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_4c, u_5a, g_7c : u_5a) were cleaved with reduced efficiency, whereas those with mismatches proximal to the cleavage site (c_2a, a_3c, c_2a : a_3c) were not cut. Analogous results were obtained for helix 1, where mismatches distal from the cleavage site (u_7a, u_8a, u_7a : u_9a) were used much more efficiently than those proximal to the cleavage site (c_4a, u_5a, g_6c, c_4a : u_5a : g_6c). 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 · G_6, c_2 · G_11, and a_3 · 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 · G_6 and c_2 · 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)IU/C)NSG(A/U/C)IU/C)(G/U/C)NNNNN-3' with high efficiency.

[Key Words: Catalytic RNA; RNA structure; catalysis]

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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 Ferrota 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...
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_{2}a:a_{3}C] was observed, whereas a substrate with an analogous mismatch distal to the cleavage site [g_{4}C:u_{5}a] 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_{+9}a:u_{+8}a:u_{+7}a] 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 [c_{4}C:u_{+5}a:C_{+4}a].

Single-base mismatches were also generated, in which G and A were changed to C, generating C:C and U:C 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_{2}a:a_{3}C] was observed, whereas a substrate with an analogous mismatch distal to the cleavage site [g_{4}C:u_{5}a] 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_{+9}a:u_{+8}a:u_{+7}a] 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 [c_{4}C:u_{+5}a:C_{+4}a].

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| Substrate | \(K_M\) (\(\mu\)M) | \(k_{cat}\) (/min) | \(k_{cat}/K_M\) (min/\(\mu\)M) | Relative \(k_{cat}/K_M\) |
|-----------|----------------|-----------------|-----------------|-----------------|
| Wild type | 0.042          | 2.2             | 52              | 1.0             |
| u_{+9}a:u_{+8}a:u_{+7}a | 1.5            | 2.9             | 1.9             | <0.005          | <0.0001       |
| c_{4}a:a_{5}a:u_{+7}a | 2.4            | 0.08            | 0.03            | 0.001           |
| g_{4}C:u_{+5}a:u_{+4}a | 0.080          | 2.5             | 31              | 0.6             |
| u_{+5}a:u_{+4}a:u_{+3}a | 0.059          | 1.5             | 26              | 0.5             |

Concentrations for u_{+9}a:u_{+8}a:u_{+7}a and g_{4}C:u_{+5}a were 0.02-5.0 \(\mu\)M and 0.01-6.0 \(\mu\)M, respectively. The ribozyme concentration was 0.002 \(\mu\)M. Concentrations for wild type, u_{+5}a, and u_{+4}a were 0.001-0.2 \(\mu\)M, and ribozyme concentration was 0.002 \(\mu\)M.
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 c₁₂ a₄ u₄ a₂ and g₄ c and by helix 2 variants c₁₂ a₃ c₃ and g₄ 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 u₄ a substitution reduced cleavage activity by a factor of three. Note that the time point chosen (30 min) is long compared with t₁/₂ 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 (a₁₂, a₁₄ and a₁, A₁) 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 x 10⁷ sequences] and in a separate experiment randomizing all eight positions of helix 2 (7 x 10⁴ 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·G₁₁, a⁻₃·U₁₂, and g⁻₄·C₁₃.

At c⁻₂·G₁₁, 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·G₁₁ 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·G₁₁, a⁻₃·U₁₂, g⁻₄·C₁₃ 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·G₁₁ 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·U₁₂, with g⁻₄·C₁₃ 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·C₁₃, but mismatches and wobble pairs are found more frequently than at the two preceding positions. At positions corresponding to u⁻₅·A₁₄, 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·G₁₁ 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-

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Figure 3. In vitro selection of active molecules after randomization of helix 2. (A) Base-pair preferences among active variants. The bar graph indicates the frequency of occurrence of individual base pairs in the population studied (119 active clones and 20 inactive clones). The wild-type sequence and the derived consensus sequence of helix 2 are shown. (B) Individual clones obtained by selection. The clones are classified based on their activity in the self-cleavage assay. The helix 2 regions from the three naturally occurring hairpin ribozyme sequences are shown, sTRSV, sARMV, and sCYMV are the self-cleaving sequences derived from the minus strand of satellite of tobacco ringspot virus, arabis mosaic virus, and chicory yellow mottle virus, respectively (Rubino et al. 1990). Sequences that differ from sTRSV are italicized. (C) Self-cleavage assay for individual clones from selection. Transcription and self-cleavage assays were performed as described in Materials and methods. (Pre-RNA) Full-length precursor RNA; (5'P and 3'P) 5'- and 3'-cleavage products. Lane numbers correspond to sequences of the following clones 402 (1), 405 (2), 406 (3), 409 (4), 411 (5), 416 (6), 418 (7), 423 (8), 425 (9), 431 (10), 438 (11), 444 (12), 445 (13), 448 (14), and 452 (15). An autoradiograph of a 10% denaturing polyacrylamide-urea gel is shown.

Random mutagenesis of ribozyme–substrate complex

The ribozyme–substrate complex was randomly mutagenized, in vitro selection was carried out, and clones with a wide range of self-cleavage activity were characterized by sequencing and self-cleavage analysis [data not shown]. Results obtained are consistent with in vitro selection following randomization of helices 1 and 2. A single clone (clone 159; Fig. 3B) was obtained that contained the wild-type sequence, except for the insertion of an additional G at G11. RNA transcripts of this clone showed no self-cleavage activity. This result suggests that additional nucleotides at the boundary between junction 1/2 (J1/2) and helix 2 may not be tolerated.

Mutational analysis of c−2 · G11 and c+4 · G6

Both lines of experiments described above—analysis of mismatched substrates and in vitro selection experiments—indicated that the base pair in helix 2 proximal to the cleavage–ligation site, involving substrate position −2 and ribozyme position 11, was the most important base pair within helix 2. The selection results provide information concerning the optimal sequence for sequential cleavage and ligation reactions of the self-cleaving construct. To confirm these results and to determine which substrates could be cleaved efficiently in trans, we constructed ribozymes containing each of the 4 bases at position 11 and substrates containing each of the 4 bases at position −2 so that the relative activity of all 16 possible base combinations at these positions could be tested. Cleavage products were observed in only 4 of the 16 nucleotide combinations [Fig. 5A,B]. For the reactive combinations, the order of observed activity was c·G > u·G > u·A > g·C. The finding that c−2 · G11 and u−2 · G11 are the optimal base combinations confirms the results obtained by in vitro selection. The extent of cleavage of the c−u mutant substrate by the
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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_{-2}a mutant substrate even at high Mg^{2+} concentrations (Fig. 5C).

All 16-bp combinations were also tested at the position of helix 1 closest to the cleavage site (c_{+4} G; 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 (Chowrrira et al. 1991; Berzal-Herranz et al. 1992) indicate that hairpin ribozymes can be engineered to efficiently cleave RNA sequences containing the sequence 5'-nnrynSghybnnnnnn-3' (Fig. 7). These changes are accom-
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 irrevocably 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}_{-4} 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 MgCl₂ 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 1 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 pGEM3Z[−] [Promega] was used for cloning cDNA from both active and inactive molecules. The Escherichia coli strain DH5α [F, endA1, hsdR17, supE44, thy-1, recA1, gyrA96, relA1, (argF-lacZΔM15)] 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 [Scharinge 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-

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loading buffer [92% formamide, 17 mM EDTA, 0.025% bromphenol 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-GUUUCGGCAGATCCTAGACCG-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'-GUCNNNNNNCGCAGATCCTAGACCG-3']. For selection following mutagenesis, the ligation substrate was randomized from position +1 to +3 and had a different PBS, PBS 6 [5'-NNNCUGUUGUAGATATCCGCAAGA-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 MgC2 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 μCi 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 μM ribozyme and 0.05 μM substrate in a volume of 10 μL. 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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