HinfI*.

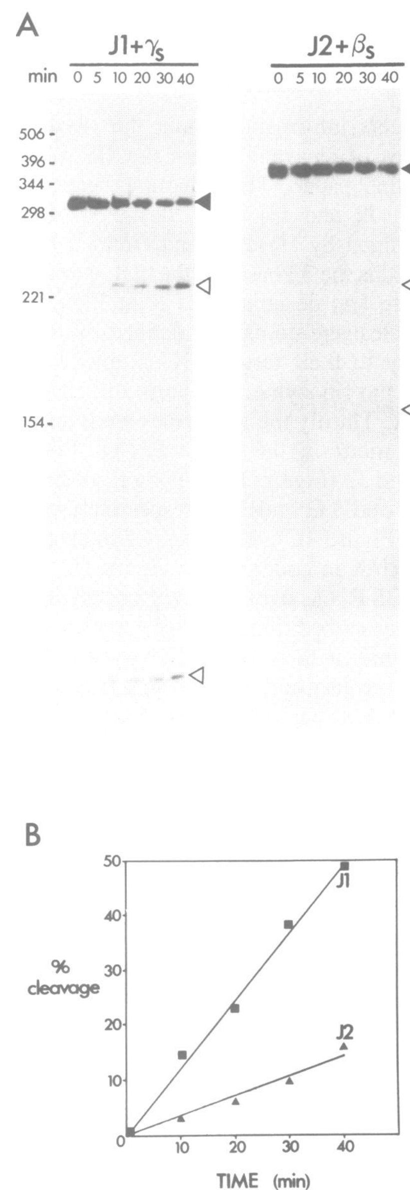

Figure 4. A time course of the cleavage of the γ_s and β_s RNAs by the J1 and J2 ribozymes respectively. **A:** Gel electrophoresis analysis of the substrate (filled arrow heads) and product (open arrow heads) RNAs after incubation at 50°C for different times. The size markers are end-labelled fragments of pBR322 digested with *HinfI*. **B:** The data in part A were plotted to show the linearity of the activities of the J1 and J2 ribozymes with time.

though the reactions were incubated at 50°C (which, in the presence of $MgCl_2$, had previously been shown to cleave 75% and 59% of the substrate respectively). Thus, both J1 and J2 endoribonuclease activities are magnesium-dependent and enhanced at higher temperatures.

J1 and J2 ribozymes cleave their RNA substrates in a time-dependent manner, but exhibit varying degrees of efficiency

The linearity of the cleavage reaction with time was examined by incubating each shortened GS RNA (at a two-fold excess) with its respective ribozyme over a 40 minute period (Figure 4). It can be seen firstly that the activities of the two ribozymes proceeded approximately linearly with respect to time despite the substrate concentrations for the J1 and J2 ribozymes declining by 49% and 16% by the end of the assay period. Secondly it is noteworthy that the activity of the J1 ribozyme was 3.3-fold higher than the activity of J2.

To examine whether the J1 and J2 ribozymes could cleave multiple substrate molecules, constant amounts of the GS RNA substrate were incubated with varying amounts of ribozyme (Figure 5). After 60 minutes it was observed that at ratios equal to or in excess of the substrate, the J1 ribozyme had cleaved essentially all of the γ_s RNA substrate (Figure 5A). In contrast, only when the J2 ribozyme was in 8-fold excess of the β_s RNA was it able to cleave essentially all of this substrate. At the highest substrate concentration it can be seen (Figure 5B) that each J1

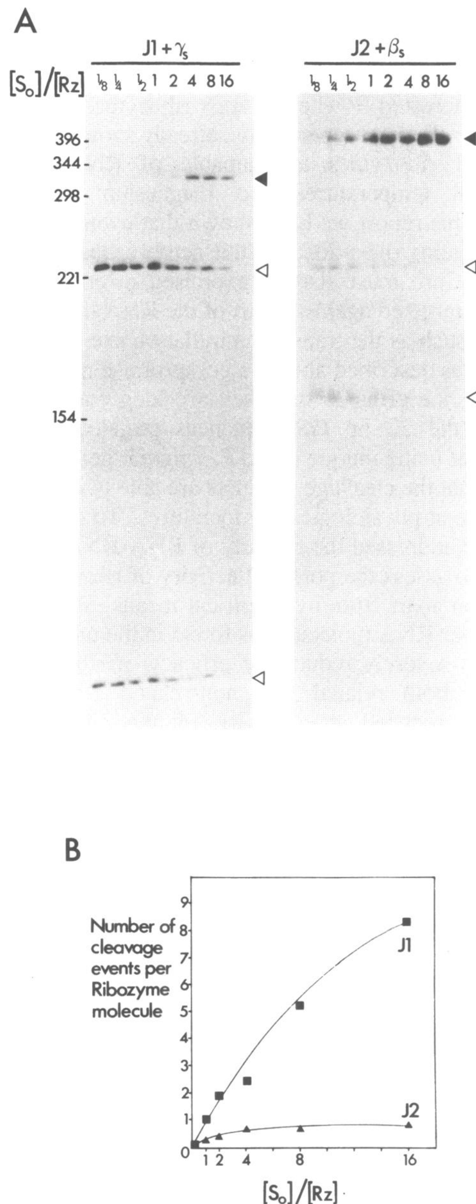

Figure 5. Activities of the J1 and J2 ribozymes at different substrate:ribozyme ($[S_o]/[Rz]$) concentrations. The γ_s or β_s substrates (at concentrations of 50 nM) were incubated at 50°C with various concentrations of the J1 or J2 ribozymes respectively to give ratios of substrate:ribozyme varying from 1:8 to 16:1. **A:** Gel electrophoresis analysis of the substrate (filled arrow heads) and product (open arrow heads) RNAs. The size markers are end-labelled fragments of pBR322 digested with *HinfI*. **B:** The data in part A were plotted to show the number of cleavage events catalysed per ribozyme molecule at the various substrate:ribozyme ratios.

and J2 ribozyme molecule was able to cleave 8 and 0.8 molecules of substrate respectively in the 60 minute assay period. Thus these two ribozymes have remarkably different catalytic potentials.

The rate of J1 mediated cleavage of the γ_s RNA is reduced in the presence of other RNAs

Further experimentation focussed on the activity of the more 'efficient' J1 ribozyme and its γ_s RNA substrate in the presence of other RNA molecules, to simulate more closely the *in vivo* conditions in which the J1 ribozyme would be expected to function.

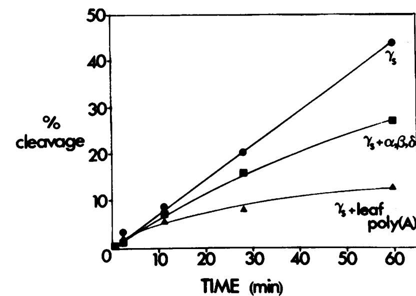

Figure 6. Effect of various RNAs on the cleavage of the γ_s RNA by the ribozyme J1. The substrate and ribozyme were incubated for various times at 50°C. One set of reactions contained an addition of a 5-fold excess of each of the α, β and δ RNAs (about 3 μg each) and another set contained an addition of 10 μg of leaf poly (A) RNA. The% cleavage of the substrate was analysed by gel electrophoresis and the data were then plotted as shown.

Initially, the potential competition from the related GS RNA molecules to the γ_s substrate was examined using the full-length *in vitro* synthesized GS α, β and δ RNAs. Each of these RNAs was added in 5-fold molar excess (about 3 μg each) to the labelled γ_s substrate which was in 2-fold excess over the J1 ribozyme. It can be seen that addition of these RNAs reduced the amount of substrate cleaved by about 40% (Figure 6). The potential inhibition of the J1 endoribonuclease activity from other RNA molecules was examined further by measuring the rate of cleavage of the γ_s substrate in the presence of 10 μg of *P. vulgaris* leaf poly(A) RNA which does not contain any endogenous γ RNA (11) but about 20 ng of δ RNA (9). The presence of the largely unrelated RNA molecules resulted in a reduction in the amount of substrate cleaved by 71%.

DISCUSSION

We have described the construction of hammerhead ribozymes that are able to discriminate between RNAs from closely-related genes. The ribozymes were designed using the guidelines described by Haseloff and Gerlach (7), and on characterisation they retain many features previously observed by these and other authors (7,8,13,14). The ribozymes were shown to cleave their substrates only at the position to which they were designed (Figures 2 and 3) and their activities were shown to proceed linearly with time (Figure 4), to be magnesium dependent and enhanced at increasing temperatures (Figure 3). We designed our ribozymes such that they had 11 bases on each flanking arm homologous to the substrate RNA. Such a design has previously been shown to cleave multiple substrate molecules even at physiological temperatures (13) but higher rates of cleavage can be achieved by using arms of only 8 (7) or 6 (14) bases. Surprisingly we found that two very similar ribozymes exhibited great variability in their catalytic ability. Ribozymes J1 and J2 are identical in the 22 bases of the catalytic domain and share 11 out of 22 bases of homology in the flanking arms. Yet J1 is able to cleave its homologous substrate at over a 3-fold higher rate than J2 and undergoes multiple rounds of cleavage events in an enzymatic manner (Figures 4 and 5). J2 is able to catalyse the cleavage of its substrate molecule, but at temperatures of 50°C and below was unable to carry out multiple cleavage events (Figures 3 and 4). The reasons for this are unclear but may relate to the different G+C content of the flanking arms and the

differences in spatial distribution of these residues. It has been suggested that the rate of cleavage by hammerhead ribozymes may be limited by the dissociation of the cleaved products from the arms of the ribozyme (14). Calculation of the T_m of the ribozyme cleavage products using the Wallace rule (15) with a modification for the greater strength of an RNA-RNA hybrid compared to a DNA-DNA hybrid (16) suggests that the cleavage product at the right-hand arm of J2 (see Figure 1) may be stable at the temperature of the assay and hence limit further rounds of catalysis. Alternatively there may be differences in the secondary structure of either the ribozymes or their RNA substrates which may account for the differences in the catalysis mediated by J1 and J2. Note that the differences we have observed here are unlikely to be due to interference from the vector sequences co-transcribed with the ribozymes, as suggested for the differences in efficacy of synthetic ribozymes derived from the self-cleaving Hepatitus delta virus (17). The vector sequence transcribed with the J1 and J2 ribozymes are identical and computor comparisons show no significant homology of this sequence with either the ribozymes or the transcribed GS RNA sequences.

The impetus for initiating this work was to assess the use of ribozymes as a strategy for investigating the individual roles of the different members of the GS gene family in *P. vulgaris* (9). At present the lack of an efficient mechanism for specific gene disruption in higher plants negates the use of mutation by reverse genetics as a strategy for this work. The expression of antisense RNA has been used as an alternative means of inhibiting gene function in higher plants but the most successful experiments have used large segments of the gene which are unable to distinguish between closely related family members (18,19). However the observation that a 41 base antisense sequence can show high, although variable, inhibition of GUS gene expression in transgenic plants (20) suggests that this technique should be investigated further for discriminating between related genes. Ribozymes have the advantages that only very small regions of homology are required plus they have the potential to be catalytic and hence could be expressed from the promoter of the gene of interest where an excess of ribozyme over substrate RNA would not be expected to ensue. To date the sequence specificity of hammerhead ribozymes has not been investigated on a range of physiological targets. We have shown here that the J1 and J2 ribozymes which were targeted against the second codon of the closely related γ and β GS RNAs respectively are entirely specific for their homologous substrate (Figure 1 and 2). Even closely related GS RNAs such as the α RNA, which contains 16 out of 22 bases of homology with the β RNA sequence, fails to be cleaved by the J2 ribozyme (Figure 2). The J3 ribozyme which was targeted against the middle of the γ RNA exploited the ability of hammerhead ribozymes to cleave after GUC, but not GUG, motifs (21). In this case even though 18 of the α and β bases are conserved within the targeted 22-base γ RNA sequence, only the substrate containing GUC at the target site was cleaved. It has been argued from work on the *Tetrahymena* ribozyme that increasing the length of the ribozyme's recognition sequence past about 7 bases would cause a decrease in the discrimination between closely-related sequences (22). However the *Tetrahymena* ribozyme appears to be able to cleave both matched and mismatched hybrids (22). It seems from the work presented here that hammerhead ribozymes may require a more exact matching before catalysing cleavage, although this suggestion needs to be tested rigorously by using point mutations in the target RNAs. However it is clear that hammerhead ribozymes can be designed which are able to distinguish between closely related genes such as the GS gene family of *P. vulgaris* described here.

We are interested in whether such ribozymes would then be active *in vivo*. Several reports have already shown that synthetic hammerhead ribozymes are capable of RNA cleavage at physiological temperatures and magnesium concentrations (13,14). In this report we have shown that even at 20°C the J1 ribozyme retains over 20% of the activity observed at 50°C (Figure 3). Care must also be exercised in ensuring that the ribozyme is targeted against a part of the RNA that is accessible to cleavage such as the translation initiation site used here (13). In addition, as described above, a compromise must be reached in designing the arms so that they are long enough to ensure specificity (the 22 bp GS sequences targeted here have a probability of being unique in the *P. vulgaris* genome) but short enough so that the cleavage products are able to dissociate from the ribozyme at physiological temperatures. To date only a few studies have addressed the stability of RNA-RNA hybrids so it is difficult to assess the potential activity of ribozymes in an *in vivo* situation apart from by empirical means. We have shown here that other RNA molecules as found in the milieu of the cell are likely to severely reduce the efficacy of ribozymes as the addition of both related and non-related RNA sequences significantly reduced the activity of the ribozyme on its homologous sequence (Figure 6). It is noteworthy however that Chang et al (23) only found an effect of total cellular RNA on the cleavage of HIV RNA by a designed ribozyme when it contained the homologous RNA. Moreover several reports have shown that ribozymes can be active *in vivo* although to be physiologically useful high ratios of ribozyme:substrate appear to be necessary (24,25,26).

Therefore in conclusion we have shown here that ribozymes are able to distinguish between closely related members of a gene family *in vitro* but it remains to be seen whether they have the potential to inactivate the function of specific genes *in vivo*.

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