Short Technical Reports

Screening for Important Base Identities in the Hairpin Ribozyme by In Vitro Selection for Cleavage

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ABSTRACT

Random mutagenesis followed by an in vitro selection procedure was shown to be capable of identifying important bases of the hairpin ribozyme for cleavage of an RNA target sequence. The selection scheme enriched the RNA population for those molecules capable of efficient site-specific self-cleavage in the absence of ligation. Cleavable mutants were selected for all positions in loop 4 except for position A_{38}, supporting the notion that A_{38} is an important base in the hairpin ribozyme. This has been confirmed by direct mutagenesis, validating the utility of this procedure. Thus, the method developed and reported here has utility for the selection of efficient hairpin ribozymes capable of highly efficient cleavage of a substrate RNA without a requirement for ribozyme-catalyzed ligation, conditions desired for many applications of catalytic RNA such as gene therapy.

INTRODUCTION

In vitro selection is a useful method to screen for structure-function relationships in RNA. It has been effective in identifying both standard and non-standard base interactions. The observation that two base positions co-vary indicates that the co-varying bases can interact with each other. The type of interaction can be deduced by the analysis of conservation of potential interactions between the bases. Selection schemes optimizing for cleaving RNA populations have previously been applied to the *Tetrahymena* ribozyme (4), hammerhead ribozyme (5,7,11) and RNase P RNA (13), and have recently been reviewed by Williams and Bartel (12).

No previous random mutagenesis/selection schemes requiring only cleavage by the hairpin ribozyme have been reported. A selection scheme for both cleavage and subsequent ligation by the hairpin ribozyme identified both required and non-required bases for these two combined activities (2). However, other reports of assays requiring only cleavage showed significant levels of cleavage activity with base substitutions determined to be inactivating based on cleavage/ligation selection (1,10). In addition, ribozymes have yielded activity when propyl linkers and abasic substitutions were incorporated at these sites (9).

For the purpose of identifying required bases and possible base interactions in the hairpin ribozyme for cleavage only, it was necessary to develop a mutagenesis/selection method for the hairpin ribozyme based only on cleavage. We have developed such a random mutagenesis/selection method. Following mutagenesis, this procedure uses the general scheme of cyclic selection/amplification—however, it is uniquely applied to the hairpin ribozyme. A template double-stranded (ds)DNA with T7 promoter (6) coding for the autocatalytic hairpin ribozyme with selected sites of random mutagenesis was transcribed, and the 3′ cleavage product was isolated, reverse-transcribed and polymerase chain reaction (PCR)-amplified with appropriate primers to restore the dsDNA template for the next generation. The method was tested by randomizing all nine bases in loop 4 of the *cis*-cleaving hairpin ribozyme, followed by selection for sequences that carried out the cleavage reaction. Previ-

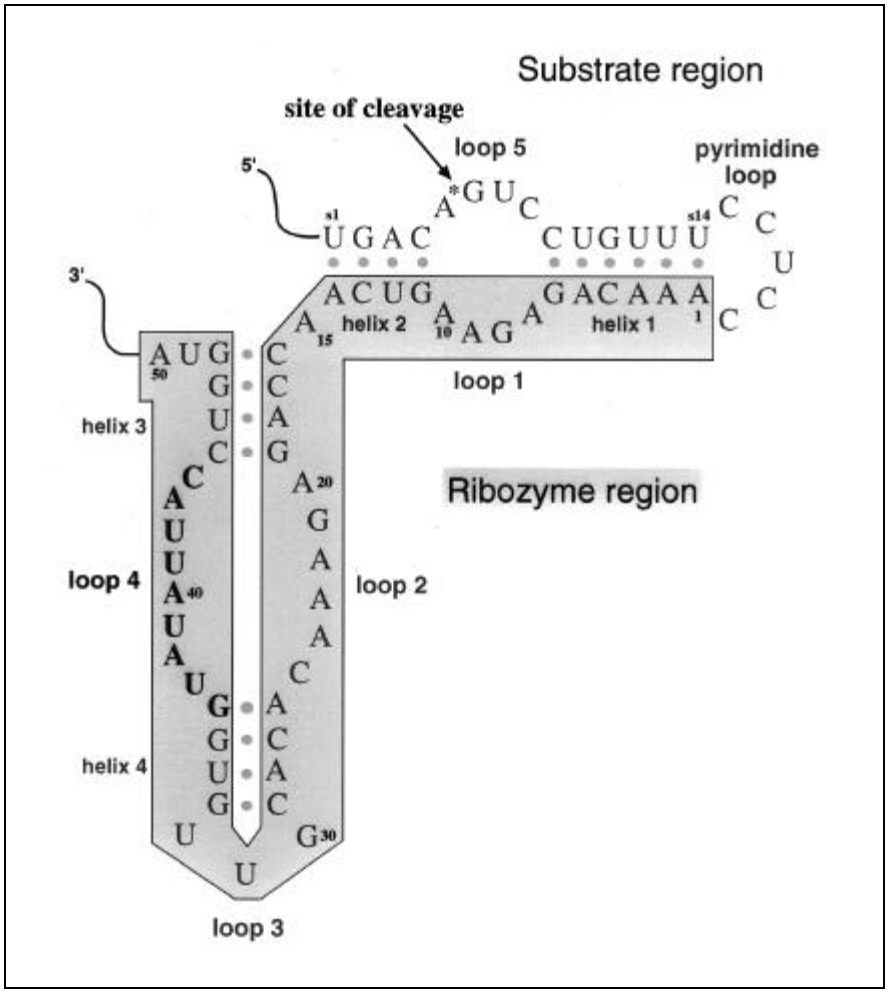

Figure 1. *Cis*-cleaving hairpin ribozyme-substrate cassette. The ribozyme is tethered to the substrate by a pentapyrimidine loop, allowing the molecule to undergo *cis* cleavage. Substrate bases s1–s14 and ribozyme bases 1–50 correspond to the native sequence minimum catalytic structure of the hairpin ribozyme found in the minus strand of the satellite RNA of tobacco ringspot virus [(-)sTRSV] (3).

ously reported experiments have shown that RNAs produced using a similar construct to the one used here were unable to carry out either *trans* cleavage or *trans* ligation to a detectable level. Thus, selected molecules are represented solely on the basis of their *cis* cleavage activity (10). The method yielded an array of clones with a frequency of base replacements that closely corresponded to catalytic activity of base mutants obtained by site-specific mutagenesis (10). Thus, this method identified important base identities of the hairpin ribozyme for cleavage.

Table 1. Primer Sequences

G_0 ssDNA template strand:

AGCTTGCATGCCTGCAGGTCGACTACCAGNNNNNNNNNCACAAC-
GTGTGTTTCTCTGGTTGACTTCTCTGTTTGGAGGAAACAGGACTG-
TCAGGATCCCTATAGTGAGTCGTATTA*

Selection primers:

Primer 1:

TAATACGACTCACTATAGGGATCCTTTTTTTTTTTTTGACAGTCC-
TGTTTCCTCCAAACAGAG

Primer 2:

GCCGAAGCTTGCATGCCTGCAGGTCGAC

*N = any nucleotide

MATERIALS AND METHODS

Oligodeoxyribonucleotide Synthesis and Purification

Template and primer DNA strands were synthesized on a Model 392 DNA/RNA Synthesizer (PE Applied Biosystems, Foster City, CA, USA). Random bases were incorporated into the DNA template by drawing from a fifth phosphoramidite bottle in which an equimolar amount of each base was combined. All synthesis products were purified using a Model 152A HPLC System with an RP-300 reverse-phase column (PE Applied Biosystems). Reverse transcription (RT) primer and PCR primers were gel-purified and the DNA visualized with UV shadowing, extracted and resuspended in H_2O to a final concentration of 20 µM for PCR primers (primers 1 and 2) and 100 pM for the RT primer (primer 2). Primer sequences are listed in Table 1.

PCR Amplification

PCR amplification was accomplished using a GeneAmp® PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA) by standard procedures. G_0 dsDNA was made by combining G_0 single-stranded (ss)DNA template strand (5 nM), primers 1 and 2 (1 µM each), 200 µM each dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 2 mM $MgCl_2$, 1× Assay Buffer B (10 mM Tris-HCl, pH 8.3 [at 25°C], 50 mM KCl) and 25 mM $MgCl_2$ solution and 5 U *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA, USA) in a final volume of 100 µL. The reaction went

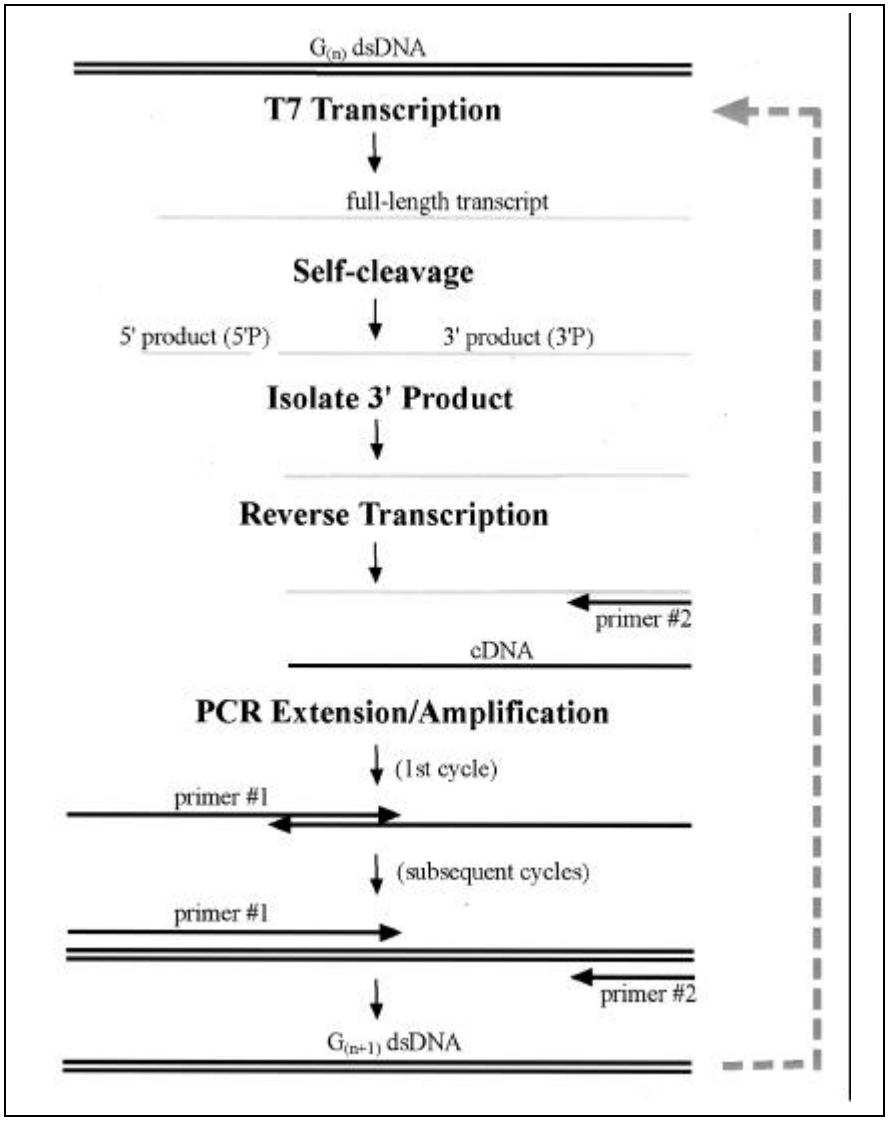

Figure 2. In vitro selection/amplification for enrichment of self-cleaving molecules. The G_n dsDNA was transcribed, the resulting RNA allowed to self-cleave and the 92-nt 3′ cleavage product containing the randomized loop 4 bases isolated. This was reverse-transcribed using primer 2 and PCR-amplified with both primers 1 and 2 to obtain the next generation [$G_{(n+1)}$] of loop 4 selection mutants. This cycle of transcription, RT and PCR amplification was repeated through four generations.

for 30 cycles, each for 30 s at 95°C, 30 s at 55°C and 120 s at 72°C. The full-size amplification product was isolated from a 3% agarose gel, phenol/chloroform-extracted and ethanol-precipitated. The resulting DNA was resuspended in 20 µL H_2O. PCR amplification of cDNA used 5 µL of RT-generated cDNA and was also done using primers 1 and 2.

In Vitro Transcription

Transcription reactions were carried out at 37°C in 50 µL of 250 ng DNA, 40 mM Tris, pH 8.0, 6 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM spermidine, 4% polyethylene glycol (PEG)-3000, 0.1% Triton® X-100, 1 mM each NTP, 10–20 µCi [α-^{32}P]CTP, 40 U RNasin® (Promega, Madison, WI, USA) and 20 U T7 RNA polymerase (Ambion, Austin, TX, USA). Two units of RNase-free DNase (Ambion) were added after 3 h and the incubation continued at 37°C for 30 min. An additional 5 µg of DNA (T3 primer), whose sequence was unrelated to the template DNA, were added with two more units of DNase, and the resulting solution was transferred to a new tube and reincubated at 37°C for 30 min. RNA was ethanol-precipitated, resuspended in 8 µL H_2O and denatured by adding 8 µL 98% formamide dye and heating to 90°C for 2 min. The sample was then snap-cooled on ice and the 3′ product was separated from the unreacted ribozymes by denaturing polyacrylamide gel electrophoresis (PAGE). The 3′ product was excised from the gel and macerated in 500 µL RNA extraction buffer consisting of 0.5 M ammonium acetate, 0.5 mg/mL sodium dodecyl sulfate (SDS) and 2 mM Na_2EDTA, shaken for 1 h and centrifuged at 14 000× *g* for 10 min. The supernatant was ethanol-precipitated, and the pellet was washed with 70% ethanol, vacuum-dried and resuspended in 20 µL H_2O.

Reverse Transcription

Primer 2 (4.75 pM) was annealed to 2.5 µL of 3′ product in 21 µL H_2O by heating the solution to 90°C and allowing it to cool to 45°C over 30 min. The resulting annealed RNA was added to 29 µL of RT buffer consisting of 35 mM Tris, pH 8.3, 35 mM KCl, 10 mM $MgCl_2$, 17 mM DTT and 860 µM each dNTP. To the RNA/buffer mixture, 15 U AMV reverse transcriptase (Amersham, Arlington Heights, IL, USA) were added, and the reaction was incubated at 48°C for 1 h. The cDNA was phenol/chloroform-extracted, ethanol-precipitated, dissolved in 20 µL H_2O and PCR-amplified as described above.

Cloning and Sequencing

After four selection cycles following the protocol outlined in Figure 2, the G_4 dsDNA was digested with *Bam*HI and *Hin*dIII, cloned into pBluescript®

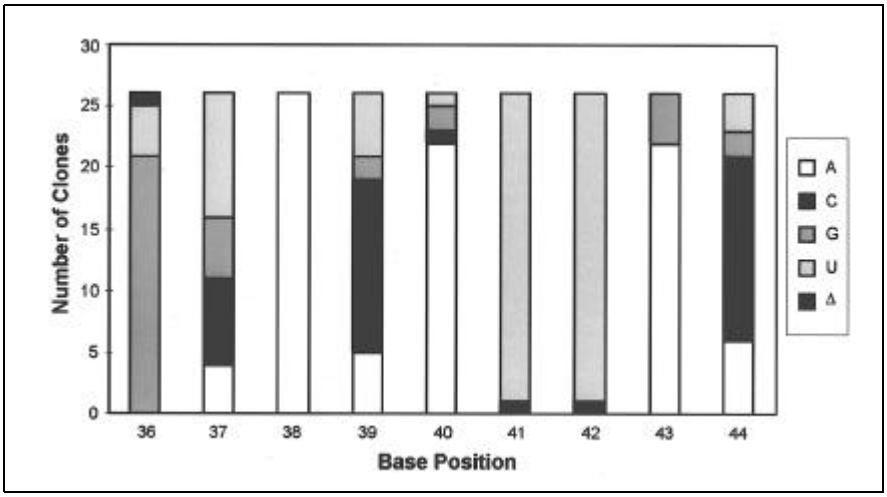

Figure 3. Frequency of selected base identities at each of the nine positions within loop 4. The sequence pool used represents the 26 different selected clones. A Δ indicates the nucleotide has been deleted from the specified position.

Table 2. Loop 4 Sequences Retrieved from Random Mutagenesis/Selection

Clone	Base Identity										
Position	36	37	38	39	40	41	42	43	44	Mutations	%
wt	G	U	A	U	A	U	U	A	C	Elsewhere	Cleavage
8*	-	C	-	C	-	-	-	-	-		95
16*	-	-	-	-	-	-	-	-	-		92
9	-	G	-	C	-	-	-	-	-		86
42	U	C	-	-	-	-	-	-	-		76
10	U	C	-	C	-	-	-	-	-	$C_{13}U$	64
7	-	G	-	C	-	-	-	-	A	$U_{32}C$	48
47	-	-	-	A	-	-	-	-	U	$A_{15}G$	46
3	U	C	-	C	-	-	-	-	A		40
21	-	A	-	C	-	-	-	-	A		35
32	U	A	-	-	-	-	-	-	-		31
18	-	C	-	C	U	-	-	-	-	$U_{32}C$	25
23	-	G	-	C	-	-	-	-	U		25
28	-	A	-	A	-	-	-	-	U		23
48	-	-	-	G	-	-	-	-	G		22
5	-	G	-	A	-	-	-	-	G	$A_{20}G$	20
15	-	-	-	C	C	-	-	-	-		19
30	-	-	-	C	-	C	-	-	-		19
33	-	-	-	C	-	-	-	-	A	$G_{19}A$	15
20	-	-	-	A	G	-	-	-	-		10
40	-	A	-	A	G	-	-	-	-		10
41	-	C	-	-	-	-	C	-	-		7
2	-	-	-	C	-	-	-	G	A		6
25	-	G	-	-	-	-	-	-	A		5
17	-	-	-	C	-	-	-	G	-		4
26*	Δ	C	-	C	-	-	-	G	-		3
31	-	-	-	G	-	-	-	G	-		2

*sequence found in two clones

Identity of selected bases in loop 4. Listed is each catalytically active loop 4 sequence selected as well as point mutations found elsewhere in the ribozyme-substrate transcript and the self-cleavage percentage attained during the one-hour transcription. A Δ indicates the nucleotide has been deleted from the specified position. Base positions 36–44 are represented by columns labeled along the horizontal axis.

II KS (Stratagene, La Jolla, CA, USA) using standard cloning procedures (8) and used to transform DH5α™ bacteria (Life Technologies, Gaithersburg, MD, USA), and individual colonies were sequenced using Sequenase® Version 2.0 (Amersham) according to the manufacturer's protocol.

Quantitation of Self-Cleavage from Selected Ribozymes

Plasmids were linearized with *Hin*dIII, phenol/chloroform-extracted, ethanol-precipitated and transcribed as above for 1 rather than 3 h, and the DNase step was omitted. Full-size transcripts and both cleavage products were excised from the gel, the radioactivity quantified and the extent of self-cleavage determined.

RESULTS

The autocatalytic hairpin ribozyme used for these studies had the substrate and ribozyme linked by a CCUCC loop (Figure 1). The mutagenesis/selection scheme (Figure 2) for cleavage of the hairpin riboyzme is summarized as follows. The original transcription template (G_0 dsDNA) contained the T7 RNA polymerase promoter (8) followed by the coding region for the autocatalytic ribozyme with randomized bases in loop 4 at positions G_{36}–C_{44}. The starting dsDNA population consisted of 4^9 (over 250 000) different sequence variants. The G_0 dsDNA was transcribed with T7 RNA polymerase so that the amount of DNA used for transcription ensured that an average of over 10^6 coding sequences were present for each individual sequence variant, resulting in a high probability that

each variant was represented in the initial population. During transcription, those molecules containing sequences in their randomized regions that supported catalysis self-cleaved resulting in two product fragments, a 24-nucleotide (nt) 5′ product and a 92-nt 3′ product. The 3′ product containing the ribozyme region was excised and the RNA reverse-transcribed. Products from the initial population of transcribed RNA were present at a level lower than could be detected. An end-labeled oligodeoxyribonucleotide that co-migrated with the product band was therefore used to identify the region of the gel from which to isolate the desired RNA product from the G_0 transcript population. The resulting cDNA was amplified using the RT primer and a primer that reestablished the T7 promoter region as well as that portion of the substrate region that was previously lost as a result of self-cleavage. The resulting PCR product was used as a template for the next generation. This cycle of transcription, RT and PCR amplification was continued for four generations (G_4), at which point the population exhibited greater than 50% self-cleavage. The PCR products from G_4 were cloned into a plasmid, self-cleaving activities assayed and sequences determined.

Of 29 clones isolated from the G_4 pool that supported self-cleavage, 26 different sequences were found (Table 2 and Figure 3). With the exception of A_{38}, no position in loop 4 showed absolute conservation of a single-base identity in all catalytically active clones. Variant clones corresponding to position G_{36} had only U in this position, consistent with this position being paired to A_{26} by either an A_{26}:G_{36} base pair or, in the variants, an A_{26}:U_{36} base pair. Both U_{37} and U_{39} showed all possible bases retrieved from random mutagenesis/selection, consistent with these being variable positions. No variant clones were identified at position 38, consistent with this being a completely conserved base. Clones isolated with variant bases at positions A_{40}–A_{43} closely reflect acceptable base substitutions at these positions. All variant clones were isolated at position C_{44}—again supporting this as a variable position.

Comparative analysis of the 26 different sequences obtained indicated that no compensatory mutations among bases within the randomized region have occurred. Thus, it is unlikely that additional base pairing exists between bases of loop 4.

DISCUSSION

Both in vitro selection and mutational analysis can provide information about specific base requirements for catalytic activity. The methods developed in this paper for identification of bases required for cleavage activity using random mutation/selection yielded results that are consistent with those obtained by site-specific mutational analysis of these same nine bases in loop 4 of the hairpin ribozyme (10). Specifically, by site-specific mutational analysis, position G_{36} was shown to interact with A_{26}. This non-canonical A:G base pair could be replaced with a Watson/Crick canonical base pair with substantial retention of activity. This explains the recovery of mutant clones in this study, which had a U in position 36. The U could pair with A_{26} in a Watson/Crick canonical base pair. Site-specific mutational analysis also showed that U_{37} and U_{39} were catalytically active for all base replacements. The results of random mutation/selection studies are consistent with this, because mutant clones were recovered that had all possible base replacements in these two positions. When any of the other three bases were put at position A_{38} by site-specific mutational analysis, no detectable activity was observed—a result supported by random mutagenesis studies in which no mutant clones were recovered at this position. Thus, this is an important base. The positions A_{40}, U_{41}, U_{42} and A_{43} had variable activity depending on the base replacement when mutated by site-specific mutation—again, consistent with results obtained by random mutagenesis. The variant clones isolated at position C_{44} closely follow base tolerances at this position, as determined previously from both nature (phylogenetic comparison) and mutational analysis (10). Thus, the methods developed here for selecting ribozymes based solely on their ability to catalyze phosphodiester

cleavage from a randomly mutagenized population yield results consistent with site-specific mutagenesis.

A selection technique requiring sequential cleavage and ligation has been previously applied to the hairpin ribozyme; however, this technique indicated that bases A_{38}, U_{41}, U_{42} and C_{44} were all required for cleavage/ligation (2). A thorough analysis of the base requirements in loop 4 for *cis* cleavage reported in this paper clearly shows that, except A_{38}, each base of loop 4 can be varied, and the ability to self-cleave is retained to some extent. Strong biases exist for the retention of U_{41} and U_{42}, but these bases are clearly not absolutely required for catalysis. The results obtained here using in vitro selection are in close agreement with those previously found by mutagenesis (10).

The sequences retrieved in this study have a wide range of catalytic activities. The three-hour transcription incubation time used to produce RNA for each generation allowed sequences supporting low cleavage rates to pass to the next generation. This lowered the selection pressure for higher cleavage efficiency while still maintaining the requirement for self-cleavage in order for a sequence to be represented in the following generation. The use of this scheme allows for inclusion of the largest diversity of bases in each position that permits self-cleavage to occur. Although no attempt was made to ensure that the transcription rate was linear throughout either the selection or self-cleavage characterization phases of the study, this does not detract from the results obtained because the goal of obtaining a diverse pool of loop 4 sequences capable of supporting self-cleavage was attained.

Random mutagenesis/selection only shows biases toward a specific sequence, and, given a larger sequence pool, one might find that a variant occurs in what otherwise would be interpreted as a required position (i.e., A_{38}). However, the power and accuracy of the random mutagenesis/selection method presented here are borne out by the fact that not a single clone varied in position A_{38}, a position shown to be invariant under identical reaction conditions by direct mutation (10).

A similar selection scheme has been used previously to select for cleavage mutants in the hammerhead ribozyme (11). Their method required an additional Sequenase-mediated polymerization step as well as utilization of GTPγS during transcription and subsequent mercury-PAGE purification of products as part of the procedure to remove deletion products that arose during the RT/amplification/transcription processes. Such modifications might prove advantageous to overcome problems if similar deletions should occur using the method reported here.

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