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# Alteration of Hairpin Ribozyme Specificity Utilizing PCR

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**We have developed a method by which a researcher can quickly alter the specificity of a *trans* hairpin ribozyme. Utilizing this PCR method, two oligonucleotides, and any target vector, new ribozyme template sequences can be generated without the synthesis of longer oligonucleotides. We have produced templates with altered specificity for both standard and modified (larger) ribozymes. After transcription, these ribozymes show specific cleavage activity with the new substrate β-glucuronidase (GUS), and no activity against the original substrate (HIV-1, 5′ leader sequence). Utilizing this technique, it is also possible to produce an inactive ribozyme that can be used as an antisense control. Applications of this procedure would provide a rapid and economical system for the assessment of *trans* ribozyme activity.**

The ability of catalytic RNA to specifically cleave and down-regulate viral or endogenous RNA holds great promise with respect to gene therapy. A number of laboratories are adapting and testing such catalytic RNA or ribozymes for use in both in vitro and in vivo systems.[1–4] Of particular interest to our laboratory is the hairpin ribozyme that is active at physiological conditions.[5,6] The only major targeting requirement is the presence of a BN*GUC sequence within the substrate, where B can be any standard nucleotide except an A; in this sequence, cleavage occurs at the asterisk. Hairpin ribozyme design therefore begins by identifying such BNGUC sites within a potential target RNA. Presently, however, there are few empirical rules for targeting domains with respect to increasing the efficiency of catalytic cleavage. Current hairpin design parameters include incorporating a 4-base complementary region 5′ to the NGUC and a 6- to 10-base complementary region on the 3′ of the NGUC sequence (Fig. 1). The optimal length of the base pairing on the 3′ end is determined by cleavage rates obtained from in vitro cleavage reactions. Using these rules and testing a number of hairpin ribozymes targeting many sequences, we have occasionally designed hairpin ribozymes that show little if any in vitro cleavage activity. Presently, there is no way to predict which target sites show decreased activity. In such cases, we need to change the ribozyme target site, transcribe new ribozyme, and determine its activity. We have therefore developed a method based on PCR by which one could rapidly alter the targeting domains of a hairpin ribozyme from one sequence to another. As a result, researchers can change hairpin ribozyme targets with great efficiency. In addition, we have designed an alternative method by which a mutant ribozyme (noncleaving ribozyme) can be produced to measure antisense inhibition versus cleavage effects of the ribozyme for in vivo applications.

## MATERIALS AND METHODS

### Target Plasmids

The plasmids used in these experiments contained hairpin ribozymes (Fig. 2A–C) that were engineered to target specific domains within the human immunodeficiency virus (HIV) 5′ leader region (pRTHIV, pRTtHIV, pRTHR). Both pRTtHIV and pRTHIV constructs include a *cis*-cleaving hairpin cassette,[2] which is located 3′ to the *trans* ribozyme. To determine whether this PCR method could amplify larger, modified ribozymes, we also used as a PCR template pRTtHIV, which incorporates a 201-nucleotide tRNA-like sequence from brome mosaic virus (BMV) between the *trans* ribozyme and the *cis*-cleaving cassette.[8–10]

### Primer Design

The primers were synthesized on a 381A or 392 Applied Biosystems DNA synthesizer and purified by HPLC. A number of primers included restriction enzyme linkers (*Xba*I and *Sst*I), which were followed by GC clamps, whereas others included the T7 promoter sequence (see Table 1).

### PCR Conditions

We used the standard PCR reaction con-

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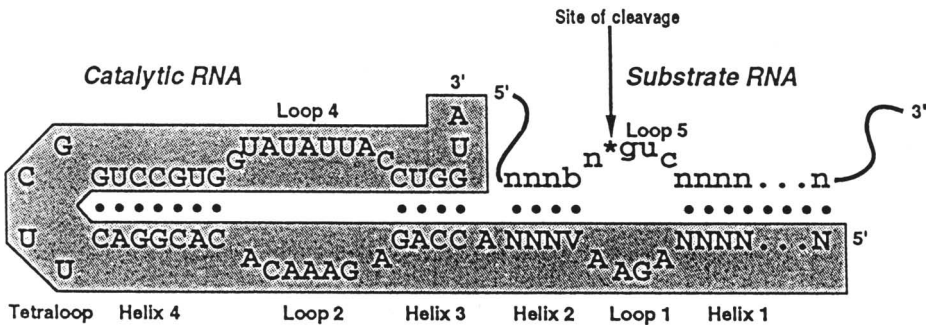


**FIGURE 1** Two-dimensional model of the tetraloop hairpin ribozyme. The ribozymes used in these studies differ from the native catalytic domain in helices 1 and 2 (targeting region) and also incorporate the addition of a 12-nucleotide sequence containing a stable tetraloop sequence,[7] which extends helix 4 and replaces loop 3.

ditions as described by the *Taq* polymerase manufacturer (Perkin-Elmer). Briefly, a 100-µl reaction included 0.1 µg of template, 100 mM each dNTP, 10 µl of PCR buffer II, 0.5 µM each primer, 2.5 units of AmpliTaq, and 2 mM $MgCl_2$. All PCR experiments were cycled at 95°C for 1 min, 53°C for 2 min, and 72°C for 3 min (with a 5-sec increase for each extension reaction) for 30 cycles.

### DNA Isolation

PCR products were isolated from a low-melting-point agarose gel, weighed, and digested with β-agarase enzyme as described by the manufacturer's instructions (New England Biolabs). The isolated products were digested overnight with *Xba*I and *Sst*I in a 50-µl reaction and cloned into a transcription vector (pTZ19U) cut with the same restriction enzymes using standard cloning techniques.[11]

### Transcription and Ribozyme Isolation

Plasmids were prepared for transcription by cutting with *Eco*RI, phenol extracted, and precipitated. The transcriptions were performed using the Ambion MAXIscript system. The conditions were as follows: In a 10-µl reaction with 0.5 µg of DNA template restricted with *Eco*RI, 1 µl each of 10 mM ATP, GTP, and UTP, 5 µl of 0.1 mM CTP, 0.5 µl of RNase inhibitor, 0.5 µl of T7 RNA polymerase, and 5 µl of [$^{32}$P]CTP. The reaction was carried out at 37°C for 2 hr. Reactions were stopped after 2 hr by the addition of formamide-loading dye. Samples were heated at 80°C for 2 min and cooled on ice for 2 min before loading on the gel. Ribozymes and substrates were isolated from 6.6% and 15% denaturing polyacrylamide gels, respectively. To extract the transcribed RNA, gel slices were ground in 300 µl of RNA extraction solution (0.5 M ammonium acetate, 0.5 mg/ml of SDS, 2 mM EDTA). The tubes were then vortexed for 1 hr and spun in a microcentrifuge tube for 20 min, and the supernatant was precipitated with 1 µl of glycogen (Boehringer Mannheim, 20 mg/ml) and 1 ml of ethanol. After an overnight precipitation at −20°C, the RNA was pelleted by centrifugation, and the resulting pellet was subsequently washed with 70% ethanol and dried. The RNA was then counted in a scintillation counter and quantitated.

### Cleavage Conditions

The ribozyme and substrate concentrations were adjusted to 0.02 and 0.04 µM, respectively. The cleavage reaction was performed as follows: A 2:1 ratio of substrate to ribozyme was obtained by adding 1 µl of ribozyme, 1 µl of substrate and 4 µl of water. From this solution, 3 µl was transferred into two new tubes to be used as 0- and 30-min time points. To the 0-time point tube, 4 µl of formamide dye was added. This was followed by the addition of 1 µl of 4× cleavage buffer (160 mM Tris at pH 7.5, 48 mM $MgCl_2$, 8 mM spermidine), to both the 0- and 30-min reactions. Incubation was then carried out for 30 min at 37°C for both tubes. After incubation, 4 µl of formamide dye was added to the 30-min tube and the products were separated on a 15% denaturing polyacrylamide gel.

## RESULTS

The hairpin ribozyme structure used in these studies includes four helices and three loops (Fig. 1). In vitro analysis has demonstrated that helices 1 and 2 can be changed such that the targeting of any mRNA is possible as long as it includes the BNGUC sequence.[12]

In these studies we have altered three plasmids, each containing a modified ribozyme (pRTHR, pTHIV, and pTtHIV). Contained within all these constructs was an HIV-1, 5′ *trans* ribozyme target[13,14] that we wanted to convert into a β-glucuronidase (GUS) mRNA targeting ribozyme. The plasmid pRTHIV contains a *trans* ribozyme sequence followed by a *cis* hairpin cassette (Fig. 2A), pRTtHIV included an additional 201-nucleotide tRNA-like sequence between a HIV *trans* ribozyme and the *cis* cassette (Fig. 2B), and pRTHR had only a *trans* ribozyme (Fig. 2C).

To design the 5′ PCR oligonucleotides, we needed to convert the information within a chosen mRNA target, in this case, GUS, a marker enzyme from *Escherichia coli*[15] on a genetic target (Fig. 3A). In this example, $B^4$ must be G, C, or U, but not A, which is based on previously established rules.[12] The GUS target follows these rules because $B^4$ is C. The next nucleotide downstream can be any standard nucleotide. The sequence GUC then follows. $N^1$–$N^4$ represent helix 2 and must remain 4 nucleotides in length. This is in contrast to helix 1, which can vary in size. To make the correct oligonucleotide, $N^1$ of the target is converted to the complement nucleotide of the ribozyme and replaces $N^1$ in the 5′ PCR oligonucleotide. This step is repeated for the rest of helix 2 ($N^1$–$N^4$) and helix 1 ($N^5$–$N^{10}$). The NGUC sequence between helix 1 and helix 2 is replaced by AGAA as needed to maintain the active loop 1 region (see Fig. 1) of the hairpin ribozyme.[12] As shown in Figure 3A, the 3′ end of the 5′ oligonucleotide has 17 bases of homology with helix 3 and loop 2 of the hairpin ribozyme.

Of equal interest to this study is the AAA sequence internal to the 17-nucleotide sequence, which was changed in another oligonucleotide (5′ mutant PCR oligonucleotide) to CGT (Fig. 3A). This mutation is known to prevent in vitro

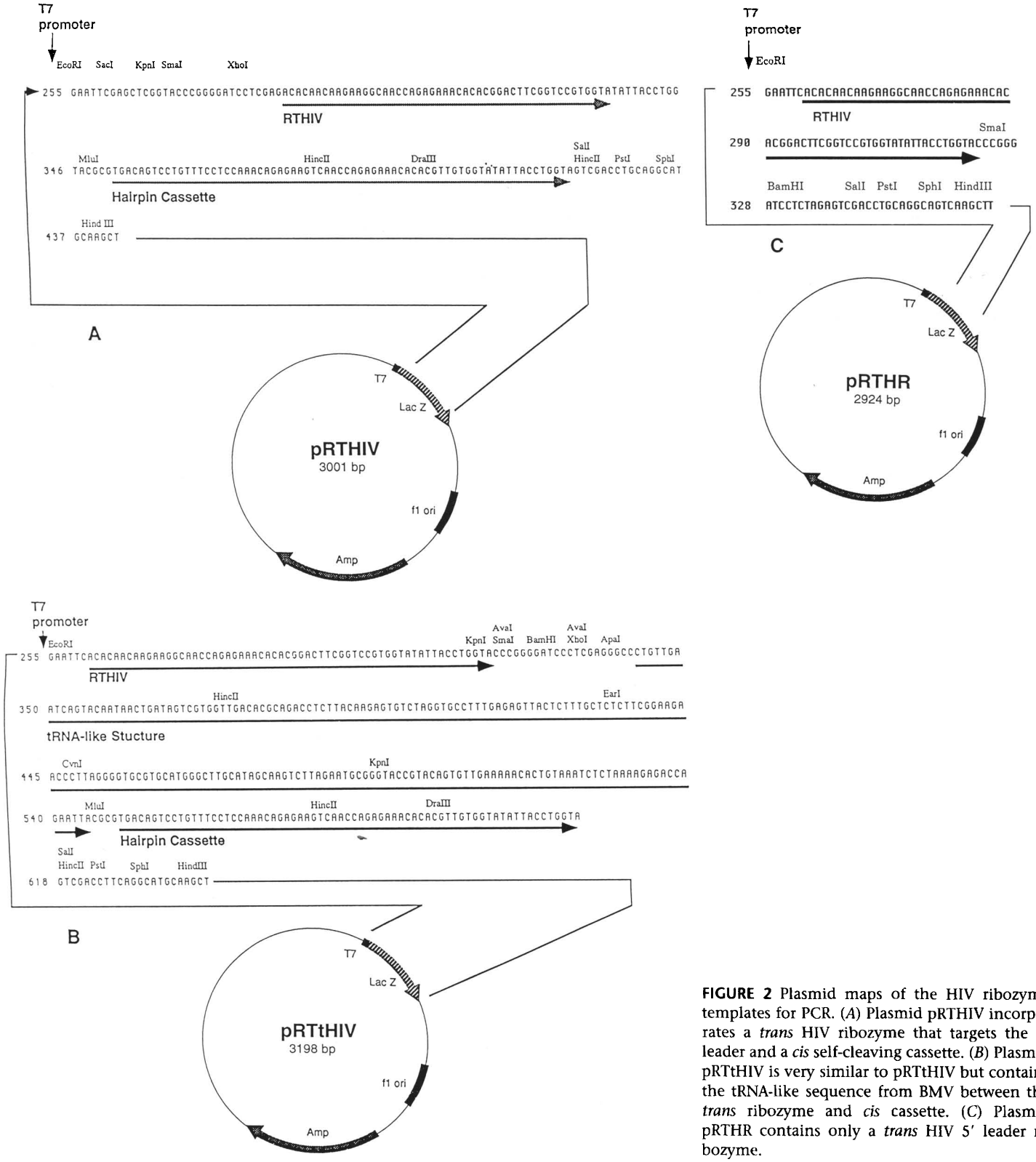


**FIGURE 2** Plasmid maps of the HIV ribozyme templates for PCR. (*A*) Plasmid pRTHIV incorporates a *trans* HIV ribozyme that targets the 5′ leader and a *cis* self-cleaving cassette. (*B*) Plasmid pRTtHIV is very similar to pRTtHIV but contains the tRNA-like sequence from BMV between the *trans* ribozyme and *cis* cassette. (*C*) Plasmid pRTHR contains only a *trans* HIV 5′ leader ribozyme.

activity and is, therefore, a good antisense control.[13] To clone any PCR fragments generated we have included a *Xba*I site on the 5′ end of both the 5′ active and mutant PCR oligonucleotides (Fig. 3B). Because the 3′ end of any *cis* or *trans* ribozyme is homologous, we designed the 3′ oligonucleotide to be complementary to the polylinker region just downstream from the *cis* cassette in pRTHIV and pRTtHIV, and to the *trans* ribozyme in pRTHR. This oligonucle-

**TABLE 1** Sequences of Oligomers Used for PCR of Ribozymes or Transcription of HIV or GUS Substrates

| Sequence | Oligomer |
|---|---|
| 5′-TAATACGACTCACTATA-3′ | T7 primer |
| 5′-GGGAGCTCCATGCCTGCAGGTCG-3′ | 3′ PCR |
| 5′-GGGTCTAGATGAGTAGAAGCATACCAGAGAAACACACGG-3′ | 5′ active PCR |
| 5′-GGGTCTAGATGAGTAGAAGCATACCAGAGCGTCACACGG-3′ | 5′ mutant PCR |
| 5′-ATGAGTGACCGCATCGCTATAGTGAGTCGTATTA-3′ | GUS substrate |
| 5′-ACACAAGAGACGGGCACGCTATAGTGAGTCGTATTA-3′ | HIV substrate |

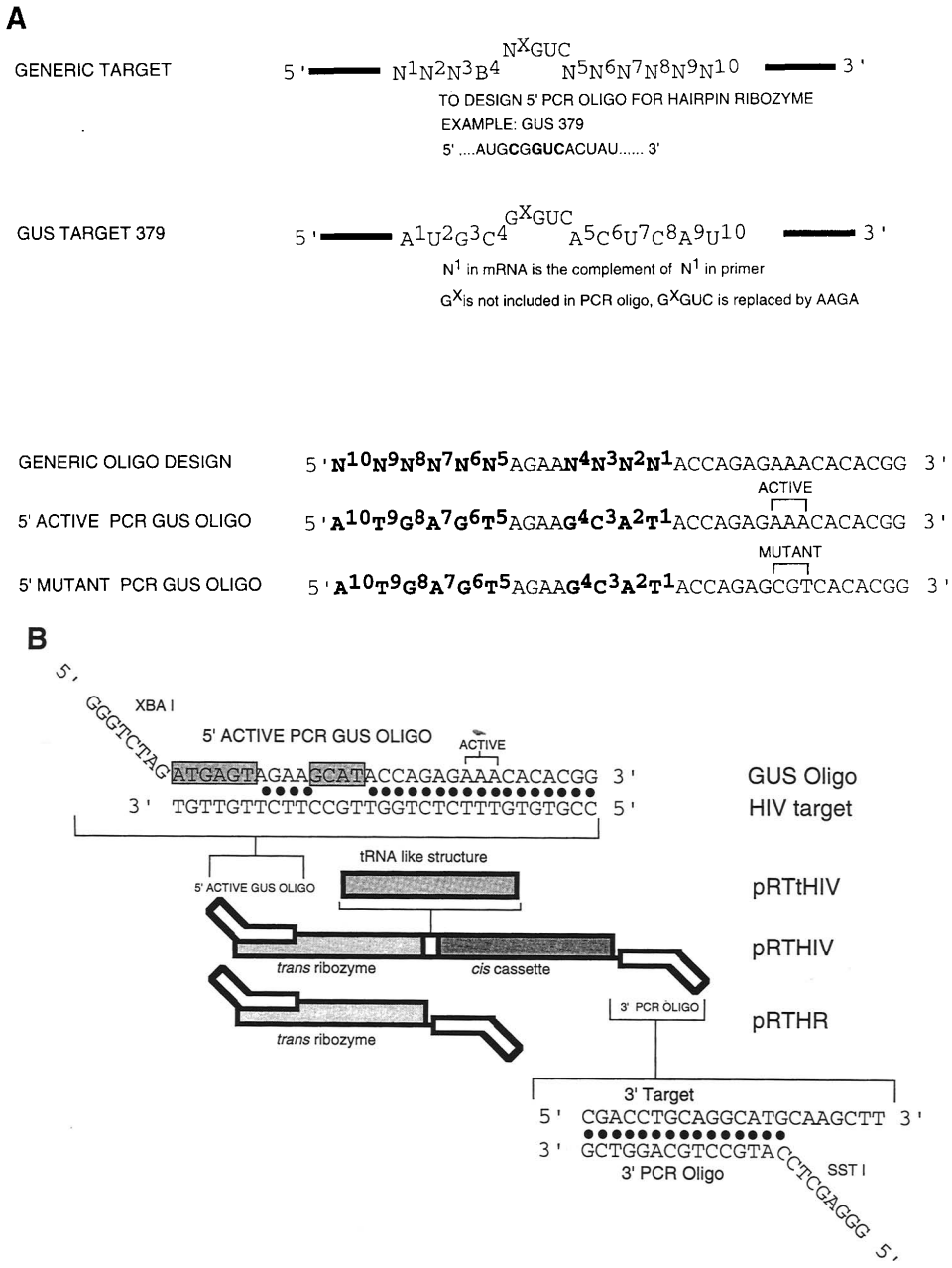


**FIGURE 3** (*A*) Strategy for determining target domains within a specific mRNA and a method by which both active and mutant 5′ PCR oligonucleotides can be designed. (*B*) Scheme by which new GUS-specific ribozymes (both modified and un-modified) are generated from a plasmid encoding the HIV 5′ leader target.

otide also includes a 3′ *Sst*I restriction site for cloning (Fig. 3B). Therefore, as designed, these oligonucleotides should be complementary to all three templates (pRTHIV, pRTtHIV, and pRTHR). Following PCR, cloning, and transcription, three modified ribozyme sequences that target GUS mRNA and not HIV 5′ leaders should be generated (Fig. 3B).

PCR amplification from the target plasmids generated bands of the expected sizes (Fig. 4). Plasmid pRTtHIV amplified the largest band of 397 bp (Fig. 4, lane 1), pRTHIV generated a band of 165 bp (Fig. 4, lane 2), and pRTHR a band of 108 bp (Fig. 4, lane 3). Similar patterns were observed when we used a mutant 5′ oligonucleotide that replaced the AAA sequence to CGT (Fig. 4, lanes 4–6). The DNA was isolated and digested with *Xba*I and *Sst*I and cloned into pTZ19U cut with the same enzymes. Colonies containing inserts were sequenced. All inserts had the selected GUS targeting domains, and no mutations were observed.

We transcribed the active and mutant *trans* GUS ribozymes that included an active *cis* cassette. Both PCR-generated sequences (active and mutant) maintained the ability to self-cleave (Fig. 5, lanes A,M) as expected. To determine the spec-

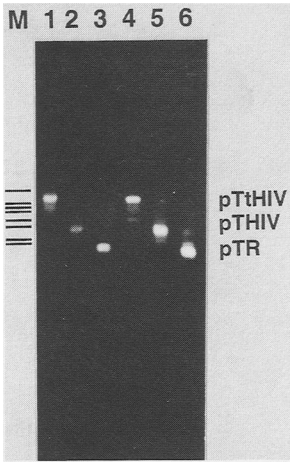


**FIGURE 4** Agarose gel analysis (2%) of PCR reactions on pRTtHIV (lanes *1,4*), pRTHIV (lanes *2,5*), and pRTHR (lanes *3,6*). Primers used were the 5′ PCR active oligonucleotide and 3′ PCR oligonucleotide (lanes *1–3*) and the 5′ PCR mutant oligonucleotide and 3′ PCR oligonucleotide (lanes *4–6*). The marker lane (M) represents the 516-, 394-, 344-, 298-, 220-, 200-, 154-, 142-, and 75-bp fragments from the 1-kb DNA ladder (GIBCO BRL, Gaithersberg, MD).

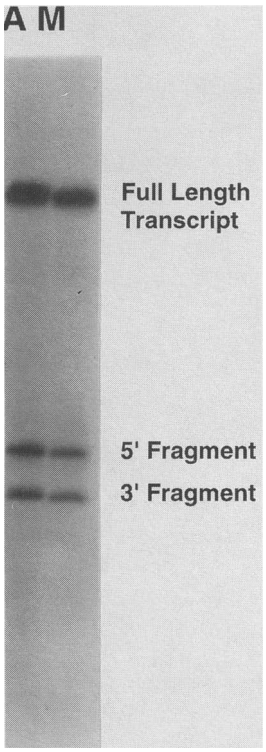


**FIGURE 5** In vitro transcription and gel analysis (15% acrylamide) of the active GUS ribozyme (lane *A*) and mutant GUS ribozyme (land *M*). The full-length transcript (191 nucleotides) should be cleaved into the active or mutant 5′ *trans* ribozyme fragment (102 nucleotides) and the 3′ fragment (89 nucleotides).

ificity of the resulting GUS ribozymes, the active and mutant *trans* ribozymes were used in cleavage assays against either the GUS or HIV substrate (Fig. 6). The active GUS ribozyme produced cleavage products after 30 min (Fig. 6, lane 2), whereas the same ribozyme was inactive against the HIV substrate (Fig. 6, lane 4). In addition, the replacement of AAA with CGT, which should result in an inactive ribozyme, also inactivated the GUS ribozyme (Fig 6, lane 6). The HIV substrate was shown to be capable of being cleaved when used in a cleavage reaction with an HIV ribozyme, and products were observed (Fig. 6, lane 10).

## DISCUSSION

We were interested in being able to efficiently alter hairpin ribozyme targeting domains to produce varied ribozymes economically. Currently, large numbers of oligonucleotides are used to study their in vitro kinetics.[5,6] It would, therefore, be useful if one could alter ribozyme specificity quickly. In this paper we have described a method by which one could identify potential targets for a hairpin ribozyme within a mRNA. Once identified, the sequence could be converted into small oligonucleotides that could generate a ribozyme with new specificity using PCR.

In these initial studies we were concerned with the balance of mismatching in the target domain (contained mostly in the 5′ end of the oligonucleotide) and the base-pairing that is needed for PCR in the 3′ end. We chose oligonucleotides that contained 17 bp of complementary sequence to ensure a successful reaction. This number of complementary bases worked with no alterations in our standard PCR reaction. For optimal utilization of this technique, that is, a less costly synthesis, we are currently reducing this 17-bp region. Our results show that by using our oligonucleotide design scheme, one can quickly alter ribozyme specificities. Even with the substitution of CGT for AAA, a substitution that reduced base-pairing by 3 bases, the PCR reaction remains robust (Fig. 4). Not only was a large quantity of DNA synthesized, but after sequencing five clones from each PCR reaction, no changes in the predicted DNA sequence were observed (data not shown). The tolerance of such mismatching in the 17-bp region suggests that the 5′ homology might be reduced as a basis for further optimization of this technique.

Although only the GUS ribozymes generated from pRTHIV were tested for cleavage in this study, we have produced PCR templates that, when transcribed, synthesize other modified *trans* ribozymes. We are currently testing the activity of GUS ribozymes transcribed from pRTtHIV and pRTHR. Previous results with the ribozyme transcribed from pRTtHIV have shown that this modified structure maintains catalytic activity when exposed to cellular lysates over nonmodified controls.[16] Therefore, the use of this structure in *trans* ribozymes could have great utility for clinical applications. This PCR method would allow us to change target domains on such modified ribozymes without the need for large oligonucleotides or extensive cloning steps.

From these results many other experiment scenarios can be envisioned. In these experiments a single targeting vector was used in combination with three

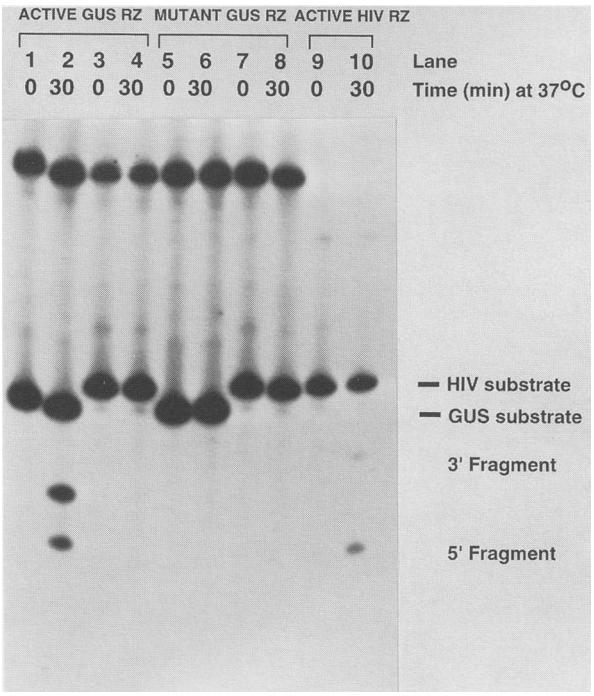


**FIGURE 6** In vitro cleavage reaction of transcribed GUS ribozymes from PCR derived reactions. (Lanes *1–4*) Active GUS ribozyme; (lanes *5–8*) the inactive GUS ribozyme; (lanes *9,10*) active HIV (cold) ribozyme. (Lanes *1,2,5,6*) GUS substrates; (lanes *3,4,7–10*) HIV substrates. Incubations were for 0 (lanes *1,3,5,7,9*) or 30 min (lanes *2,4,6,8,10*) at 37°C.

PCR oligonucleotides (5′ active, 5′ mutant, and 3′ PCR oligonucleotide). Alternatively, a dual vector system might be more efficient, in which a truncated 5′ oligonucleotide could amplify from an individual plasmid containing an active or mutant target. This could reduce the size of the 5′ oligonucleotide needed. Other possibilities include engineering a T7 promoter on the 5′ oligonucleotide for transcription without cloning.[17]

## ACKNOWLEDGMENTS

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