Substrate selection rules for the hairpin ribozyme determined by in vitro selection, mutation, and analysis of mismatched substrates

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Substrate recognition by the hairpin ribozyme has been proposed to involve two short intermolecular helices, termed helix 1 and helix 2. We have used a combination of three methods (cleavage of mismatched substrates, in vitro selection, and site-specific mutational analysis) to systematically determine the substrate recognition rules for this RNA enzyme. Assays measuring substrate cleavage in *trans* under multiple turnover conditions were conducted using the wild-type ribozyme and substrates containing mismatches in all sites potentially recognized by the ribozyme. Molecules containing single- and multiple-base mismatches in helix 2 at sites distant from the cleavage site ($g_{-4}c$, $u_{-5}a$, $g_{-4}c : u_{-5}a$) were cleaved with reduced efficiency, whereas those with mismatches proximal to the cleavage site ($c_{-2}a$, $a_{-3}c$, $c_{-2}a : a_{-3}c$) were not cut. Analogous results were obtained for helix 1, where mismatches distal from the cleavage site ($u_{+7}a$, $u_{+8}a$, $u_{+9}a$, $u_{+7}a : u_{+8}a : u_{+9}a$) were used much more efficiently than those proximal to the cleavage site ($c_{+4}a$, $u_{-5}a$, $g_{+6}c$, $c_{+4}a : u_{-5}a : g_{+6}c$). In vitro selection experiments were carried out to identify active variants from populations of molecules in which either helix 1 or helix 2 was randomized. Results constitute an artificial phylogenetic data base that proves base-pairing of nucleotides at five positions within helix 1 and three positions within helix 2 and reveals a significant sequence bias at 3 bp ($c_{+4} \cdot G_6$, $c_{-2} \cdot G_{11}$, and $a_{-3} \cdot U_{12}$). This sequence bias was confirmed at two sites by measuring relative cleavage rates of all 16 possible dinucleotide combinations at base pairs $c_{+4} \cdot G_6$ and $c_{-2} \cdot G_{11}$. The strong sequence preference suggests that tertiary structure (e.g., base triples) or alternative secondary structures at these sites may be important for ribozyme function. Together with results from previous work, we conclude that hairpin ribozymes can be engineered to cleave substrates containing the sequence 5'-NN(G/A)(U/C)N↓G(A/U/C)(U/C)(G/U/C)NNNN-3' with high efficiency.

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Small catalytic RNA molecules, or ribozymes, have been identified that function as sequence-specific endonucleases (Symons 1992), acting in a manner that is superficially analogous to the action of restriction enzymes. Substrate recognition by ribozymes results from RNA-RNA interactions, including simple Watson-Crick base pairs, as well as nonstandard base pairs and other tertiary interactions (Barford and Cech 1989; Michel et al. 1989; Michel and Westhof 1990; Been and Perrotta 1991; Pyle et al. 1992). Because the rules for RNA-RNA interactions are better understood than those for protein-nucleic acid interactions, it is possible to manipulate the sequence specificity of ribozymes by altering the structure of their substrate-binding domains (Been and Cech

1986; Waring et al. 1986; Zaug et al. 1986; Uhlenbeck 1987; Haseloff and Gerlach 1988; Hampel et al. 1990).

Engineered ribozymes are useful reagents for RNA inactivation studies for the elucidation of gene function in cultured cells (Sioud et al. 1992) and transgenic organisms. Additionally, they may serve as gene-specific therapeutic agents for the treatment of viral and genetic diseases (Cech 1988). The development of engineered ribozymes depends on a detailed understanding of the mechanism of interaction between ribozymes and their cognate substrates. In addition, it is important to empirically assess the specificity of ribozymes, for example, to determine the extent to which sequence variation in the substrate affects cleavage efficiency.

Binding between the hairpin ribozyme and its cognate substrate involves two short intermolecular helices of 6 bp (helix 1) and 4 bp (helix 2), separated by an internal

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loop that includes 4 substrate nucleotides (Fig. 1A; Feldstein et al. 1990; Hampel et al. 1990). In the form shown in Figure 1A, the hairpin ribozyme may potentially interact with a maximum of 14 nucleotides. The functional importance of helices 1 and 2 is supported by the construction and analysis of compensatory sequence changes (Feldstein et al. 1990; Hampel et al. 1990; Chowrira et al. 1991). These experiments have shown that the specificity of the hairpin ribozyme can be altered. However, such specificity changes may be accompanied by unacceptable decreases in catalytic activity (S. Joseph and J. Burke, unpubl.). These results indicate that the sequence of helices 1 and 2 can strongly affect catalytic activity even if the predicted secondary structure is preserved.

Here, we present experiments designed to elucidate molecular details of the interaction between the hairpin ribozyme and its substrate. Three complementary experimental strategies have been employed: (1) We have analyzed the ability of the ribozyme to cleave a series of variant substrates, each containing single- or multiple-base substitutions; (2) an *in vitro* selection method (Fig. 1B) (Berzal-Herranz et al. 1992) has been applied to identify sequences necessary for optimal cleavage and ligation

activities in *cis*; and (3) sequence preferences resulting from the selection experiments have been critically tested by assaying *trans*-cleavage activity of panels of variant ribozymes against a series of variant substrates. Our results permit the formulation of a set of comprehensive substrate selection rules for the hairpin ribozyme.

Results

Cleavage assays using mismatched substrates

Analysis of ribozyme activity against mismatched substrates was used to identify those sites within the substrate where the hairpin ribozyme was capable of distinguishing between the naturally occurring base and mismatched bases. Four substrates containing multiple-base changes in helices 1 and 2 were tested, as were 10 substrates containing single-base mismatches in the two substrate-binding helices. Results are shown in Figure 2A and Table 1.

Results of cleavage assays under multiple turnover conditions for substrate variants containing multiple-base mismatches show that mismatches proximal to the

Figure 1. Hairpin ribozyme and *in vitro* selection. (A) Secondary structure model of the hairpin ribozyme-substrate complex. The ribozyme sequence is shown in uppercase letters, and the substrate sequence is in lowercase letters. The arrow indicates the cleavage-ligation site. Substrate nucleotides to the 3' side of the cleavage site have positive numbers; those to the 5' side have negative numbers. Nomenclature is as follows: The four helical regions are H1-H4. (X/Y) RNA segment joining the 3' end of helix X to the 5' end of helix Y. The shaded regions indicate the sequences studied in this work. For *in vitro* selection experiments, the substrate was linked to the ribozyme by a pentacytidine linker (broken lines, shown) or by PBS 4 (not shown). (B) *In vitro* selection strategy. Shown is *in vitro* selection of active and inactive hairpin ribozyme variants, as described (Berzal-Herranz et al. 1992). (x) Mutation sites. (P1-P3) Sequences that serve as primer-binding sites for cDNA synthesis and polymerase chain reaction amplification.

Figure 2. Cleavage assays using mismatched substrates. (A) Autoradiograph of the cleavage of substrates with single-base mismatches. (RZ) Ribozyme; (S) substrate; (3'P and 5'P) 3'- and 5'-cleavage products. (Lane S) Wild-type substrate; (lane RZ) ribozyme; (lane S + RZ) wild-type substrate and ribozyme; (lanes u_{+9a} to u_{-5a}) variant substrates with single-base mismatches at the indicated positions were incubated with ribozyme. The *trans*-cleavage reactions were performed as described in Materials and methods. (B) Efficiency of substrate cleavage. The bar graph indicates percent relative cleavage of single-base mismatched substrates. Cleavage efficiencies were determined from at least three independent experiments as described in Materials and methods. For comparison, the cleavage efficiency of substrates with single-base substitutions in J2/1 are included (Chowrira et al. 1991). (C) Time course of cleavage. The variant substrates were incubated with ribozyme as described in Materials and methods, for increasing amounts of time. Aliquots of the reaction mixture were taken at the indicated time point, added to equal volume of loading buffer, and immediately placed on crushed dry ice before electrophoresis.

substrate cleavage site eliminate cleavage activity, whereas those distal to the cleavage site reduce but do not eliminate cleavage (Table 1). Within helix 2, no cleavage of a substrate with a 2-base mismatch proximal to the cleavage site (c_{-2a} : a_{-3c}) was observed; whereas a substrate with an analogous mismatch distal to the cleavage site (g_{-4c} : u_{-5a}) was cleaved, but the cleavage efficiency (k_{cat}/K_M) was reduced by three orders of magnitude relative to wild type. In helix 1, a 3-base mismatch distal from the cleavage site (u_{+9a} : u_{+8a} : u_{+7a}) was cut inefficiently; its cleavage efficiency is reduced 25-fold relative to wild type. No cleavage activity was detected against a helix 1 variant with a three-base mismatch proximal to the cleavage site (g_{+6c} : u_{+5a} : C_{+4a}).

Single-base mismatches were also generated, in which G and A were changed to C, generating C · C and U · C

Table 1. Kinetic parameters for mismatched substrates

Substrate	K_M (μM)	k_{cat} (/min)	k_{cat}/K_M (/min/ μM)	Relative k_{cat}/K_M
Wild type	0.042	2.2	52	1.0
u _{+7a} : u _{+8a} : u _{+9a}	1.5	2.9	1.9	0.04
c _{+4a} : u _{+5a} : g _{+6c}	—	—	<0.005	<0.0001
c _{-2a} : a _{-3c}	—	—	<0.005	<0.0001
g _{-4c} : u _{-5a}	2.4	0.08	0.03	0.001
u _{+9a}	0.080	2.5	31	0.6
u _{-5a}	0.059	1.5	26	0.5

Concentrations for u_{+9a} : u_{+8a} : u_{+7a} and g_{-4c} : u_{-5a} were 0.02–5.0 μM and 0.01–6.0 μM , respectively. The ribozyme concentration was 0.002 μM . Concentrations for wild type, u_{+9a}, and u_{-5a} were 0.001–0.2 μM , and ribozyme concentration was 0.002 μM .

mismatches in the ribozyme–substrate complex. C and U were changed to A, resulting in G · A and A · A mismatches. Results (Fig. 2A,B) show very strong discrimination against mismatched substrates at 6 of 10 positions in the two helices. For both helices 1 and 2, these positions represent the three positions closest to the cleavage sites, as defined by helix 1 variants $c_{+4}a$, $u_{+5}a$, and $g_{+6}c$ and by helix 2 variants $c_{-2}a$, $a_{-3}c$, and $g_{-4}c$. All six of these single-base mismatches reduce cleavage activity by a minimum of 20-fold in this single time-point assay. These results establish strong substrate discrimination by the hairpin ribozyme at positions -2 through -4 and at positions $+4$ through $+6$. A more moderate effect was seen at position $+7$, where the $u_{+7}a$ substitution reduced cleavage activity by a factor of three. Note that the time point chosen (30 min) is long compared with $t_{1/2}$ for the wild-type substrate under these conditions (~ 3 min); therefore, actual rate differences are greater than would be estimated by results depicted in Figure 2A and B alone.

Although the single time-point experiments described above serve to identify those sites where single-base mismatches have the greatest inhibitory effect, they do not effectively measure substrate discrimination at the distal sites, that is, positions $+7$ through $+9$ in helix 1 and position -5 in helix 2. To examine the effect of the less inhibitory mismatches and to quantitate the effects of all single-base mismatches, we carried out time course experiments to measure initial cleavage rates. Results (Fig. 2C) showed that the ribozyme discriminates mismatched substrates at all 10 positions in helices 1 and 2. Discrimination at substrate positions -5 , $+8$, and $+9$ is clearly evident, and an order of discrimination within the two helices ($-2 > -3$, -4 , $+5$, $+6 > +7 > +8 > -5$, $+9$) is defined. Additional confirmation that discrimination is observed even at distal positions -5 and $+9$ was obtained through steady-state kinetic analysis (Table 1). Each of the mismatches at these positions ($a_{-5} \cdot A_{14}$ and $a_9 \cdot A_1$) resulted in a twofold reduction in cleavage efficiency (k_{cat}/K_M).

In vitro selection

In vitro selection experiments were carried out in which the sequences of helices 1 and 2 were randomized by introducing equimolar quantities of all four nucleoside phosphoramidites during synthesis of each of the 12 bases of helix 1 (generating a theoretical initial pool of 2×10^7 sequences) and in a separate experiment randomizing all eight positions of helix 2 (7×10^4 sequences). *In vitro* selection experiments were also carried out following random mutagenesis of the ribozyme–substrate complex as described (Materials and methods). Both active and inactive clones were selected from each experiment for further analysis by cloning, sequencing, and *in vitro* self-cleavage assays (Fig. 1B) (Berzal-Herranz et al. 1992).

Randomization of helix 2 Following randomization of helix 2, clones containing cDNAs encoding constructs

with a wide range of self-cleavage activities were characterized by sequencing (Fig. 3A,B) and self-cleavage assays (Fig. 3C). For active clones, base-pair frequencies for each position in helix 2 are summarized in Figure 3A. Among the clones obtained from the selection of active helix 2 variants that showed the highest levels of cleavage activity, evidence was obtained for base-pairing at the three positions proximal to the reaction site—those corresponding to $c_{-2} \cdot G_{11}$, $a_{-3} \cdot U_{12}$, and $g_{-4} \cdot C_{13}$.

At $c_{-2} \cdot G_{11}$, a very strong sequence bias was observed favoring both base-pairing and the wild-type sequence, G at ribozyme position 11 and C at substrate position -2 . The strong preference for the $c_{-2} \cdot G_{11}$ pair extended even to those molecules emerging from the helix 2 active selection, which showed moderate and low levels of activity (Fig. 3B,C). Among the higher activity variants containing sequences other than $c_{-2} \cdot G_{11}$, a $u_{-2} \cdot G_{11}$ wobble pair was always present. The selection experiments indicate that base-pairing at these positions is required for even very low levels of cleavage activity. Inspection of the sequences of clones obtained with low activity levels suggests that in helix 2 a $c_{-2} \cdot G_{11}$ pair may be sufficient for low levels of cleavage and ligation activity even if all other positions of helix 2 are mismatches or wobble pairs.

Selection results for other positions in helix 2 are as follows. Strong support is provided for a base pair at $a_{-3} \cdot U_{12}$, with $g_{-3} \cdot C_{12}$ favored and a strong bias in favor of a purine at substrate position -3 and a pyrimidine at ribozyme position 12. Each of the three naturally occurring variants also shows a substrate purine and ribozyme pyrimidine at these positions (Fig. 3B) (Rubino et al. 1990). The selection experiments support a base pair at positions corresponding to $g_{-4} \cdot C_{13}$, but mismatches and wobble pairs are found more frequently than at the two preceding positions. At positions corresponding to $u_{-5} \cdot A_{14}$, a random distribution of nucleotides is observed. Taken by themselves, the selection results do not support the need for a base pair between substrate position -5 and ribozyme position 14. These results are consistent with *in vitro* selection done under high stringency (Materials and methods), all of the variants had $c_{-2} \cdot G_{11}$ base pair, and the overall stability of helix 2 was greater (data not shown).

Randomization of helix 1 Helix 1 was randomized, and clones with a wide range of self-cleavage activities were obtained by *in vitro* selection. In one respect, the helix 1 selection differs from the helix 2 experiment. Sequences to the 3' side of the substrate cleavage site are lost during the cleavage step of the selection protocol (Fig. 1B). Because of this limitation, the substrate sequences obtained have been selected in only a single ligation step. The ligation substrate has positions $+4$ to $+9$ randomized.

Selection results strongly support the importance of 4 bp in helix 1 (pairing of substrate positions $+4$ through $+7$ with ribozyme positions 3 through 6) for efficient ligation (Fig. 4). In addition, moderately strong support is obtained for pairing between the bases at substrate posi-

Figure 4. In vitro selection of active molecules following randomization of helix 1. (A) Base-pair preferences among active variants. The bar graph indicates the frequency of occurrence of individual base pairs in the population studied (24 active clones and 13 inactive clones). The wild-type sequence and the derived consensus sequence of helix 1 are shown. (B) Individual clones obtained by selection. The clones were classified based on their activity in the self-cleavage assay. The helix 1 regions from the three naturally occurring hairpin ribozyme structures in sTRSV, sARMV, and sCYMV are shown.

wild-type ribozyme could be increased by high magnesium concentrations (Fig. 5C). In contrast, the wild-type ribozyme could not cleave the c₋₂a mutant substrate even at high Mg²⁺ concentrations (Fig. 5C).

All 16-bp combinations were also tested at the position of helix 1 closest to the cleavage site (c₊₄·G₆; Fig. 6). At these positions, cleavage products were detected in six combinations. The most active combinations were c·G > g·C > u·A. In addition, low levels of activity were detected for u·G, a·U, and g·U. The results for helix 1 above correspond closely to those obtained from

in vitro selection, where observed base-pair frequencies in the most active molecules were also c·G > g·C > u·A.

Discussion

Results of studies reported here and elsewhere (Chowrira et al. 1991; Berzal-Herranz et al. 1992) indicate that hairpin ribozymes can be engineered to efficiently cleave RNA sequences containing the sequence 5'-nnryn↓ghybnnnn-3' (Fig. 7). These changes are accom-

Figure 5. Mutational analysis of c₋₂ : G₁₁. (A) Cleavage in trans of all possible base combinations. (RZ) Ribozyme; (S) substrate RNA; (3'P and 5'P) 3'- and 5'-cleavage products. Substrates with cytidine (c₋₂,wt), uridine (c₋₂u), adenine (c₋₂a), or guanine (c₋₂g) at position -2 were incubated with ribozymes that had a guanine (G, wt), uridine (U), adenine (A), or cytidine (C) at position 11 in the ribozyme. The cleavage reactions were carried out exactly as described in Materials and methods for 30 min. An autoradiograph of a denaturing 20% polyacrylamide gel is shown. (B) Relative cleavage efficiencies. The relative cleavage efficiencies of all 16 possible ribozyme-substrate combinations are shown. The cleavage efficiencies were obtained from at least three independent experiments. (C) Magnesium concentration effects. The effect of Mg²⁺ on a mismatch [c₋₂a] and a wobble [c₋₂u] base pair were studied by incubating the substrates with ribozyme in the standard reaction buffer containing 5, 10, 50, or 100 mM MgCl₂ as described in Materials and methods. The plot shows the percentage cleavage of wild-type and variant substrates (c₋₂a and c₋₂u) with increasing Mg²⁺ concentration.

		Substrate c_{+4}			
		g	a	u	c
Ribozyme G_6	G	0	0	8	100
	A	0	0	34	0
	U	3	3	0	0
	C	63	0	0	0

Figure 6. Mutational analysis of $c_{+4} : G_6$. The nucleotides at position +4 in the substrate and position 6 in the ribozyme were changed to three other nucleotides, and the cleavage efficiencies of all possible base pairs were analyzed. The reactions were carried out as described in Materials and methods. The relative cleavage efficiencies of all 16 possible ribozyme–substrate combinations are shown.

plished by the manipulation of the sequence of ribozyme positions 1–6 and 11–14. However, we do not expect that ribozymes engineered according to these rules will always exhibit high levels of catalytic activity, because some such changes may inhibit correct folding of the ribozyme or may result in the formation of substrate structures that inhibit binding to the ribozyme (Fedor and Uhlenbeck 1990). Nevertheless, our results indicate that hairpin ribozymes with a very broad range of specificities can be developed, such that any mRNA of interest is expected to have numerous potential target sites.

Previously, it has been shown that in vitro selection is a powerful method for identifying sequences and structural elements important for ribozyme activity (Green et al. 1990; Robertson and Joyce 1990; Berzal-Herranz et al. 1992). Our results validate the use of in vitro selection methods to identify important sequences and structures within the substrate and the substrate-binding domain of the ribozyme. A priori, it might be expected that significant differences might exist between the structural requirements for optimal activity in *trans*-cleavage and those for recovery among the active cDNA clones by in vitro selection, as the selection experiment involves self-cleavage followed by ligation. In particular, the substrate sequences to the 5' side of the cleavage site are covalently tethered to the ribozyme throughout the selection experiments. Thus, base substitutions that destabilize helix 2 would be expected to affect *trans*-cleavage more than *cis*-cleavage and ligation. In addition, substrate sequences to the 3' side of the cleavage site that are selected as active need only to have undergone a single ligation step, because the sequences present in the initial transcripts are irretrievably lost during the cleavage event in each round of selection. Nevertheless, all of the essential predictions arising from the selection experiments (Figs. 3A and 4A) were confirmed by mutational analysis (Figs. 5 and 6) and activity assays results using mismatched substrates. Therefore, our results show that in vitro selection represents a powerful tool

for the analysis of ribozyme–substrate interactions. We are currently using this method to increase the activity of ribozymes targeted to specific viral RNA sequences.

How selective is the hairpin ribozyme for its target? Our experiments demonstrate that the ribozyme can cleave some, but not all, targets containing single-base mismatches. The ribozyme exhibits the highest selectivity for properly base-paired structures at 6 bp. These sites represent the 3 bp proximal to the cleavage site in both helices 1 and 2. Mismatches within the helices at positions distal to the cleavage site are more highly tolerated by the ribozyme, although kinetic analysis showed that mismatches at those positions where the ribozyme is most highly tolerant ($A_1 \cdot u_{+9}$ and $A_{14} \cdot u_{-5}$) still resulted in a twofold decrease in cleavage efficiency. In all, eight positions within the substrate are strongly discriminated by the ribozyme (substrate positions -4 , -3 , -2 , $+1$, $+4$, $+5$, $+6$, and $+7$). Furthermore, at low $MgCl_2$ concentration the hairpin ribozyme can discriminate significantly between a $c_{-2} \cdot G_{11}$ base pair and a $u_{-2} \cdot G_{11}$ wobble pair (Fig. 5C). This is consistent with results from in vitro selection of helix 2 under high stringency (low magnesium conditions), where all of the active variants had a $c_{-2} \cdot G_{11}$ base pair.

Our results contrast with studies of the *Tetrahymena* group I ribozyme using mismatched substrates. This work showed that mismatches can enhance the cleavage efficiency under multiple turnover conditions (Zaug et al. 1988; Herschlag and Cech 1990b), because product dissociation is rate-limiting when the normal substrate is used (Herschlag and Cech 1990a). Because product dissociation is not rate-limiting for the hairpin ribozyme cleavage reaction under the conditions used, mismatches in helices 1 and 2 reduce the cleavage rate.

Our work shows that the hairpin ribozyme is highly selective. We conservatively estimate that the frequency of occurrence of an efficiently usable substrate site for a ribozyme with a fixed target sequence is equal to or less than one site per 100 kb. This estimate was made assuming an unstructured RNA molecule containing equal frequencies of all 4 bases. RNA structure and the occlusion of recognition sites by bound proteins are expected to reduce this frequency substantially (Fedor and Uhlenbeck 1990). Although the substrate selectivity of the more widely used hammerhead ribozyme (Symons 1992) has not been studied as rigorously as that of the hairpin,

Figure 7. Substrate selection rules for the hairpin ribozyme. The substrate selection rules for the hairpin ribozyme as determined by mutational analysis and in vitro selection strategies. N is A, U, G, or C; H is A, U, or C; B is U, C, or G; V is A, C, or G; Y is U or C; and R is A or G.

the selectivities of the two ribozymes are likely to be similar, as each ribozyme uses two intermolecular RNA duplexes flanking the cleavage site. Thus, the hairpin ribozyme is selective enough that *in vivo* applications should be feasible and so this ribozyme may prove to be an important tool for *in vivo* RNA inactivation studies.

Materials and methods

Plasmids and bacterial strains

Plasmid pGEM3Zf(–) (Promega) was used for cloning cDNA from both active and inactive molecules. The *Escherichia coli* strain DH5 α (F, *endA1*, *hsdR17*, *supE44*, *thy-1*, *recA1*, *gyrA96*, *relA1*, (Δ *argF-lacZYA*)U196, 080 Δ *lacZM15*) was used as host for the individual clones.

Oligonucleotides and mutagenesis

All DNA and chimeric DNA–RNA oligonucleotides were synthesized using standard solid-phase phosphoramidite chemistry on an Applied Biosystems 392 DNA–RNA synthesizer and were purified as described (Scaringe et al. 1990; Chowrira and Burke 1991). Ribonucleoside phosphoramidites were obtained from Glen Research.

Randomization of selected sequences was obtained by incorporation of equimolar quantities of the four DNA or RNA monomers, as appropriate, during chemical synthesis (Berzal-Herranz et al. 1992). Mutagenesis of the ribozyme–substrate complex was also carried out using contaminated phosphoramidites. Inactive molecules were selected following mutagenesis to give approximately one mutation per molecule. For mutagenesis of the ribozyme (50 nucleotides), this level of mutagenesis was obtained by mixing DNA monomers such that 0.66% contamination with each of the three mutagenic nucleotides was obtained. For mutagenesis of the substrate sequence (14 nucleotides), DNA monomers were mixed such that 2.3% contamination with each of the three mutagenic nucleotides was obtained.

Active molecules were selected following mutagenesis to give approximately five mutations per molecule. This level of mutagenesis was obtained by mixing monomers such that 3.3% contamination with each of the three mutagenic nucleotides was obtained for ribozyme sequence. For mutagenesis of substrate, RNA monomers were mixed such that 12% contamination with each of the three mutagenic nucleotides was obtained.

In vitro selection

The template for transcription of variant pools of self-cleaving molecules was assembled as described (Berzal-Herranz et al. 1992). The template for selection following mutagenesis had primer binding site 4 (PBS 4, 5'-GCGTGATCACCAAGCTTG-CG-3') linking the ribozyme and substrate instead of the pentacytidine linker (Fig. 1A,B). PBS 4 has *Bcl*I and *Hind*III restriction enzyme sites, which allow ribozyme activity assays to be performed *in trans*. Transcription of the templates, RNA self-cleavage, and purification of both the active and inactive variants were carried out as described (Fig. 1B) (Berzal-Herranz et al. 1992). RNA-catalyzed ligation was done by incubating the 5'-cleavage product containing the ribozyme and the 5'-substrate fragment (terminating with a 2',3'-cyclic phosphate) with a large excess (100 pmoles) of a ligation substrate at 4°C in cleavage–ligation buffer for 20 min (Berzal-Herranz et al. 1992). Reactions were terminated by the addition of an equal volume of

loading buffer (92% formamide, 17 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol). Ligation products were gel purified. The ligation substrate for helix 2 selection was a mixed RNA–DNA polymer containing the sequence corresponding to the 3'-cleavage product and PBS 3 (5'-GUCCU-GUUUCGACAGATCTCTAGACGC-3', with ribonucleotides in italics and deoxyribonucleotides in roman type). In helix 1 selection, the ligation substrate was randomized from position +4 to +9 (5'-GUCNNNNNNCGACAGATCTCTAGACGC-3'). For selection following mutagenesis, the ligation substrate was randomized from position +1 to +3 and had a different PBS, PBS 6 (5'-NNNCUGUUUAGATATCCGTCGACAAG-3'). Amplification, sequencing of active and inactive variants, and ribozyme self-cleavage assays were performed as described (Berzal-Herranz et al. 1992).

High-stringency *in vitro* selections were performed by carrying out the transcription and self-cleavage reactions in transcription buffer containing 6 mM MgCl₂ for 30 min; the ligation reactions were done in cleavage–ligation buffer containing 2 mM MgCl₂ for 10 min after the RNAs were renatured for 3 min on ice.

Trans-cleavage reactions

The RNAs were synthesized by *in vitro* transcription, internally labeled by including 50 μ Ci of [α -³²P]CTP in the transcription mixture, and gel-purified as described (Chowrira and Burke 1991). The ribozyme and substrate were separately denatured by heating to 90°C and renatured by rapid cooling on ice for 30 min in standard reaction buffer (12 mM MgCl₂, 2 mM spermidine, 40 mM Tris at pH 7.5). Reactions were initiated by mixing ribozyme and substrate on ice and incubating at 37°C for 30 min. Each reaction contained 0.01 μ M ribozyme and 0.05 μ M substrate in a volume of 10 μ l. Reactions were stopped by adding an equal volume of loading buffer. Reactions were analyzed on 7 M urea–20% polyacrylamide gel, dried, and directly quantitated using a Betascan radioanalytic imaging instrument (Betagen).

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