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Efficient *trans*-cleavage of a stem–loop RNA substrate by a ribozyme derived from *Neurospora* VS RNA

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We have constructed a ribozyme containing 144 nucleotides of *Neurospora* VS RNA that can catalyze the cleavage of a separate RNA in a true enzymatic manner ($K_m \approx 0.13$ μM, $k_{cat} \approx 0.7$/min). Comparison of the rates of *cis*- and *trans*-cleavage, as well as the lack of effect of pH on the rate of cleavage, suggest that a rate-limiting step, possibly a conformational change, occurs prior to cleavage. The minimum contiguous substrate sequence required for cleavage consists of one nucleotide upstream and 19 nucleotides downstream of the cleavage site. Unlike most other ribozymes which interact with long single-stranded regions of their substrates, the minimal substrate for the VS ribozyme consists mostly of a stable stem–loop, which would appear to preclude its recognition simply via extensive Watson–Crick base pairing.

Key words: cleavage/*Neurospora*/plasmid/RNA/ribozyme

Introduction

A small number of RNAs isolated from a variety of natural sources have been found to possess a self-cleavage activity that is involved in processing multimeric transcripts into monomers, apparently as part of the replication cycle. Several different RNA sequences and secondary structures appear to be capable of such activity. These include: the hammerhead, found in several plant viral satellite RNAs, a viroid RNA and the transcript of a nuclear satellite DNA of a newt (Forster and Symons, 1987; reviewed by Symons, 1992); the hairpin (or paper clip) in the minus strand of the satellite of tobacco ringspot virus and related viruses (Buzayan *et al.*, 1986; Feldstein *et al.*, 1990; Hampel *et al.*, 1990; Feldstein and Breuning, 1993); the genomic and antigenomic RNAs of hepatitis delta virus (HDV; Kuo *et al.*, 1988; Sharmeen *et al.*, 1988; Perrotta and Been, 1991, 1993); and VS RNA in the mitochondria of certain *Neurospora* isolates (Saville and Collins, 1990).

In their natural contexts the ribozymes mentioned above, as well as others such as Group I (Cech, 1990) and Group II introns (Michel *et al.*, 1989), perform intramolecular *cis*-cleavage and, in some cases, ligation reactions. Structure–function studies of Group I introns (Szostak, 1986; Zaug *et al.*, 1986) and later hammerhead (Uhlenbeck, 1987), hairpin (Feldstein *et al.*, 1990; Hampel *et al.*, 1990) and HDV (Branch and Robertson, 1991; Perrotta and Been, 1992; Wu *et al.*, 1992) ribozymes have been facilitated by altering these RNAs to perform intermolecular *trans*-cleavage reactions. In a *trans*-cleavage reaction one RNA, the substrate, contains the site to be cleaved; a separate RNA, the ribozyme, provides the sequences required to catalyze the cleavage. One naturally occurring *trans*-acting ribozyme has been discovered, the RNA component of RNase P, which cleaves pre-tRNA precursors *in trans* (Guerrier-Takada *et al.*, 1983). *Trans*-cleavage reactions of most ribozymes have been designed such that binding of the substrate occurs via formation of multiple Watson–Crick base pairs with the ribozyme. Non-Watson–Crick and tertiary interactions are also involved in substrate binding and may be essential for proper binding (Pyle *et al.*, 1992; Smith *et al.*, 1992; Dib-Haij *et al.*, 1993; Guerrier-Takada and Altman, 1993).

With hammerhead, hairpin and Group I ribozymes it has been found that very few specific nucleotides in the substrate are required for *trans*-cleavage, provided that the adjacent region(s) are complementary to the binding site on the ribozyme. This property has allowed the engineering of ribozymes that can cleave sequences other than those recognized by the naturally occurring ribozyme. Some engineered ribozymes also function *in vivo* in non-native host cells, which has raised the possibility of their use as therapeutic agents in dominant inherited disorders and against retroviruses and RNA viruses (reviewed by Castanotto *et al.*, 1992).

We are studying the VS catalytic RNA which is found in the mitochondria of certain natural isolates of *Neurospora* (Collins and Saville, 1990). VS performs the same type of RNA cleavage as hammerhead, hairpin and HDV ribozymes, leaving products with 2′,3′-cyclic phosphate and 5′-OH termini (Saville and Collins, 1990), but it is different in sequence, secondary structure, choice of cleavage site and functional properties from these other ribozymes (Collins and Olive, 1993; Guo *et al.*, 1993; T.L.Beattie, J.E.Olive and R.A.Collins, submitted). An understanding of the structure and function of VS RNA may therefore provide unique information about the ways that RNAs can catalyze reactions and about the nature of RNA active sites.

We report here that the catalytic region of VS RNA can cleave a separate substrate RNA at a specific target site. The minimal substrate forms a stable hairpin with few and only short single-stranded regions potentially available for recognition by the ribozyme via Watson–Crick pairing. Nonetheless, the low K_m of the VS *trans*-cleavage reaction (0.13 μM) suggests that the VS ribozyme binds its substrate quite efficiently, implying that substrate recognition involves multiple, including tertiary, interactions.

Results

VS RNA can cleave a separate RNA substrate in trans

The *trans* reaction described below was constructed before we had determined the secondary structure of VS RNA. In the absence of structure-based predictions about where to divide the normally *cis*-cleaving RNA to establish a *trans*-cleaving system, we used several restriction fragments of VS DNA cloned in a T7 promoter vector to construct pairs of non-overlapping regions of VS RNA. One member of each pair, the substrate (S; see Materials and methods), contained the expected cleavage site, following nucleotide G620 (numbered as in Saville and Collins, 1990); the other, the enzyme or ribozyme (Rz; see Materials and methods), contained the remainder of the VS sequence, terminating at the *Ssp*I site at nucleotide 783. In preliminary experiments (Saville, 1991; Guo, 1992) these transcripts were mixed at an ~1:1 ratio and incubated under conditions known to support *cis*-cleavage (Collins and Olive, 1993). Most combinations showed little or no cleavage, however, almost complete cleavage of a 32 nucleotide substrate RNA that terminates at the *Ava*I site (nucleotide 639) was observed during a 1 h incubation with a ribozyme that begins at the *Ava*I site and ends at the *Ssp*I site (nucleotide 783); no cleavage was observed in the absence of ribozyme (Figure 1). The electrophoretic mobilities of the two cleavage products, P1 and P2, were approximately those expected for cleavage after nucleotide 620 (confirmed below), which is the site of intramolecular *cis*-cleavage of VS RNA. We chose to examine this *trans*-cleavage reaction in further detail.

Trans-cleavage occurs at the same site as cis-cleavage

To determine the precise site of cleavage, S, P1 and P2 were labeled at their 5′ ends and sequenced by partial enzymatic digestion using RNases T1 or U2 (Figure 2). Cleavage products of a mutant substrate containing a single base substitution 3′ of the cleavage site (A621U) were also characterized to resolve possible ambiguities due to anomalous migration of some bands (see below). Because S and P1 are identical in sequence from the 5′ end to the cleavage site, all RNase sequencing bands co-migrated, as expected (Figure 2A–C). Full-length P1 co-migrated with the 13 nucleotide RNase T1 fragment of S that terminates at G620, which is the site of intramolecular *cis*-cleavage in VS RNA. Also, the 3′ end of P1 was found to be guanosine 2′,3′-cyclic phosphate (Figure 2F), indicating that both the location and chemical pathway of *trans*-cleavage are the same as in the *cis*-cleavage reaction.

As expected from the finding of a cyclic phosphate at the 3′ end of P1, a 5′ hydroxyl group was found at the 5′ end of P2, as evidenced by its end-labeling by [γ-^{32}P]ATP and T4 polynucleotide kinase without prior phosphatase treatment. Alkaline hydrolysis ladders of 5′ end-labeled P2 contained only 18 of the expected 19 bands. This is the result of a compression artefact involving the formation of a very stable stem–loop structure in the longer RNAs; this will be described in detail below. Nonetheless, the 5′ terminal nucleotides of P2 derived from cleavage of S and the A621U mutant were A and U respectively (Figure 2G and H), confirming that cleavage occurred between nucleotides 620 and 621, as in the *cis*-cleavage reaction.

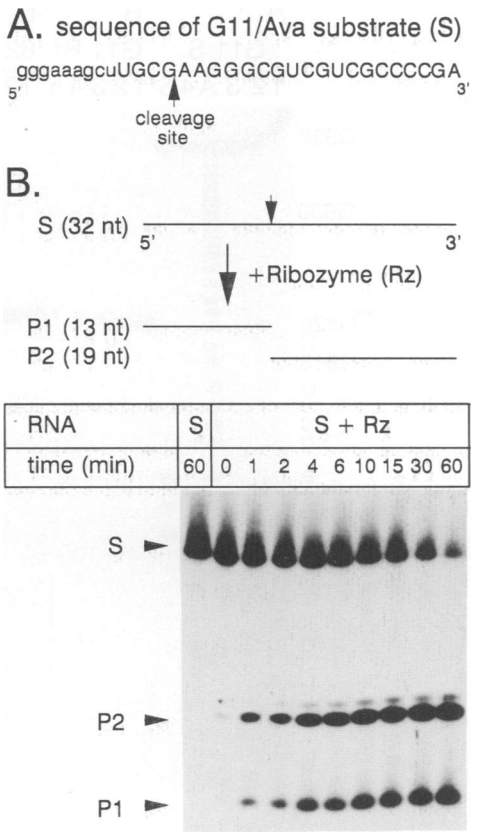

Fig. 1. The *trans*-cleavage reaction. (**A**) The nucleotide sequence of the G11/*Ava*I substrate RNA (S) transcribed from clone G11 which had been linearized by digestion with *Ava*I. VS nucleotides 617–639 (numbered as in Saville and Collins, 1990) are indicated in upper case; vector nucleotides are in lower case. (**B**) Time course of *trans*-cleavage of S by the *Ava*I ribozyme (Rz). ^{32}P-Labeled S and unlabeled Rz (0.05 μM each) were incubated in 50 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$ at 37°C. Aliquots were removed at the times indicated and analyzed by electrophoresis and autoradiography (see Materials and methods for details). The left lane contains a control incubation in the absence of Rz. P1 and P2 are the cleavage products of S.

Minimal length of the substrate RNA

To determine the minimal sequence required downstream of the cleavage site, we used essentially the approach described by Forster and Symons (1987) and used for other riboyzmes (Feldstein *et al.* 1989; Perotta and Been, 1992). 5′-End-labeled G11/*Ssp*I RNA was partially hydrolyzed by treatment at high pH, then incubated with or without the ribozyme (Figure 3A). Incubation in the absence of ribozyme (lane 2) confirmed our previous finding that full-length G11/*Ssp*I RNA and deletion derivatives lacking ⩽10 nucleotides at the 3′ end can *cis*-cleave (Guo *et al.*, 1993). Incubation with the ribozyme resulted in the disappearance, or at least decrease in intensity, of bands corresponding to RNAs terminating at nucleotide 639 or longer (lane 4). A few RNAs were not cleaved to completion under these conditions, indicating that they are relatively poorer substrates. The minimal length substrate terminates at residue 639, which by coincidence corresponds precisely to the RNA used in Figure 1, which was synthesized by run-off transcription

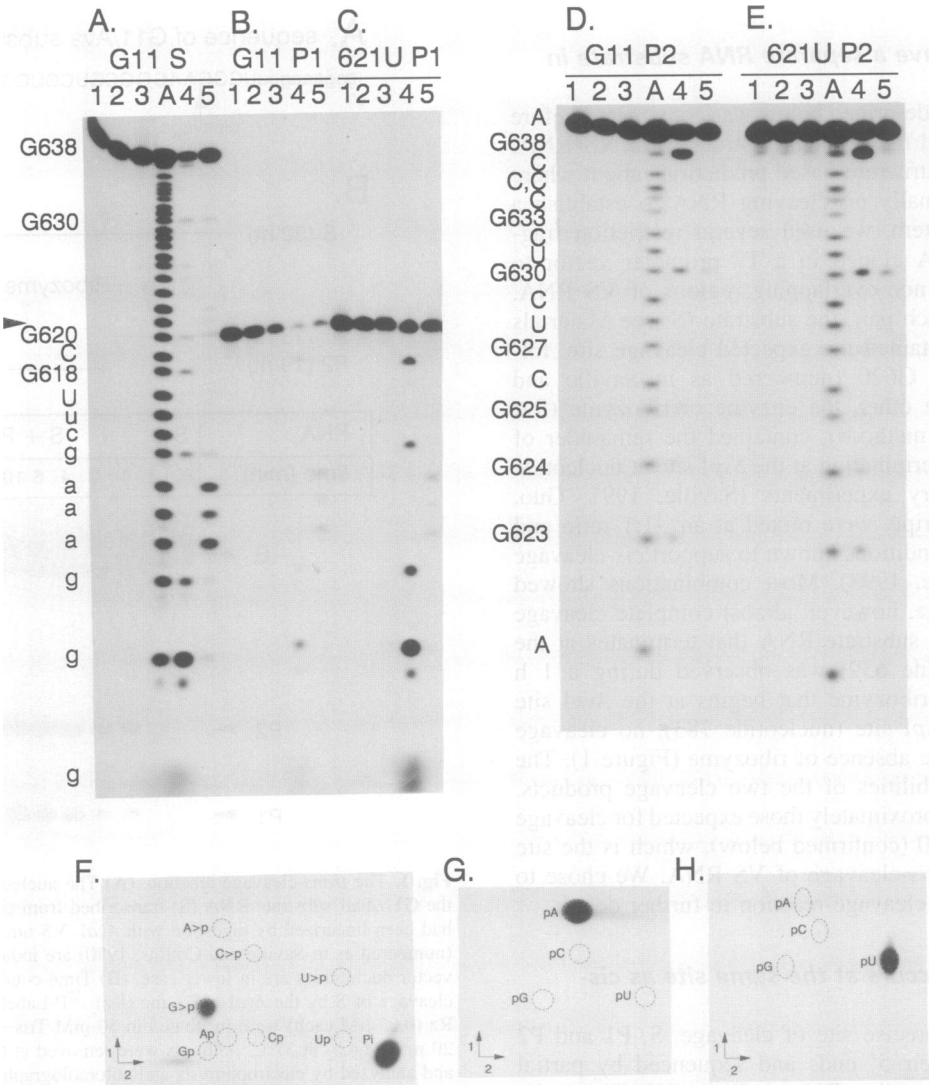

Fig. 2. Characterization of the *trans*-cleavage products. (**A**–**E**) Ribonuclease T1 and U2 sequencing of 5′ end-labeled S and cleavage products P1 and P2 from clone G11 and mutant 621U. Lanes 1–3 are controls (untreated or incubated in T1 or U2 buffer without RNases respectively); lanes A, partial alkaline hydrolysis ladder; lanes 4, RNase T1; lanes 5, RNase U2. Nucleotide sequences are indicated to the left of the figure. VS nucleotides are in upper case, vector nucleotides in lower case; the site of cleavage is indicated by the arrowhead. (**F**–**H**) Thin layer chromatography of: (F) the 3′ nucleotide of G11 P1; (G) the 5′ nucleotide of 5′ phosphorylated G11 P2; (H) the 5′ nucleotide of 5′ phosphorylated 621U P2.

of a template linearized at the *Ava*I site. Thus only 19 nucleotides downstream of the cleavage site are required for *trans*-cleavage by the *Ava*I ribozyme.

A parallel experiment using 3′ end-labeled RNA showed that only a single nucleotide upstream of the cleavage site is required for *trans*-cleavage (Figure 3B). Taken together with the results from 5′ end-labeled RNA (Figure 3A), these data show that the minimum contiguous region of the native RNA required for *trans*-cleavage consists of one nucleotide upstream of the cleavage site and 19 nucleotides downstream.

The minimal substrate RNA consists mostly of a stable hairpin loop

RNA structure prediction using the MFOLD program of Zuker and collaborators (Zuker, 1989; data not shown) suggests that the most thermodynamically reasonable structure of the substrate RNA would be the hairpin-containing structure drawn in Figure 4B. During the characterization of the *trans*-cleavage products (Figures 1 and 2) we noted several observations that were consistent with such a structure. P2 migrated faster than expected relative to size markers for a 19 nucleotide RNA, suggesting that it contained a structure that was not fully denatured even in a gel containing 8.3 M urea (Figure 1 and data not shown). Certain guanosine (623–625, 627 and 633) and adenosine (621 and 622) residues in S and P2 were cleaved weakly or not at all by RNases T1 and/or U2, even though sequencing reactions were performed under putatively denaturing conditions of 50°C, 1 mM EDTA and 7 M urea (Figure 2A–E). Only 18 of the expected 19 bands were observed in the 5′ end-labeled partial alkaline hydrolysis products of P2 (in the gel shown in Figure 2D and E, the first nucleotide, A621, has been run off the gel, so only 17 bands are shown). Also, the spacing and intensity of bands at C634–C636 was uneven (Figure 2D and E), typical of compression artefacts during electrophoresis caused by a secondary structure in the

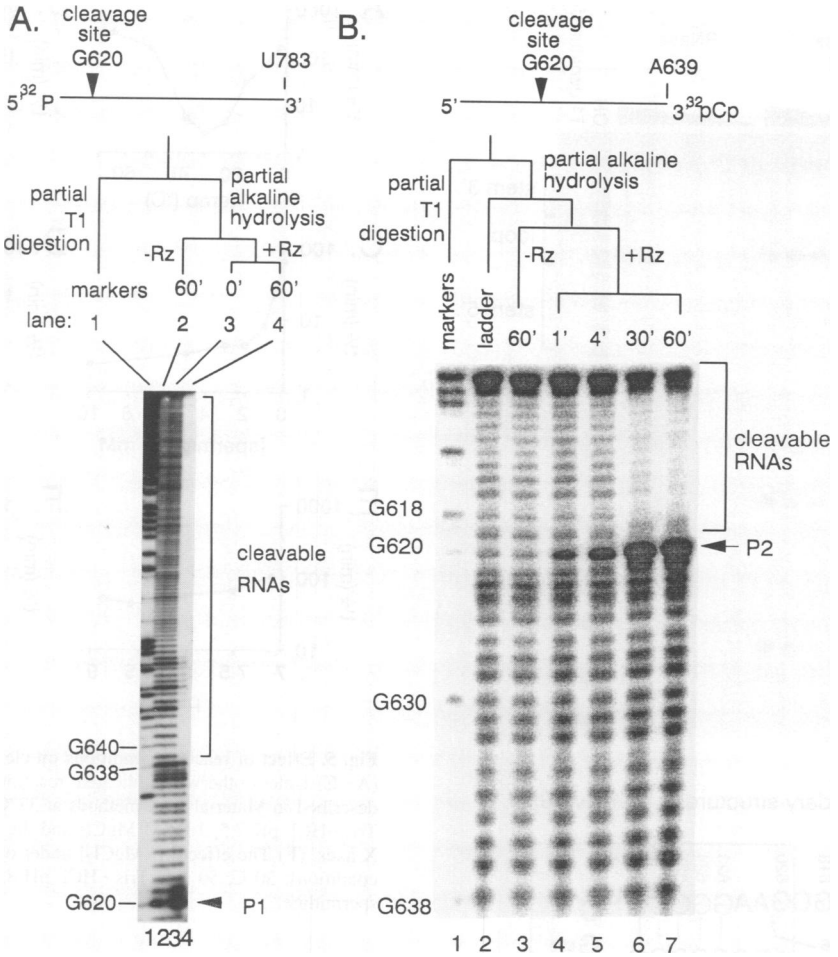

Fig. 3. Minimal substrate requirements for *trans*-cleavage of VS substrates by the *Ava*I ribozyme. End-labeled substrate RNAs, diagrammed at the top of each panel, were treated as indicated in the flow charts. (**A**) 5′-End-labeled G11/*Ssp*I substrate. Substrate (0.3 μM) was incubated with Rz (0.3 μM) in 50 mM Tris−HCl, pH 7.5, 20 mM $MgCl_2$ at 37°C for 0 or 60 min. (**B**) 3′-[^{32}P]pCp-End-labeled G11/*Ava*I substrate. Substrate (0.05 μM) and Rz (1.5 μM) were incubated as in (A) and samples were withdrawn at the times indicated in lanes 4–7. To facilitate interpretation, relevant guanosines are indicated in the T1 marker lane. P1 and P2 mark the position of the detectable (end-labeled) cleavage product in (A) and (B) respectively.

longer molecules. The pattern and intensity of bands is consistent with the interpretation that the two RNAs in the alkaline ladder that terminate at C635 and C636 were compressed; the RNA terminating at C635 is denatured, like those shorter than it (below it in the gel), while the RNA ending at C636 extends the putative helix by one base pair, thereby preventing its denaturation under these electrophoretic conditions. The hairpin-containing RNA co-migrates with the shorter, but denatured, product terminating at C635.

Complementary results were obtained from RNase sequencing of 3′ end-labeled S and P2 (data not shown), i.e. the same guanosines and adenosines were resistant to cleavage by the RNases and the alkaline hydrolysis ladder had a compression involving the two RNAs with 5′ ends at G623 and G624 (those complementary to C636 and C635).

Nuclease probing under native conditions is also consistent with the formation of a stable hairpin loop in S (Figure 4A and data not shown). Guanosines upstream of the ribozyme cleavage site at G620 appeared to be single-stranded, as they were susceptible to cleavage by RNase T1, although G620 itself was only weakly cleaved. The only strong T1 cleavages downstream of G620 were at G630 in the loop and G638 in the 3′ single-stranded region. Guanosines in the stem were resistant to T1 cleavage. The RNase T1 data are not consistent with an alternative structure in which two substrate RNAs dimerize to form a partially self-complementary duplex. In such a duplex, G630 would be paired and inaccessible to RNase T1. RNase T2, which has specificity for single-stranded regions (Knapp, 1989), cleaved in the loop (U628, C629) and at some positions upstream of the stem (U617, C619) and weakly at A621, A622. The weak T1 and T2 cleavages at several positions (620–622) adjacent to the Watson–Crick base pairs in the hairpin stem may indicate that some structure extends beyond that drawn in Figure 4B.

Reaction conditions

We have investigated the effects of several variables that would be expected to affect RNA structure and that have been found to affect the cleavage rates of other ribozymes. We arbitrarily chose an equimolar ratio of S and Rz (0.05 μM each) for most initial investigations; more

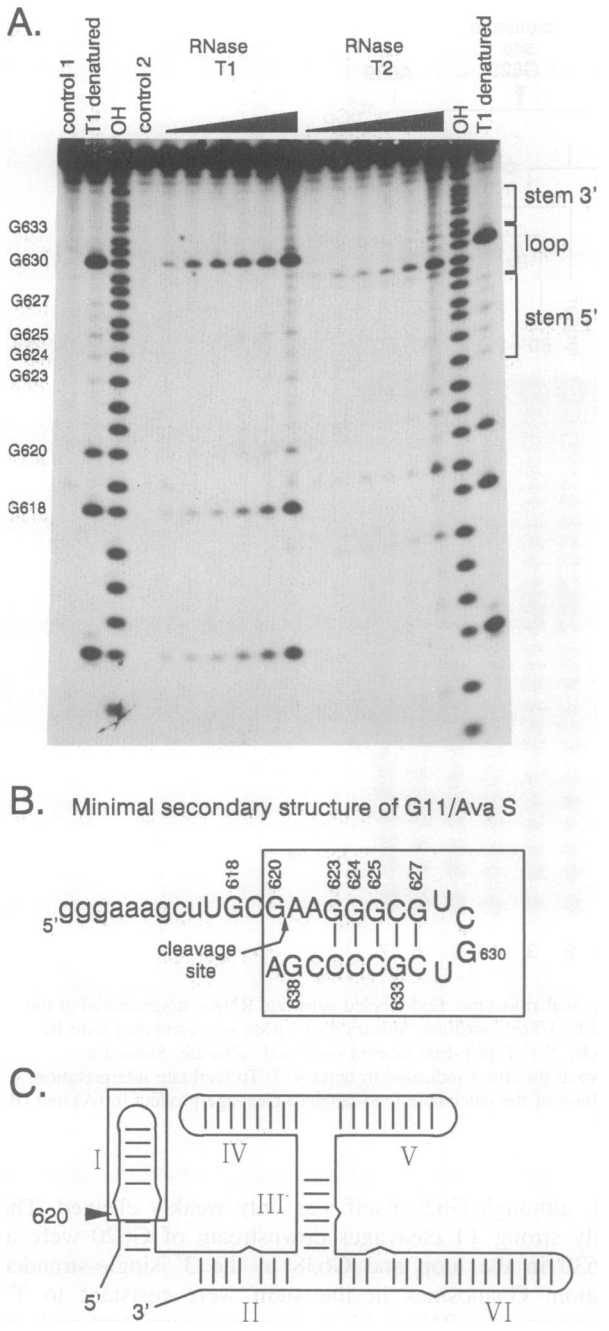

Fig. 4. Structure of S and Rz RNAs. (**A**) Limited RNase T1 and T2 digestion. 5'-End-labeled S was partially digested with RNase T1 or T2 at 30°C in native buffer (50 mM Tris−HCl, pH 8.0, 25 mM KCl, 10 mM $MgCl_2$, 2 mM spermidine) for 0.5–30 min. A guanosine marker lane (labeled T1 denatured) was prepared by RNase T1 sequencing in denaturing buffer (see Materials and methods for details). Controls 1 and 2 contain RNA incubated in the absence of RNase in denaturing buffer or native buffer respectively. OH indicates a partial alkaline hydrolysis ladder. Nucleotides contributing to the stem−loop structure are indicated on the right. (**B**) Minimal secondary structure of S. VS nucleotides are in upper case, vector nucleotides in lower case. The box indicates the minimal length of the substrate determined from deletion experiments shown in Figure 3. (**C**) Proposed secondary structure of the minimal *cis*-cleaving region of VS RNA (T.L.Beattie, J.E.Olive and R.A.Collins, submitted). The box encloses the minimal substrate for the *trans* reaction (boxed in B). Rz consists of the sequences downstream of the substrate, forming stems II−VI (see Materials and methods).

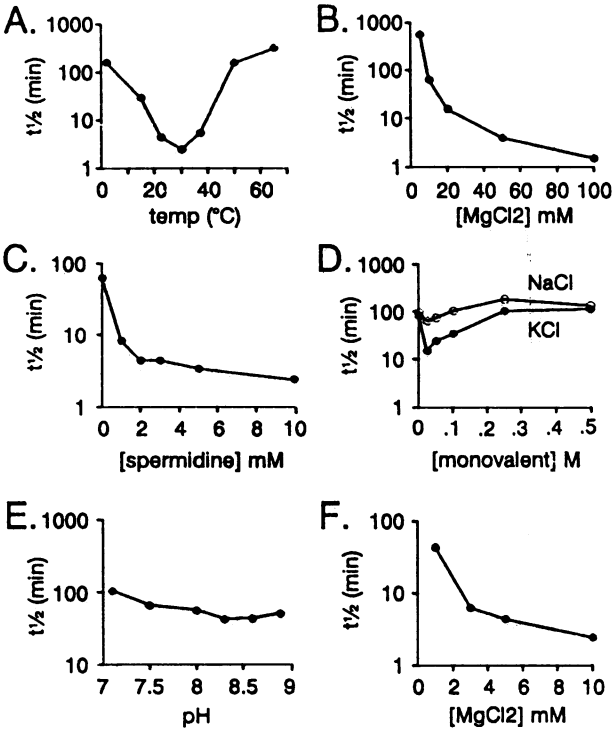

Fig. 5. Effect of reaction conditions on cleavage of S by Rz. (**A**–**E**) Unless otherwise indicated, reactions were carried out as described in Materials and methods at 37°C in the presence of 50 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$ and the variables indicated on the X axes. (**F**) The effect of [$MgCl_2$] under otherwise 'optimized' conditions: 30°C, 50 mM Tris−HCl, pH 8.0, 25 mM KCl, 2 mM spermidine.

detailed analyses specifically under either steady-state or single turnover conditions are described later.

Cleavage rate increased with temperature until an optimum was reached around 30°C and then decreased sharply above 40°C (Figure 5A). No reaction was observed in the absence of a divalent cation and reaction rate increased with increasing $MgCl_2$, reaching a maximum around 100 mM when magnesium was the only cation present (Figure 5B). To determine whether some of the $MgCl_2$ was acting simply as a structural counterion, the effects of spermidine, NaCl, and KCl were investigated in the presence of a subsaturating concentration of $MgCl_2$ (10 mM). In the presence of 10 mM $MgCl_2$, spermidine at 1 mM or greater enhanced the rate of cleavage nearly 10-fold compared with the same reaction without spermidine (Figure 5C). Low concentrations of KCl (<100 mM) also stimulated the reaction rate up to ~10-fold. Perhaps surprisingly, NaCl had almost no effect (Figure 5D). These observations are similar to the effects of cations observed previously on the rate of *cis*-cleavage of VS RNA (Collins and Olive, 1993). The stimulation by low concentrations of some other cations suggests that some $MgCl_2$ was acting as a counterion which could be replaced more effectively by spermidine or potassium.

The rate of reaction showed only a small pH dependence. The nearly 100-fold increase in the hydroxide concentration between pH 7.1 and 8.9 resulted in only a 2-fold increase in rate (Figure 5E). The effect of pH specifically under single turnover conditions is described later.

Finally, the effect of $MgCl_2$ was re-assayed under

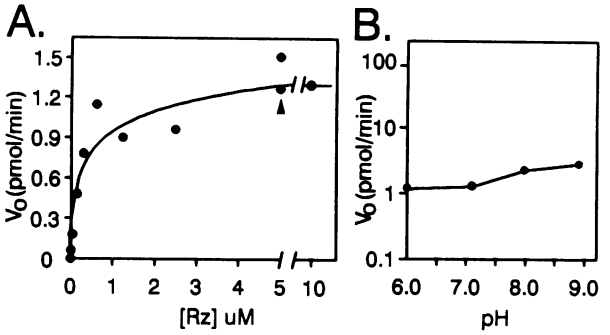

Fig. 6. Effects of pH on the rate of *trans*-cleavage under single turnover conditions. (**A**) Determining single turnover conditions. Initial rates of cleavage of S (0.13 μM) by increasing concentrations of Rz were determined. The arrowhead indicates the ribozyme concentration (5 μM) used to determine the effects of pH on cleavage rate. (**B**) S (0.13 μM) was incubated with 5 μM Rz at the indicated pH. Reactions were carried out as described in Materials and methods.

'optimized' reaction conditions containing 50 mM Tris, pH 8.0, 2 mM spermidine, 25 mM KCl and incubated at 30°C (Figure 5F). Under these conditions 10 mM $MgCl_2$ allowed the same rate of cleavage as a reaction containing 70 mM $MgCl_2$ under suboptimal conditions (compare Figure 5F and B). Thus, the combined effects of temperature, pH and cations other than magnesium enhanced cleavage substantially compared with the arbitrary conditions used for the reactions shown in Figure 1. However, no reaction was observed in the absence of $MgCl_2$, indicating that neither spermidine nor KCl can replace magnesium in cleavage.

Effects of pH under single turnover conditions

The *trans*-cleavage reaction rate showed only a small pH dependence at equimolar concentrations of ribozyme and substrate (Figure 5E). However, these experiments were performed at subsaturating concentrations of $MgCl_2$ and they were probably not under single turnover conditions. Consequently, it was possible that some step in the reaction other than the actual cleavage step itself may have been the rate-limiting step, thereby masking the effect of increased hydroxide ion concentration. To investigate this possibility, single turnover conditions were established empirically under optimized reaction conditions by measuring the initial rates of *trans*-cleavage of 0.13 μM substrate by increasing concentrations of ribozyme. Figure 6A shows that the initial rate of cleavage increased with ribozyme concentration up to ~2.5 μM and subsequently leveled off, suggesting that the reaction was approaching single turnover conditions. The cleavage rate as a function of concentration of $MgCl_2$ was re-investigated using 0.13 μM S and 5 μM Rz and found to be essentially the same shape as in Figure 5F (data not shown); a concentration of 25 mM $MgCl_2$ was chosen to ensure that magnesium was not limiting. *Trans*-cleavage reactions using 0.13 μM S and 5 μM Rz over a range of pH showed only a minor enhancement in reaction rate (Figure 6B).

Steady-state reaction kinetics

To determine if Rz is capable of multiple turnover, it was incubated with an ~20-fold molar excess of S (Figure 7A). If each ribozyme molecule cleaved only a single substrate, a maximum of 5% of S could be cleaved. In contrast, we observed that cleavage continued at a constant rate until ~40% of S was cleaved and then decreased slowly as the concentration of available uncleaved S decreased. This indicated that Rz behaved like a true enzyme in that it was capable of multiple rounds of cleavage. Also, as expected of an enzyme, the initial rate of cleavage was directly proportional to the concentration of the ribozyme under conditions of substrate excess (Figure 7B).

The *trans*-cleavage reaction exhibits a saturation curve with respect to substrate concentration that is typical of Michaelis–Menten kinetics (Figure 7C). A K_m of 0.13 μM and k_{cat} of 0.7/min were obtained from these data. These values have been observed to vary by a factor of ~2 when experiments were repeated with different batches of riboyzme over a period of 2 years.

Discussion

We have modified the natural intramolecular *cis*-cleavage reaction of VS RNA by constructing a ribozyme containing 144 nucleotides of VS RNA that is capable of an intermolecular *trans*-cleavage reaction. This ribozyme acts as a true enzyme in cleaving a 32 nucleotide substrate RNA. In the presence of excess substrate, the initial rate of cleavage was proportional to ribozyme concentration and a single ribozyme molecule could cleave multiple substrate molecules. The ribozyme was specific in cleaving a single phosphodiester bond, the same one as cleaved in the natural *cis*-cleavage reaction. The *trans*-cleavage reaction exhibits Michaelis–Menten kinetics, with $K_m \approx 0.13$ μM and $k_{cat} \approx 0.7$/min. Fedor and Uhlenbeck (1990) have noted that k_{cat} values in the range of 1/min and K_m values in the nanomolar range are characteristic of many diverse ribozymes. However, these measurements of kinetic parameters, including ours, used ribozymes removed from their natural sequence contexts and intracellular environment. Also, the rate-limiting steps may be different in the reactions of the different ribozymes. The similarities in K_m and k_{cat} may therefore be coincidental, unless there is some interesting but unrecognized feature of biology that results in selection for steady-state kinetic parameters in this range.

The shortest contiguous region of VS RNA that functions as a substrate in the *trans* reaction described here contains a single nucleotide upstream of the cleavage site and 19 nucleotides downstream. Our previous characterization of the *cis*-cleavage reaction also showed that only a single nucleotide is required upstream of the cleavage site (Guo *et al.*, 1993); in this respect VS is similar to HDV ribozymes, which also require only a single upstream nucleotide for *cis*- or *trans*-cleavage (Perrotta and Been, 1990, 1992, 1993). Based on minimum free energy predictions, aberrant electrophoretic mobility and the pattern of accessibility to single-strand-specific nucleases, we conclude that the VS substrate RNA consists mostly of a stable stem–loop structure flanked by three nucleotides on the 5′ and 3′ ends, some of which may be involved in non-Watson–Crick structure (Figure 4B). Although this structure appears to predominate under the conditions of the reaction, we do not know that it is necessary for cleavage, nor that the RNA remains in this structure throughout the reaction. Indeed, analysis of the effect

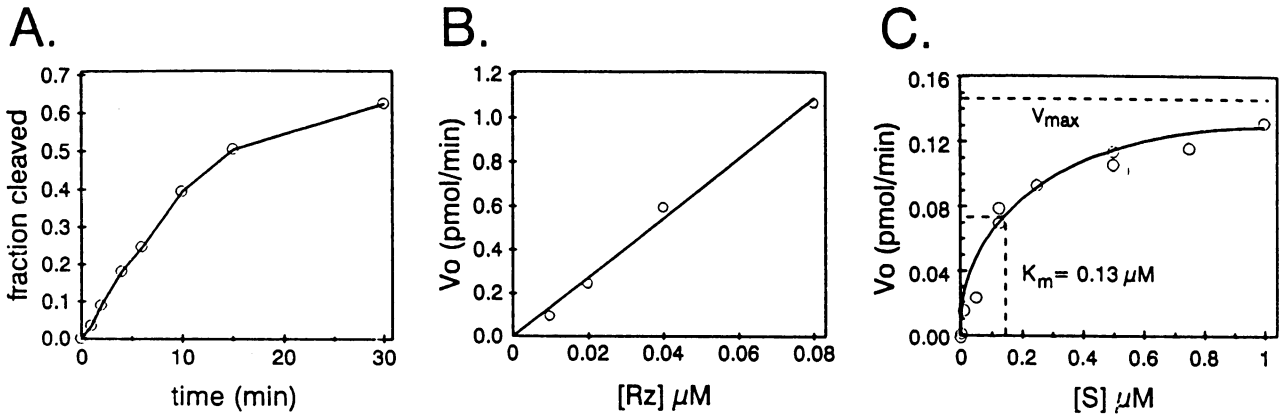

Fig. 7. Steady-state kinetics. Unless otherwise stated, reactions were carried out as described in Materials and methods at 30°C in 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM spermidine, 25 mM KCl). (**A**) Rz is capable of multiple turnover. An ~20-fold molar excess S (≈0.8 μM) was incubated with Rz (0.04 μM) and the fraction of substrate cleaved was determined at different times. (**B**) Rate of cleavage is proportional to ribozyme concentration in the presence of excess substrate. S (0.75 μM) was incubated with 0.01, 0.02, 0.04 or 0.08 μM Rz and the initial rates of cleavage (V_0) were measured. (**C**) Determination of K_m and V_{max}. Initial rates of cleavage of various concentrations of S by 0.01 μM Rz were determined. Substrate concentrations were 2, 10, 50, 125, 250, 750 and 1000 nM. k_{cat} was calculated as V_{max} (0.14 pmol/min/20 μl) divided by [Rz] (0.2 pmol/20 μl).

of base substitution mutations on *cis*-cleavage in this stem–loop region (J.Olive and R.A.Collins, unpublished results) has revealed a complex pattern of effects on cleavage rate. Disruption of some base pairs in the stem by certain single base substitutions has little or no effect on *cis*-cleavage. However, at some positions the identity of one of the bases in a particular pair is critical; even when the compensating substitution is made in the complementary position to restore the helix, cleavage is not restored. We have just begun to study the effect of these mutations on *trans*-cleavage. Our current information suggests that specific bases at specific positions are more important than simply the presence of a stem–loop structure.

The stem–loop structure of the VS substrate RNA leaves no long regions available for Watson–Crick pairing with the ribozyme. Our working model for the structure of the ribozyme, inferred from the recently determined secondary structure of the minimal *cis*-cleaving region of VS RNA (see Figure 4C), predicts that the ribozyme also has no long single-stranded regions. This is in contrast to most *trans*-acting ribozymes derived from hammerhead, hairpin, HDV and Group I intron RNAs, which have been designed to interact with single-stranded regions of their substrates via formation of one or two intermolecular helices flanking the site to be cleaved. In retrospect, it is fortunate that we constructed the *trans* reaction in an empirical fashion, rather than designing it based on knowledge of the secondary structure of VS RNA. Given the precedents from most other ribozymes, it is unlikely that we would have chosen to divide the VS sequence where we did and expect the two RNAs to interact well. Indeed, after we determined the secondary structure of VS RNA, we attempted to design a *trans*-reaction analogous to other ribozymes, by dividing the VS sequence within loop I, however, this combination of RNAs showed no activity (A.Kawamura and R.A.Collins, unpublished results). This could be due to inactive RNA conformations in the ribozyme and/or substrate, or it may indicate that sequences in loop I are important for formation of the active structure.

In addition to base pairing, tertiary interactions are known or suspected to contribute to substrate binding by several ribozymes (Pyle *et al.*, 1992; Dib-Haij *et al.*, 1993). In fact, tertiary interactions alone are sufficient to allow very weak (K_m > 0.1 mM) but specific binding of the P1 stem–loop of a Group I intron to its catalytic core (Doudna and Szostak, 1989). RNase P also recognizes substrates that contain substantial secondary structure and have very limited potential for Watson–Crick pairing with the ribozyme (Smith *et al.*, 1992; Westhof and Altman, 1994). We are currently undertaking more direct measurements of the binding of wild-type and mutant substrates to investigate the idea that the high apparent affinity of the VS ribozyme for its substrate, inferred from its low K_m (0.13 μM), is due to multiple interactions.

The temperature optimum of the *trans*-cleavage reaction is substantially lower than for the *cis*-cleavage reaction (30 versus ~45°C) and activity drops off much more sharply at higher temperatures (cf. Collins and Olive, 1993). The retention of activity at higher temperatures in the *cis*-cleavage reaction indicates that the active site of the ribozyme does not begin to denature until at least 45°C. The lower optimum temperature of the *trans*-cleavage reaction may reflect decreased binding of the substrate at higher temperatures.

We noted in our previous characterization of the VS RNA *cis*-cleavage reaction that the cleavage rate was essentially unaffected by pH (Collins and Olive, 1993). Consistent with this observation, the *trans*-cleavage reaction described here also showed little, if any, pH dependence, even when examined under single turnover conditions. These observations differ from results examining the rate of the chemical cleavage step of hammerhead ribozymes (Dahm and Uhlenbeck, 1993), RNase P (Guerrier-Takada *et al.*, 1986; Smith and Pace, 1993; Beebe and Fierke; 1994) and *Tetrahymena* Group I intron (Herschlag *et al.*, 1993; Herschlag and Khosla, 1994). For these ribozymes, the rate of the cleavage step was found to increase with increasing pH. Failure to observe pH dependence in VS RNA could mean that OH^- is not involved in the cleavage reaction, that the reaction

proceeds via a novel mechanism or, more likely, that the VS *trans* reaction is not limited by the rate of the chemical cleavage step under these conditions, but rather by some step that precedes actual cleavage.

One interesting candidate for such a rate-limiting step would be a conformational change in the substrate and/or ribozyme following binding. At saturating ribozyme concentration, the pseudo-first-order rate constant for *trans*-cleavage of S (≈0.6/min) is ~10-fold higher than the rate of *cis*-cleavage of G11 RNA under similar conditions (determined from plots of fraction of uncleaved RNA versus time of the data used for Figure 6 and Collins and Olive, 1993). Since we envision that the *trans*-cleavage reaction recreates essentially the same RNA conformation as in the *cis*-cleavage reaction, the higher rate suggests that the cleavable conformation may be more easily attained when S (stem−loop I; Figure 4C) is not constrained by covalent attachment to the ribozyme core. In support of this idea, we have also found that the rate of *cis*-cleavage of G11 RNA can be increased several fold by increasing the distance between stem−loop I and the ribozyme core (A.Andersen and R.A.Collins, unpublished results). These observations are consistent with the idea of at least one conformational change involving the substrate stem−loop occurring during the reaction.

The observation that the VS ribozyme can recognize a substrate that contains a stable secondary structure may be useful from the perspective of ribozyme engineering. Among the limitations to modifying hammerhead, hairpin or Group I intron riboyzmes to cleave non-native target RNAs is the requirement that the target site be in a single-stranded region to allow recognition via base pairing with the ribozyme. Because the cleavage site for the VS ribozyme is adjacent to a stable secondary structure, the VS ribozyme may have unique properties that can be adapted to cleaving certain RNAs that are not accessible to the action of other ribozymes. We are currently investigating the substrate recognition requirements, including whether substrate secondary structure is required, or simply tolerated, by the ribozyme.

Materials and methods

DNA templates and synthesis of RNAs

Fragments of VS DNA were cloned into vectors pTZ18R or 19R (Pharmacia). Clone G11 (see Guo *et al.*, 1993) contains VS nucleotides 617–881, numbered as in Saville and Collins (1990); the cleavage site is between G620 and A621. Substrate RNAs were transcribed (see below) from G11 or its derivatives which had been linearized at the *Ava*I site (nucleotide 639) or the *Ssp*I site (nucleotide 783) to make RNAs designated G11/*Ava*I and G11/*Ssp*I respectively. These RNAs begin with nine vector nucleotides (5′-gggaaagcu) followed by VS sequence. A site-directed mutant of G11, clone 621U, which contains a single A→U substitution immediately following the self-cleavage site, was also used.

Clone A-3 contains VS sequences downstream of the *Ava*I site (nucleotides. 640–881) in a derivative of pTZ19R that lacks the *Xba*I and *Sph*I sites in the multiple cloning site (constructed for reasons unrelated to the project described here). Transcripts of clone A-3 digested with *Ssp*I (VS nucleotide 783) begin with 9 vector nucleotides (5′-gggaaagcu) followed by 144 nucleotides of VS RNA; this RNA is designated the *Ava*I ribozyme, or Rz.

RNAs were prepared by *in vitro* T7 RNA polymerase transcription from linearized plasmid DNAs. Transcription reactions (usually 300 µl) contained 10–20 µg appropriately linearized template, 1 mM each NTP (Pharmacia), 5 mM dithiothreitol, 1× T7 polymerase buffer [Bethesda Research Laboratories; 40 mM Tris−HCl, pH 8.0, 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine–$(HCl)_3$], 300 U RNAguard (Pharmacia), 150–200 U T7 RNA polymerase (Bethesda Research Laboratories) for 2 h at 37°C. Radioactive transcripts were prepared as above except an additional 30 µCi [α-^{32}P]GTP (or, for specific experiments, ATP or UTP) was added. Samples were subsequently treated with DNase I (5 U/µg DNA template; Pharmacia) for 15 min, then EDTA was added to 10 mM. RNAs were extracted with phenol:chloroform:isoamyl alcohol, chloroform:isoamyl alcohol (CIA) and ethanol-precipitated in the presence of 0.3 M sodium acetate, pH 5.2.

Precipitated RNAs were dissolved in water and two volumes of sequencing dye (95% formamide, 0.5× TBE, 0.1% xylene cyanol, 0.1% bromphenol blue), heated at 75°C for 3 min and fractionated by electrophoresis on denaturing polyacrylamide gels (40:1 acrylamide:bis-acrylamide) of appropriate concentration containing 8.3 M urea and 1× TBE (135 mM Tris, 45 mM boric acid, 2.5 mM EDTA). RNAs were visualized either by autoradiography or UV shadowing. Bands of interest were excised, eluted overnight at 4°C in water and filtered to remove residual polyacrylamide. RNAs were precipitated with ethanol in the presence of 0.3 M sodium acetate and dissolved in water. Concentrations were determined spectrophotometrically, assuming 1 OD_{260} corresponds to an RNA concentration of 40 µg/ml.

End-labeling of RNAs

RNAs were labeled at 5′ termini using T4 polynucleotide kinase and [γ-^{32}P]ATP or at 3′ termini using T4 RNA ligase and 5′-[^{32}P]pCp. End-labeled RNAs were fractionated on denaturing polyacrylamide gels and detected by autoradiography.

In order to remove 5′-triphosphates prior to 5′ end-labeling, some RNAs were treated with 1 U calf intestinal alkaline phosphatase (Boehringer Mannheim) in a 10 µl reaction containing 50 mM Tris−HCl, pH 8.0, 0.1 mM EDTA at 55°C for 30 min. Reactions were terminated by extraction with phenol:CIA and CIA.

RNA sequencing

End-labeled RNAs were partially digested with RNase T1 or U2 essentially as described by Donis-Keller *et al.* (1977) in denaturing buffer (7 M urea, 50 mM citric acid, 1 mM EDTA, 0.05% xylene cyanol, 150 µg/ml yeast tRNA) and adjusted with NaOH to pH 5 for T1 digests and pH 3.5 for U2. Reactions were terminated by chilling in ice. To prepare partial alkaline hydrolysis products, labeled RNA and 0.5 µg/µl yeast carrier tRNA were heated to 95°C for 5–10 min in 0.15 M NH_4OH, lyophilized and dissolved in denaturing buffer. RNAs were separated by electrophoresis on denaturing 20% polyacrylamide gels.

Structure probing

Limited partial digestion of 5′ end-labeled S was performed under native conditions essentially as described by Knapp (1989). Labeled RNA (10–100 ng) was dissolved in 27 µl of 50 mM Tris−HCl, pH 8.0, 25 mM KCl, 10 mM $MgCl_2$, 2 mM spermidine–$(HCl)_3$, 2.5 µg/µl yeast tRNA. RNase T1 (4.5 U) or T2 (0.225 U) was added and the reactions were incubated at 30°C. Samples (3 µl) were removed at 0.5, 2, 3, 8, 15 and 30 min (RNase T1) or 2, 3, 5, 8, 15 and 30 min (RNase T2), mixed with an equal volume of stop solution (9 M urea, 10% glycerol, 0.05% xylene cyanol) and frozen in dry ice. Products were subjected to electrophoresis on denaturing 20% polyacrylamide gels followed by autoradiography.

Analysis of 5′ and 3′ termini

Identification of 5′ and 3′ terminal nucleotides by P1 nuclease digestion and two-dimensional thin layer chromatography of appropriately labeled RNAs was carried out as described previously (Shugar, 1967; Silberklang *et al.*, 1979; Saville and Collins, 1990).

Trans-cleavage reactions

Trans-cleavage of S by the *Ava*I ribozyme (Rz) was carried out following pre-incubation of gel-purified S and Rz in the appropriate 1× reaction solution for 2 min. Reactions were initiated by addition of ribozyme to substrate in a final volume of 20 µl. In a typical reaction, 10 aliquots of 1.5 µl were removed at specified times, terminated by addition of 13.5 µl of stop mix (70% formamide, 7 mM EDTA, 0.4× TBE, 0.07% xylene cyanol, 0.07% bromphenol blue) and stored on ice. Samples were fractionated by electrophoresis on denaturing 20% polyacrylamide gels.

The effects of temperature, pH, $MgCl_2$ and spermidine–$(HCl)_3$ on the *trans*-cleavage reaction were analyzed by incubating equimolar concentrations of Rz and S (0.05 µM each) in solutions as described in the figure legends. For no good reason, the effects of NaCl and KCl were examined at 0.05 µM Rz and 0.125 µM S. A final study of the

effects of $MgCl_2$ under otherwise 'optimized' conditions was performed at 30°C, 50 mM Tris–HCl, pH 8.0, 2 mM spermidine, 25 mM KCl.

Experiments to establish single turnover conditions (Figure 7A) were performed at 30°C in 50 mM Tris–HCl, pH 7.1, 25 mM $MgCl_2$, 25 mM KCl, 2 mM spermidine. Analyses of the effect of pH under single turnover conditions (Figure 6B) were performed as above, except that the concentrations of Rz and S were 5 μM and 0.13 μM respectively. Tris–HCl (50 mM) was used for pHs 7.1–8.9; 16.5 mM PIPES/44 mM Tris (Smith and Pace, 1993) was used for pH 6.

Amounts of substrate and products were quantitated using a PhosphorImager and ImageQuant version 3.0 software (Molecular Dynamics, Sunnyvale, CA). Estimates of initial cleavage rates were derived from plots of fraction of substrate cleaved versus time using Grafit software (Erithacus Software Ltd, Staines, UK). Up to 90% of the substrate could be cleaved in 60 min at approximately equimolar concentration of ribozyme, with the curve indicating the presence of ~10% unreactive starting material. Curves were not adjusted to 100% completion and the nature of the unreactive substrate has not been characterized further.

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