Reprinted from Nature, Vol. 334, No. 6183, pp. 585-591, 18 August 1988
© *Macmillan Magazines Ltd., 1988*

Simple RNA enzymes with new and highly specific endoribonuclease activities

Jim Haseloff & Wayne L. Gerlach

CSIRO Division of Plant Industry, Canberra, Australia

In vitro mutagenesis of sequences required for the self-catalysed cleavage of a plant virus satellite RNA has allowed definition of an RNA segment with endoribonuclease activity. General rules have been deduced for the design of new RNA enzymes capable of highly specific RNA cleavage, and have been successfully tested against a new target sequence.

CERTAIN naturally occurring RNA molecules possess the property of self-catalysed cleavage[1]. One class of this reaction is shared by a number of small circular RNA molecules which replicate in plants, either alone (viroid RNAs) or dependent on a helper virus (satellite RNAs). Self-cleavage has been demonstrated *in vitro* for avocado sunblotch viroid (ASBV)[2] and the satellite RNAs of tobacco ringspot virus (sTobRV)[3,4] and lucerne transient streak virus (sLTSV)[5] and appears to be an essential and unique part of the life cycle of these RNAs. During replication, circular forms of the RNAs are thought to act as templates for the production of longer than unit-length RNA transcripts[6-8], and the subsequent self-catalysed cleavage of these concatameric transcripts gives rise to unit-length progeny. In addition to these replicating RNAs, *in vitro* self-catalysed cleavage has now been observed in RNA transcripts of certain tandemly repeated DNA sequences from newt[9].

These self-catalysed RNA cleavage reactions share a requirement for divalent metal ions and neutral or higher *p*H, and

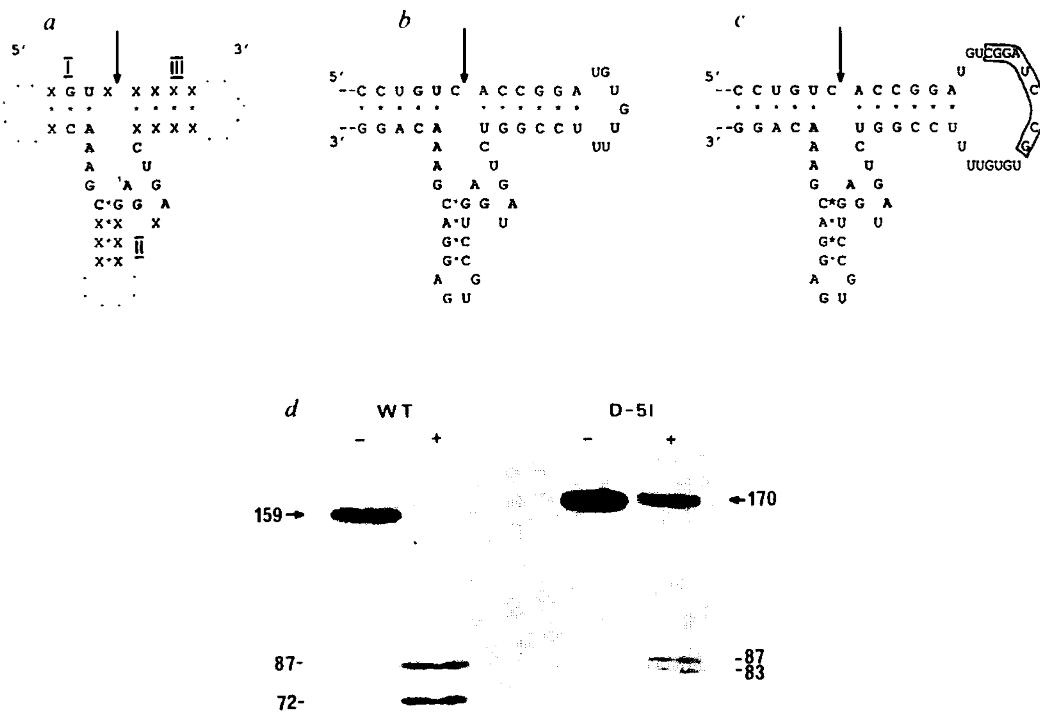

Fig. 1 A mutated self-cleavage domain which is active. *a*, The conserved structures associated with naturally-occurring RNA cleavage sites in ASBV[2,11], newt satellite DNA transcripts[9] and the satellite RNAs of TobRV[4], LTSV[5], velvet tobacco mottle virus[22], Solanum nodiflorum mottle virus[22] and subterranean clover mottle virus[23] are summarized. Nucleotide sequences which are absolutely conserved between these structures are shown, while others are represented as X. The three RNA helices (I, II and III) vary in length from 2 to 7 base pairs. Base-pairing is represented by (*) and the site for RNA cleavage is arrowed. ([1] an extra U is positioned after this residue in LTSV (+) strand.) *b*, The conserved 52-nucleotide structure associated with self-cleavage of (+) strand sTobRV RNA is shown schematically. We refer to this as the wild-type cleavage structure. Conserved residues are highlighted, and the site for cleavage is arrowed. *c*, The D-51 *in vitro* mutant of sTobRV possesses an altered self-cleavage domain, containing an insertion of 8-nucleotides (CGGAUCCG, shown boxed) together with a flanking duplication of three nucleotides (UGU, 7-9). *d*, Subcloned *Hae*III fragments of wild-type sTobRV and the D-51 *in vitro* mutant were each transcribed in both (−) and (+) orientations and radiolabelled transcripts were fractionated by PAGE. The positions of uncleaved 159 and 170 base transcripts from the wild-type (WT)and mutant (D-51) sequences are arrowed; sizes of cleavage products are shown.

Methods. 97 and 108 base-pair *Hae*III fragments containing the sites for self-cleavage were excised from sequenced plasmid clones containing wild-type and D-51 sTobRV sequences, respectively. The fragments were each ligated into the *Sma*I site of pGEM 4 and screened to obtain both orientations of the insert. The plasmids were linearized using *Eco*RI, and (+) and (−) strand RNAs of lengths 159 and 170 bases were transcribed using 200 units ml^{-1} T7 RNA polymerase in 50 mM Tris-HCl *p*H 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 1,000 unit ml^{-1} RNasin, 500 μM ATP, CTP and GTP with 200 μM [α^{32}P]UTP RNAs were fractionated by 10% polyacrylamide, 7 M urea, 25% formamide gel electrophoresis,and autoradiographed.

result in the production of RNA with termini possessing 5', hydroxyl and 2', 3' cyclic phosphate groups[3,5,9,10]. The cleavage reactions presumably result from RNA conformation bringing reactive groups into close proximity. The sites of cleavage are specific and associated with domains of conserved sequence and secondary structure. For sTobRV (ref. 4 and J.H. and W.L.G., unpublished results), ASBV[11] and sLTSV[12], it is known that precisely these conserved regions are required for cleavage, and may thus be directly involved in the reaction. A consensus of the domains associated with known RNA self-cleavage reactions is shown schematically in Fig. 1*a*. They consist of three branched RNA helices which flank the susceptible phosphodiester bond and two single-strand regions which are highly conserved in sequence. The helices are numbered I, II and III, following the convention of Forster and Symons[5], but the structures are redrawn with the sequences immediately adjacent to the site of cleavage shown across the top of the diagram. In the different self-cleavage domains, the base-paired stems are variously terminated by a small single-strand loop, or connected to the remainder of the viroid or satellite RNA molecule.

The self-catalysed cleavage of these RNAs is normally an intra-molecular reaction, that is a single molecule contains all the RNA-encoded functions required for cleavage. For example, cleavage of sTobRV (J.H. and W.L.G., unpublished results) and sLTSV RNAs[12] requires single contiguous sequences of about 52 nucleotides in length, which contain the conserved structures shown in Fig. 1*a*. The self-cleavage of ASBV in contrast requires the participation of two sequences which are widely separated on the intact RNA. Similar sequences have been transcribed *in vitro* as separate 19- and 24-nucleotide RNA fragments[11]. When mixed, the two fragments may base-pair (via stems I and II) to form the conserved structure associated with self-cleavage (Fig. 1*a*) and efficient cleavage of the 24-nucleotide RNA was observed. The 19-nucleotide fragment remained unaltered and could participate in many cleavage reactions, and therefore possessed the properties of an RNA enzyme. In this case, however, the cleaved RNA also contained conserved sequences and secondary structure, making this system unsuitable as a general model for the design of ribonucleolytic RNA enzymes with wide sequence specificity. Similarly, the more complex RNA component of RNAse P[13] and shortened forms of the self-splicing ribosomal RNA intervening sequence from *Tetrahymena thermophila*[14,15], which have both been shown to act as endoribonucleases, have so far proved of limited practical use for the design of new endonuclease activities. The term 'ribozyme' has been used to describe such RNAs with catalytic activity[15].

We have taken the single self-cleaving domain from the (+) strand of sTobRV and dissected its RNA substrate and enzyme activities. An RNA substrate was defined which possessed little conserved sequence and no essential secondary structure. Inspection of the separated substrate and ribozyme activities, and comparison with other naturally occurring self-cleaving domains, led to a model for the design of oligoribonucleotides which possess new and highly sequence-specific endoribonuclease activities. This model was successfully tested by the design and construction of ribozymes targeted against three sites within the Tn9 chloramphenicol acetyl-transferase (CAT) messenger RNA sequence.

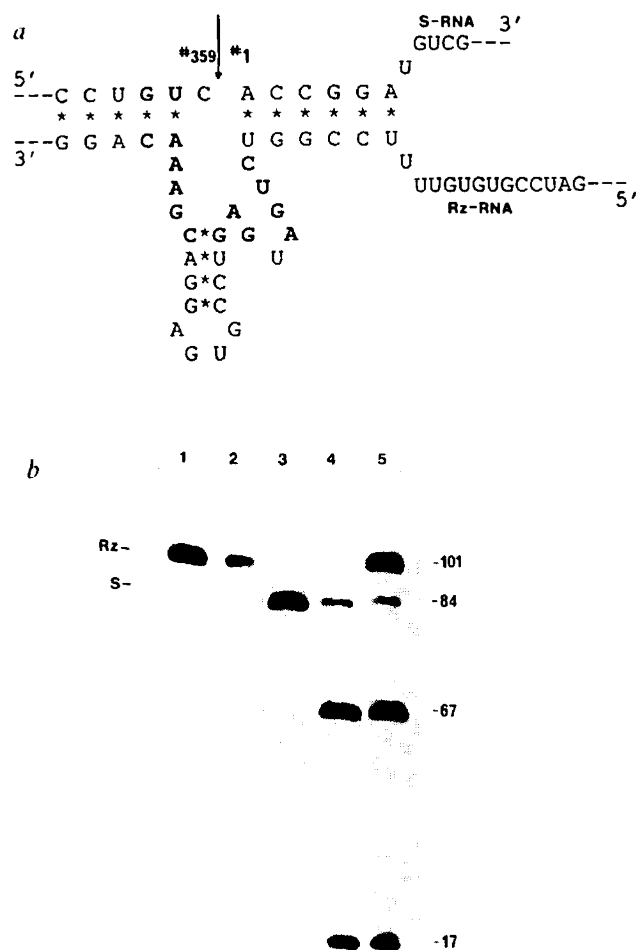

Fig. 2 Separation of substrate and catalytic activities. *a*, The inserted nucleotides in the D-51 mutant (Fig. 1*c*) contain a *Bam*HI restriction endonuclease site. *Bam*HI was used to split the mutant DNA, and the two sequences were subcloned and transcribed separately *in vitro*. The RNA transcripts are shown schematically, with potential base-pairings between the RNAs indicated (*). The fragment containing the arrowed site for cleavage was termed S-RNA, and the other Rz-RNA. *b*, [^{32}P]Rz transcript (101 bases) was incubated alone (lane 1), and with unlabelled S-RNA (lane 2). [^{32}P]S RNA was incubated alone (lane 3), and with unlabelled and [^{32}P]Rz RNAs (lanes 4 and 5, respectively). The sizes of the two products are consistent with cleavage of the S-RNA (84 bases) at the normal site between nucleotides 359 and 1, to give 5' and 3' proximal fragments of 67 and 17 nucleotides, respectively.
Methods. Isolated D-51 *Hae*III fragment was digested with *Bam*HI and the Rz and S fragments ligated into *Sma*I-*Bam*HI digested pGEM3 and 4, respectively. T7 DNA polymerase treated, *Kpn*I-digested Rz-pGEM3 and *Xba*I digested S-pGEM4 were transcribed using SP6 RNA polymerase under the same conditions used for T7 RNA polymerase (Fig. 1), either without radiolabel or in the presence of 200 μM [α-^{32}P]UTP. The Rz and S RNAs were incubated alone in 50 mM Tris-HCl *p*H 8.0, 20 mM $MgCl_2$ at 50 °C for 60 min, fractionated on a 10% polyacrylamide, 7 M urea, 25% formamide gel and autoradiographed.

A mutant that self-cleaves

Encapsidated forms of the satellite RNA of tobacco ringspot virus (+strand sTobRV) have been shown to undergo self-catalysed cleavage at a specific phosphodiester bond[3] between bases 359 and 1. RNA transcripts of sTobRV made *in vitro* also undergo efficient self-cleavage[4], and *in vitro* mutagenesis and transcription of cloned sTobRV complementary DNA has led to mapping of the sequences required for the reaction (J.H. and W.L.G., unpublished results). In these experiments, cloned sTobRV cDNAs were mutagenized using an oligonucleotide linker insertion protocol. A library of sTobRV mutants resulted, and nucleotide sequence analysis showed that each mutant contained an inserted *Bam*HI linker sequence (CGGATCCG) together with flanking duplicated or deleted sTobRV sequences. The mutants were transcribed *in vitro* and the RNAs assayed for their ability to undergo cleavage. The only mutations affecting cleavage of (+) strand sTobRV were those which altered a 52-nucleotide sequence adjacent to the cleaved bond. This sequence contains the domain of conserved sequence and possible secondary structure required for self-cleavage in other RNAs (Fig. 1*a*,*b*).

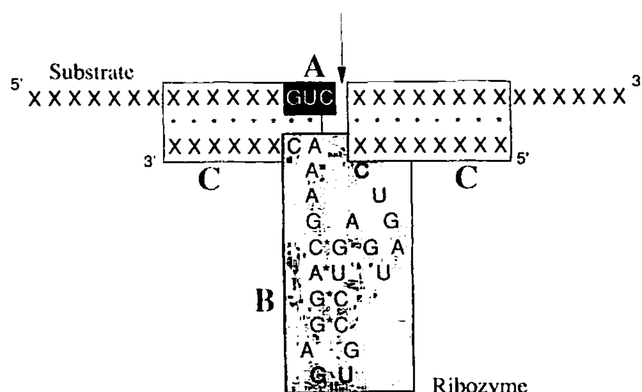

Fig. 3 Model for design of ribozymes. Three structural domains are boxed. (A) contains conserved sequences in the RNA substrate, immediately adjacent to the site of cleavage. (B) comprises the highly conserved sequences maintained in the ribozyme, and the regions (C) consist of flanking helices, with base-pairing between the substrate and ribozyme RNAs.

One mutant, designated D-51, which contained an alteration in this domain proved an exception (Fig. 1*c*). It contained an 8-nucleotide *Bam*HI linker sequence inserted between three duplicated sTobRV nucleotides numbered 7 to 9, yet still underwent cleavage. The wild-type and D-51 cleavage domains were subcloned as 97 and 108 base-pair *Hae*III fragments, respectively, and RNAs were transcribed in both orientations *in vitro*. As expected, no cleavage of (−) strand RNA transcripts was observed, but (+) strands of both the wild-type and D-51 sequences underwent cleavage (Fig. 1*d*), with cleavage of the D-51 RNA being somewhat less efficient than that of the wild-type. The extra 11-nucleotides contained in the D-51 mutant were precisely located within a proposed single-strand loop in the conserved structural domain associated with self-cleavage (Fig. 1*c*). This provided further experimental support for the structural model, and suggested that this single-strand loop region may not be required for cleavage.

Separation of substrate and nuclease activities

Using the *Bam*HI restriction endonuclease site inserted into D-51, flanking *Hae*III-*Bam*HI and *Bam*HI-*Hae*III fragments were obtained and each was subcloned into an *Escherichia coli* plasmid suitable for *in vitro* RNA transcription. This allowed us to effectively eliminate the mutated single-strand loop from the self-cleavage domain, splitting the region into two RNA segments (Fig. 2*a*). The smaller *Hae*III-*Bam*HI transcript contained sTobRV nucleotides 321 to 9, including the actual site of cleavage, and was termed the substrate or S-RNA. The *Bam*HI-*Hae*III transcript containing sTobRV nucleotides 7 to 48 was termed the ribozyme or Rz-fragment. These were separately transcribed with and without radiolabelled ribonucleotide as tracer. Both the S- and Rz-RNAs showed no significant degradation when incubated alone (Fig. 2*b*, lanes 1 and 3) under conditions suitable for highly efficient self-cleavage (50 °C, 20 mM $MgCl_2$, *p*H 8.0). The Rz-RNA also appeared unaltered after incubation with the S-RNA (Fig. 2*b*, lanes 2 and 5), but efficient cleavage of the S-RNA occurred (Fig. 2*b*, lanes 4 and 5) producing two fragments. The product sizes were consistent with cleavage at the normal site. These data show that the S-RNA acted as a substrate for ribonucleolytic cleavage by the Rz-RNA, which apparently acted in a catalytic fashion.

The substrate defined above contains none of the highly conserved secondary structures and few of the conserved sequences thought to be essential for cleavage. Instead, base-pairing between the sTobRV enzyme and substrate RNA segments (via stems I and III) may result in formation of an RNA complex with the required properties for cleavage. Inspection of the separated substrate and enzyme activities derived from the sTobRV (+) strand, and comparison with other naturally occurring self-cleavage sites has led to a model for the design and construction of novel ribozymes.

Design of new ribozymes

A model showing the proposed minimal structural requirements for ribozyme-catalysed RNA cleavage is presented in Fig. 3, and consists of three elements. First, a region (A) containing the sequence GUC adjacent to the site for cleavage is brought into close proximity to a second region (B) of highly conserved sequence and secondary structure. Third, flanking regions (C) of base-paired RNA helix stabilize this interaction. The model provides the basis for the *de novo* design of ribozymes with new sequence-specific endoribonuclease activities by consideration of three main parameters.

(1) Before a ribozyme can be designed, a particular site for cleavage must be chosen within the target RNA. In naturally occurring self-cleavage, the site in the RNA substrate is 5′ flanked by several nucleotides which are highly conserved (A). This sequence, GUC, usually immediately precedes the site of cleavage. In one exception, GUA precedes the active site of cleavage in the (−) strand of sLTSV[5]. Alteration of a self-cleaving sequence similar to that found in transcripts of newt satellite DNA also showed that the residues immediately preceding the cleavage site could be changed[16]. When the normal sequence GUC was changed to GUA or GUU, cleavage activity remained

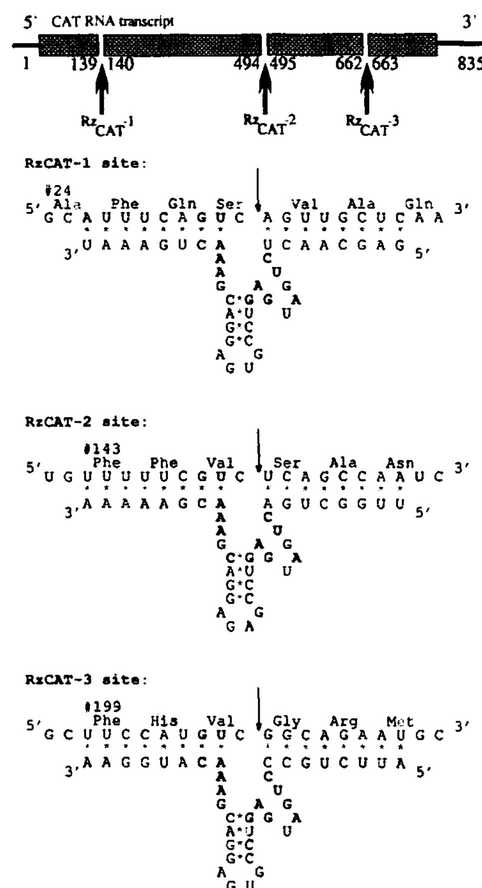

Fig. 4 Design of ribozymes targeted against the CAT gene transcript. Following the design rules described in this paper, ribozymes, termed $Rz_{CAT}1$, 2 and 3, were targeted against three sites within an 835 base *in vitro* transcript of the CAT gene. The relative locations of the cleavage sites on the transcript are shown schematically with the flanking bases numbered. The three ribozyme sequences are shown with the actual target sequences. Amino-acid sequences of the CAT gene are numbered and the predicted sites for RNA cleavage arrowed. $Rz_{CAT}1$ and 3 contain 24 base sequences derived from (+) strand sTobRV (region B, Fig. 3), while $Rz_{CAT}2$ contains a single U to A change in this region.

essentially unaltered, but no cleavage occurred after the sequence GUG. Further, cleavage was shown after the sequences CUC and, to a lesser extent, AUC and UUC, provided that the conserved base-pairing (Fig. 1*a*) was maintained. In the experiments described below, the sequence GUC was used as the sole requirement in the target RNA sequence for design of a corresponding enzyme. Future experiments will determine the full extent of the sequence requirements for efficient RNA cleavage.

(2) The region (B) contains sequences highly conserved in naturally occurring cleavage domains. In Fig. 1*a*, those nucleotides which are conserved in all known self-cleavage domains are specified, while only the positions and required base-pairing of the other nucleotides are indicated. The lengths of the base-paired stem II and associated loop do not appear to be conserved and the loop may be dispensible, as for ASBV. In this case, an RNA enzyme might be active as two halves, or subunits, each held to the other by formation of an extended base-paired stem II. But, conserved tertiary folding within this domain might not be evident from sequence comparisons, being maintained by compensating changes in nucleotide sequence, and initially we have used sequences similar to those of sTobRV for this region.

(3) The catalytic region and RNA substrate are held together by flanking regions of base-pairing (C) which must allow accurate positioning of the enzyme relative to the potential cleavage site in the substrate. The extent and type of base-pairing will directly affect the specificity, affinity (K_m) and turnover (k_{cat}) of an RNA enzyme. For the enzymes described below, a size of eight base-pairs has been arbitrarily chosen for each of the flanking regions (C).

Testing of synthetic ribozymes

To test these principles an indicator gene, chloramphenicol acetyl transferase (CAT)[17], was used to provide RNA transcripts as a target for cleavage by three newly designed RNA enzymes. Expression of the CAT gene can provide antibiotic resistance

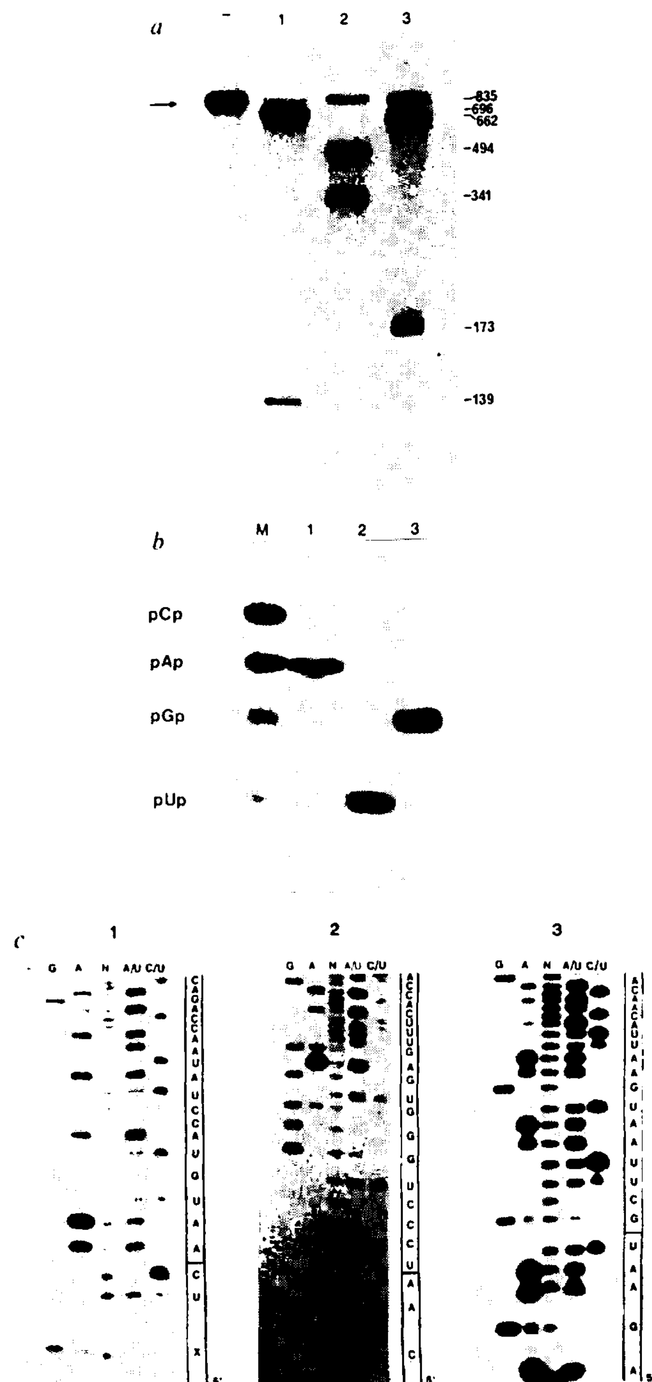

Fig. 5 Cleavage of the CAT gene transcript. *a*, The [^{32}P]-labelled CAT RNAs were gel fractionated after incubation alone (−) or with one of the three ribozymes $Rz_{CAT}1$, 2 and 3 (lanes 1, 2 and 3, respectively). The location of the full-length transcript is arrowed. CAT mRNAs incubated with each of the ribozymes underwent efficient cleavage. In each case only two fragments were produced after incubation with a given ribozyme, and their nucleotide sizes were consistent with the predicted sites for cleavage (that is, 139 and 696, 494 and 341, 662 and 173 base fragments were the 5′ and 3′ products from $R_{zCAT}1$, 2, 3-catalysed cleavage respectively). *b*, 5′ Terminal base analysis. The 3′ fragments produced by ribozyme cleavage of CAT mRNA were [5′-^{32}P]-kinased, gel purified, subjected to complete nuclease digestion and the released terminal residues were fractionated by *p*H 3.5 PAGE. The 5′ terminal nucleotides, determined by reference to markers (lane M), were A, U and G for the fragments produced by $Rz_{CAT}1$, 2 and 3 (lanes 1, 2 and 3, respectively). *c*, 5′ terminal sequence analysis. [5′-^{32}P]-labelled CAT mRNA fragments were subjected to base-specific partial ribonucleolytic cleavage. The products were polyacrylamide gel fractionated to allow 5′ terminal sequence determination. Panels 1, 2 and 3 correspond to products of $Rz_{CAT}1$, 2 and 3, respectively. Base specific cleavages are shown above each lane except for lane N which is a size ladder control. Sequences from 4 to 6 to about 25 nucleotides 3′ of the cleavage site are indicated for each fragment, and are consistent with the expected sites for cleavage by each ribozyme.

Methods. The CAT gene was obtained from pCM4[24] and subcloned as a *Bam*HI fragment into pGEM-blue. This plasmid was linearized with *Hin*dIII and CAT gene transcripts were obtained using T7 RNA polymerase with 200 μM [α-^{32}P]UTP. Ribozyme sequences were synthesized as oligodeoxynucleotides using automated phosphoamidite chemistry. The 47-, 48- and 44-mer oligonucleotides, $Rz_{CAT}1$, 2 and 3, respectively, were kinased, ligated with phosphatase treated, *Eco*RI-*Pst*I cut pGEM4 and incubated with the Klenow fragment of DNA polymerase I before bacterial transformation. *Eco*RI-linearized plasmids were transcribed with T7 RNA polymerase to produce ribozyme RNAs. Ribozymes were incubated with CAT transcript in 50 mM Tris-HCl *p*H 8.0, 20 mM $MgCl_2$ at 50 °C for 60 min, and the products fractionated by 5% polyacrylamide 7 M urea, 25% formamide gel electrophoresis before autoradiography. Ribozyme-cleaved CAT mRNA fragments (2 μg) were incubated in 50 mM Tris-HCl *p*H 9.0, 10 mM dithiothreitol with 50 μCi [γ-^{32}P]ATP and 5 units T4 polynucleotide kinase for 30 min at 37 °C. Radiolabelled fragments were purified on a 5% polyacrylamide gel and digested with an equal volume of 500 units ml^{-1} RNase T1, 25 units ml^{-1} RNase T2 and 0.125 mg ml^{-1} RNAse A in 50 mM ammonium acetate *p*H 4.5 for 120 min at 37 °C. Products were fractionated on a 20% polyacrylamide gel containing 25 mM sodium citrate *p*H 3.5 and 7 M urea[25], and were detected by autoradiography. Terminal sequences were determined directly using the partial enzymatic digestion technique[26].

in bacteria, plants and animals and this can be easily assayed. The coding region of the CAT gene transcript was scanned for the occurrence of the sequence GUC as potential sites for RNA cleavage. Three such sites were arbitrarily chosen, and their flanking sequences used to define the eight-nucleotide regions of complementary sequence within the ribozymes, which flanked conserved sequences based on those of the sTobRV (+) strand self-cleavage site (Fig. 4).

The ribozyme sequences (Rz_{CAT} 1, 2 and 3) were synthesized as single-stranded oligodeoxyribonucleotides, with the addition of sequences containing half-sites for *Eco*RI (AATTC) and *Pst*I (CTGCA) restriction endonucleases at the 5′ and 3′ termini, respectively. These additional sequences are partly complementary to the overhanging termini left by *Eco*RI and *Pst*I digestion of double-stranded DNA. The kinased oligomers were incubated with *Eco*RI-*Pst*I digested, phosphatase-treated plasmid DNA in the presence of DNA ligase and DNA polymerase, and after transformation the resulting clones were screened for presence of the *Eco*RI-*Pst*I insert. This procedure allowed efficient cloning of the ribozyme sequences using single-strand oligomers, obviating the need for synthesis of a second complementary oligomer.

The ribozyme sequences and the CAT gene were cloned downstream of promoters for T7 RNA polymerase, and this enzyme was used to obtain RNA transcripts *in vitro*. When the 835-nucleotide CAT transcript was incubated with any one of the three ribozymes, efficient and highly sequence-specific cleavage occurred producing two RNA fragments (Fig. 5*a*). The conditions required for ribozyme-catalyzed cleavage were similar to those observed for the naturally-occurring cleavage reactions[3,5,9,11] with more efficient cleavage occurring at elevated *p*H, temperature and divalent cation concentrations (data not shown). When present in molar excess, the three ribozymes catalysed almost complete cleavage of the CAT RNA substrate after 60 minutes in 50 mM Tris-HCl *p*H 8.0, 20 mM $MgCl_2$ at 50 °C (Fig. 5*a*). Under similar conditions with 0.1 μM substrate and 3 μM ribozyme, the $T_{1/2}$ of CAT mRNA substrate was 3.5, 3.5 and 2.5 min, in the presence of Rz_{CAT} 1, 2 and 3, respectively. The ribozyme sequences were inactive in the form of oligodeoxyribonucleotides or against the complement of the substrate RNA (data not shown).

The sizes of the CAT RNA fragments produced by each ribozyme were consistent with the predicted sites for cleavage. The 3′ terminal cleavage fragments from each ribozyme-catalysed reaction were isolated and 5′ ^{32}P-kinased. Efficient kinasing of the fragments indicated that they possessed 5′ terminal hydroxyl groups, similar to those produced in naturally-occurring cleavage reactions. Terminal nucleotide analysis of these fragments demonstrated that cleavage of CAT sequences by Rz_{CAT} 1, 2 and 3 occurred precisely before nucleotides A, U and G, respectively (Fig. 5*b*) and RNA sequence determination confirmed that cleavage had occurred at the expected locations within the CAT RNA (Fig. 5*c*).

Enzymatic catalysis

To demonstrate that these ribozymes cause cleavage of the CAT mRNA substrate in a catalytic manner, each was incubated with a molar excess of substrate, under conditions which should favour both efficient cleavage and product dissociation. Figure 6 shows the results of an experiment where, after 75 min at 50 °C, *p*H 8.0 in 20 mM $MgCl_2$, 10 pmol of Rz_{CAT}1 had catalysed specific cleavage of 163 pmol of a truncated CAT mRNA substrate. On average, each ribozyme had participated in greater than 10 cleavage events. Similar results were obtained for Rz_{CAT} 2 and 3 (data not shown), and thus each acts as an RNA enzyme.

Mechanism of cleavage

All three RNA enzymes which were designed according to the principles outlined above have been demonstrated to catalyse effective and precise cleavage of their target RNA sequences. It is therefore likely that these design principles are generally valid, as is the model upon which they are based. The model contains two main assumptions: (1) that catalysis requires that the RNA enzyme and substrate interact through base-pairing (although tertiary interactions presumably exist between enzyme and substrate, they do not need to be considered in the design), and (2) that base-pairing allows precise positioning of a conserved domain (B, Fig. 3) adjacent to a site for cleavage. It may not only be useful, but of functional significance, to consider this conserved domain as the catalytic section or active site of the RNA enzymes.

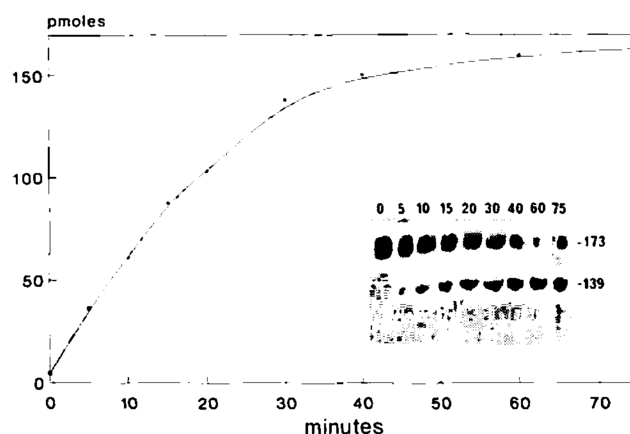

Fig. 6 Enzymatic catalysis. Rz_{CAT}1 (15 pmol) was incubated for varying times with a 20-fold molar excess of truncated (173 base) CAT mRNA containing the site for Rz_{CAT}1 cleavage. Rz_{CAT}1 catalysed cleavage of the CAT RNA to form 5′ and 3′ fragments, of 139 and 34 bases, respectively. The inset shows the accumulation of the 139 base fragment with time, after PAGE. The amounts of the 139 base fragment were quantified and plotted. After 75 min at 50 °C some non-specific cleavage of RNA was noticed due to the extreme conditions, but 70% of the remaining intact RNAs (163 pmol) had accumulated as the 139 base fragment.

Methods. *Pvu*II-linearized pGEM-blue containing the CAT gene sequence, and *Eco*RI-linearized pGEM4 containing Rz_{CAT}1 were transcribed *in vitro* with T7 RNA polymerase and 200 μM [α-^{32}P]UTP. 300 pmol of 173 base long, truncated CAT mRNA and 15 pmol of Rz_{CAT}1 were mixed in 100 μl of 50 mM Tris-HCl *p*H 8.0, 20 mM $MgCl_2$. 10 μl aliquots were incubated for varying times at 50 °C. Samples were fractionated by 7 M urea, 5% PAGE, stained with toluidine blue, the bands were excised, and amounts of radiolabelled RNA were determined.

The RNA enzyme- and related self-catalysed cleavage reactions appear to proceed via concomitant cleavage of the susceptible 3′,5′phosphodiester linkage and formation of termini containing 2′,3′cyclic phosphodiester and 5′ hydroxyl groups[3,5,9,11], a mechanism similar to that occurring during base-catalysed hydrolysis of RNA. There exists a previously described case of similar, highly specific RNA cleavage for which detailed structural information is available. During X-ray crystallographic studies of yeast $tRNA^{phe}$, heavy-atom derivatives were sought. When the $tRNA^{phe}$ crystals or solutions were incubated with lead (Pb(II)) salts, specific cleavage of the RNA occurred between nucleotides 17 and 18 (refs 18, 19). The cleavage was *p*H dependent, with increased rates observed around neutral *p*H, and produced termini containing 2′,3′ cyclic phosphodiester and 5′ hydroxyl groups. Three Pb(II) ions were located within the $tRNA^{phe}$ molecule and one, covalently bound to bases 59 and 60 within the TΨC-loop, was positioned in close proximity to the cleaved bond in the D-loop. Brown *et al.*[18] suggested that this Pb(II) ion acted as a source of metal-bound hydroxyl ions near neutral *p*H, as Pb(II) bound water molecules possess a pK_a of ~*p*H 7.4. The perhaps fortuitously but precisely positioned $(Pb\text{-}OH)^+$ moiety could abstract a proton from the 2′ hydroxyl group of nucleotide 17, thus initiating nucleophilic attack of the phosphate atom in the adjacent phosphodiester bond between nucleotides 17 and 18, and resulting in formation

of a 2',3' cyclic phosphate group and 5' hydroxyl leaving group. A similar mechanism has been proposed for the initial step in cleavage by the protein RNase A, where the precisely positioned imidazole of histidine-12 acts as a general base to deprotonate the 2' hydroxyl of the RNA substrate[20].

The ribozyme- and self-catalysed cleavage reactions discussed in this paper require divalent metal ions for activity, are *p*H-dependent, produce similar terminal groups, and thus may involve similar mechanisms to those responsible for (PbII)-catalysed $tRNA^{Phe}$ cleavage. In addition, the involvement of such mechanisms would be consistent with the simple structural properties of the ribozymes outlined above. For example, the domain of a highly conserved sequence within the ribozyme might constitute a metal-ion binding site. The flanking base-paired regions joining the ribozyme and substrate would serve to both stabilize the structure of this binding site and bring a bound metal ion in close proximity to the 2' hydroxyl group adjacent to the susceptible phosphodiester bond. The precisely positioned metal-bound hydroxyl group could then abstract the proton from the 2' hydroxyl group and initiate cleavage of the substrate RNA in a fashion similar to that seen in $tRNA^{Phe}$ cleavage. Alternatively, cations may play a structural role in catalysis, stabilizing the interaction between substrate and reactive group(s) present in the RNA enzyme. Detailed structural and kinetic studies are required to test such models for ribozyme action, and may provide a further basis for manipulation of the substrate specificity, and catalytic activity of ribozymes.

Potential applications

The RNA enzymes described in this paper have been shown to be capable of efficient and specific cleavage of RNA sequences *in vitro*, and are based on RNA self-cleavage reactions which occur normally *in vivo*. Accordingly, the ability to design and use specific endoribonucleases for cleavage of particular RNA species or target sequences may prove useful both *in vitro* and *in vivo*.

Ribozymes could be used for the *in vitro* manipulation of RNAs either to produce large quantities of particular RNA fragments or, by using ribozymes with lesser specificities, as a means of physically mapping RNAs. In addition, it may be possible to construct ribozymes as two halves, with the halves held together by extended base-pairing. Each half of such a ribozyme would be specific for one side of a particular cleavage site. By combining ribozyme halves it would be possible to generate new substrate specificities. Given that certain self-catalysed cleavage reactions are reversible to some extent[3,21], it may also be possible to isolate and splice together specific RNA fragments using this approach.

A major potential application for these highly sequence-specific endoribonucleases is in cleavage, and thereby inactivation, of gene transcripts *in vivo*. It is now possible to express foreign genes in a number of bacterial, fungal, plant and animal species either through stable chromosomal transformation of a particular genotype, transient expression via episomal or viral vectors, or by microinjection. Such methods could also be used for the introduction and expression of ribozyme sequences. Genes are universally expressed via RNA transcripts, giving rise to structural/functional RNAs or messenger RNAs for polypeptide synthesized. Essentially any RNA is a potential substrate for cleavage by a ribozyme. Provided that the transcribed sequences of the gene are known, it should be possible to target one or more ribozymes against specific RNA transcripts. Expression *in vivo* of such ribozymes and cleavage of the transcripts would in effect inhibit expression of the corresponding gene. This 'anti-gene' activity of the ribozymes could provide a basis for various gene and viral therapies and analyses.

We are grateful to Tim Close for pCM4 plasmid and Kim Newell and Paul Whitfeld for synthesis of oligonucleotides. We thank George Bruening for helpful discussion, and Mark Young, Jim Tokuhisa, John Watson, Bob Symons and Jim Peacock for comments on the manuscript. J.H. was supported by a Queen Elizabeth II Research Fellowship.

Received 13 June; acceped 30 June 1988.

1. Cech, T. R. *Science* **236**, 1532-1539 (1987).
2. Hutchins, C. J., Rathjen, P. D., Forster, A. C. & Symons, R. H. *Nucleic Acids Res.* **14**, 3627-3640 (1986).
3. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R. & Bruening, G. *Science* **231**, 1577-1580 (1986).
4. Buzayan, J. M., Gerlach, W. L. & Bruening, G. B. *Proc. natn Acad. Sci. U.S.A.* **83**, 8859-8862 (1986).
5. Forster, A. C. & Symons, R. H. *Cell* **49**, 211-220 (1987).
6. Kiefer, M. C., Daubert, S. D., Schneider, I. R. & Bruening, G. B. *Virology* **121**, 262-273 (1982).
7. Bruening, G. B., Gould, A. R., Murphy, P. J. & Symons, R. H. *FEBS Lett.* **148**, 71-78 (1982).
8. Hutchins, C. J. *et al. Plant molec. Biol.* **4**, 293-304 (1985).
9. Epstein, L. M. & Gall, J. G. *Cell* **48**, 535-543 (1987).
10. Buzayan, J. M., Gerlach, W. L., Bruening, G. B., Keese, P. & Gould, A. R. *Virology* **151**, 186-199 (1986).
11. Uhlenbeck, O. C. *Nature* **328**, 596-600 (1987).
12. Forster, A. C. & Symons, R. H. *Cell* **50**, 9-16 (1987).
13. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. *Cell* **35**, 849-957 (1983).
14. Zaug, A. J., Been, M. D. & Cech, T. *Nature* **324**, 429-433 (1986).
15. Kim, S-H. & Cech, T. R. *Proc. natn Acad. Sci. U.S.A.* **84**, 8788-8792 (1987).
16. Koizumi, M., Iwai, S. & Ohtsuka, E. *FEBS Lett.* **228**, 228-230 (1988).
17. Alton, N. K. & Vapnek, D. *Nature* **282**, 864-869 (1979).
18. Brown, R. S., Hingerty, B. E., Dewan, J. C. & Klug, A. *Nature* **303**, 543-546 (1983).
19. Brown, R. S., Dewan, J. C. & Klug, A. *Biochemistry* **24**, 4785-4801 (1985).
20. Wlodawer, A., Miller, M. & Sjolin, L. *Proc. natn Acad. Sci. U.S.A.* **80**, 3628-3631 (1983).
21. Buzayan, J. M., Gerlach, W. L. & Bruening, G. *Nature* **323**, 349-352 (1986).
22. Haseloff, J. & Symons, R. H. *Nucleic Acids Res.* **10**, 3681-3691 (1982).
23. Haseloff, J. thesis, Univ. Adelaide (1983).
24. Close, T. J. & Rodriguez, R. *Gene* **20**, 305-316 (1982).
25. Dewachter, R. & Fiers, W. *Analyt. Biochem.* **49**, 184-197 (1974).
26. Donis-Keller, H., Maxam, A. M. & Gilbert, W. *Nucleic Acids Res.* **4**, 2527-2538 (1980).

Printed in Great Britain by Turnergraphic Limited, Basingstoke, Hampshire