A systematic study of selectively modified, 36-mer hammerhead ribozymes has resulted in the identification of a generic, catalytically active and nuclease stable ribozyme motif containing 5 ribose residues, 29–30 2'-O-Me nucleotides, 1-2 other 2'-modified nucleotides at positions U4 and U7, and a 3'-linked nucleotide “cap.” Eight 2'-modified uridine residues were introduced at positions U4 and/or U7. From the resulting set of ribozymes, several have almost wild-type catalytic activity and significantly improved stability. Specifically, ribozymes containing 2'-NH₂ substitutions at U4 and U7, or 2'-C-allyl substitutions at U4, retain most of their catalytic activity when compared to the all-RNA parent. Their serum half-lives were 5-8 h in a variety of biological fluids, including human serum, while the all-RNA parent ribozyme exhibits a stability half-life of only ~0.1 min. The addition of a C3'-linked nucleotide “cap” (inverted T) did not affect catalysis but increased the serum half-lives of these two ribozymes to ~260 h at nanomolar concentrations. This represents an overall increase in stability/activity of ~53,000–80,000-fold compared to the all-RNA parent ribozyme.

Trans-acting ribozymes exert their activity in a highly specific manner and are therefore not expected to be detrimental to non-targeted cell functions. Because of this specificity, the concept of exploiting ribozymes for cleaving a specific target mRNA transcript is now emerging as a therapeutic strategy in human disease and agriculture (Cech, 1992; Bratty et al., 1993). Improvements in the chemical synthesis of RNA (Scaringe et al., 1990; Wincott et al., 1995) have led to the ability to similarly modify ribozymes containing the hammerhead ribozyme core motif (Usman and Cedergren, 1992; Yang et al., 1992) (Fig. 1). Yang et al. (1992) demonstrated that 2'-O-Me modification of a ribozyme at all positions except G5, G8, A9, A15.1, and G15.2 (see numbering scheme in Fig. 1) led to a catalytically active molecule having a greatly decreased kcat value in vivo, but a 1000-fold increase in nuclease resistance over that of an all-RNA ribozyme when tested in a yeast extract. In another study (Paolella et al., 1992), a persubstituted 2'-O-allyl-containing ribozyme with ribose residues at positions U4, G5, A6, G8, G12, and A15.1 showed a 5-fold decrease in catalytic activity compared to the all-RNA ribozyme (based on kcat/Km), while the stability of this ribozyme in bovine serum was increased substantially (30% intact material after 2 h compared to a ~1-min half-life for the all-RNA ribozyme). Shimayama et al. (1993) found it necessary to introduce 2 additional phosphorothioate linkages at positions C3, U4 and to replace U7 by A or G in a phosphorothioate-DNA/RNA chimera containing 21 phosphorothioate (P=S)1 substitutions (13 P=S DNAs in StemLoop II plus 5 and 3 P=S DNAs in Stems I and III, respectively). These ribozymes showed a 100-fold increase in stability relative to the all-RNA ribozyme, but the catalytic activities of these chimeras were reduced 15-fold (U7 → A7) and 42-fold (U7 → G7) compared to the wild-type ribozyme. Substitution of all pyrimidine nucleotides in a hammerhead ribozyme by their 2'-amino or 2'-fluoro analogs resulted in a 25-50-fold decrease in activity and a 1200-fold increase in stability in rabbit serum compared to the unmodified ribozyme (Pieken et al., 1991).

The above data suggest that a strategy of uniform modification cannot be directly applied to ribozymes, since it is necessary to preserve a reasonable level of catalytic activity and therefore to leave some residues, especially in the catalytic core, unmodified. We have constructed a generic, catalytically active, nuclease stable hammerhead ribozyme motif that contains only 5 ribose residues; the remaining residues consist of 2'-O-Me nucleotides with one or two other 2'-modified sugars at positions U4 and/or U7 (Figs. 1 and 2). Two of these ri-

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1 The abbreviations used are: P=S, phosphorothioate; 2'-F, 2'-deoxy-2'-fluorouridine; 2'-NH₂, 2'-deoxy-2'-aminouridine; iT, 3'-linked thyridine; Rz, ribozyme; t1/2, time required to cleave 50% of a short matched substrate; t50, time required to degrade 50% of the full-length ribozyme; kcat/Km, maximum ribozyme cleavage rate under single turnover (enzyme excess) conditions; Kcat, Michaelis constant under single turnover (enzyme excess) conditions.
bozymes (containing 2'-NH2 modifications at U4 and U7 or 2'-C-allyl modifications at U4) have almost wild-type catalytic activity and a 5-8 h half-life in human serum at nanomolar concentrations. The addition of a 3'-3' linked thymidine nucleotide to these ribozymes maintains their catalytic activity and increases their half-lives in serum to >260 h.

EXPERIMENTAL PROCEDURES

Synthesis of Ribozymes—Automated RNA synthesis and deprotection was carried out on an Applied Biosystems model 394 DNA/RNA synthesizer using the method of Scaringe et al. (1990), modified according to Wincott et al. (1995). Syntheses were carried out at 25 μmol on a derivatized aminomethyl polystyrene solid support (Applied Biosystems). A 5-min coupling step was used for 2'-O-protected RNA (Pharmacia Biotech Inc.) and modified phosphoramidites (Figs. 2).1 A 2.5-min coupling step was used for 2'-O-Me RNA (Milligen/Biosearch). Average coupling yields, determined by colorimetric quantitation of trityl fractions, were 97.5–99%. Phosphorothioate linkages at the 3'- and 5'-ends of Rz 5 were introduced by a sulfurization step2 with Beaucage’s reagent (Iyer et al., 1990). Ribozymes were gel-purified, eluted, ethanol-precipitated, rinsed twice with 70% ethanol, dried, and resuspended in TE buffer. Nucleoside Composition—The nucleoside compositions of the ribozymes were confirmed by nuclease digestion of the ribozyme and analysis by reverse phase high performance liquid chromatography. The ribozymes were converted to nucleosides by incubation of 0.3 A260 units of ribozyme with 10 units of P1 nuclease (EC 3.1.30.1; Boehringer Mannheim) and 2 units of calf intestinal alkaline phosphatase (EC 3.1.3.1; Boehringer Mannheim) in 30 mM NaOAc, 1 mM ZnSO4, at pH 5.2 (total volume = 100 μl) overnight at 50°C. The digested material was injected directly onto a C18 column (Ramin, Dynamax, ODS 4 × 250 mm), and nucleosides were separated by an octanitrile gradient buffered with 50 mM potassium phosphate, pH 7.0. The retention times were compared with monomer standards.

Radiolabeling of Ribozymes and Substrates—Ribozymes and substrates were 5'-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. For internal labeling, ribozymes were synthesized in two halves with the junction 5' to the GAA sequence in Loop II (Fig. 1). The 3'-half-ribozyme portion was 5'-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP, and was then ligated to the 5'-half-ribozyme portion using T4 RNA ligase. Labeled ribozymes were isolated from half-ribozymes and unincorporated label by gel electrophoresis. Ribozyme Activity Assay—Ribozymes and 5'-P2'-labeled substrate were heated separately in reaction buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl2) to 95°C for 2 min, quenched on ice, and equilibrated to the final reaction temperature (37°C or as indicated) prior to starting the reactions. Reactions were carried out in enzyme excess, and were started by mixing ~1 μM substrate with the indicated amounts of ribozyme (5–200 nM for the initial screens) to a final volume of 50 μl. Aliquots of 5 μl were removed at 1, 5, 15, 30, 60, and 120 min, quenched in formamide loading buffer, and loaded onto 15% polyacrylamide, 8 μM urea gels. The fraction of substrate and product present at each time point was determined by quantitation of scanned images from a Molecular Dynamics PhosphorImager. Ribozyme cleavage rates were calculated from plots of the fraction of substrate remaining versus time using a double exponential curve fit (Kaleidagraph, Synergy Software). The fast portion of the curve was generally 60–90% of the total reaction, so that observed cleavage rates (kobs) and activity half-times (t1/2) were taken from fits of the first exponential. Detailed kinetic analyses of Rzs 1, 2, 25, and 26 were performed in the same way except that reactions were carried out at 25°C and pH 6.5 to slow down the reactions and to enable more accurate determination of kinetic parameters. Plots of kcat versus ribozyme concentration were fit to the Michaelis-Menten equation using a non-linear, least squares routine (Kaleidagraph, Synergy Software) to determine values for kcat and Km. Values for the combined parameter, kcat/Km, were confirmed by performing cleavage reactions at low ribozyme concentration (5–20 nM), then determining kcat/Km from the initial slope of the kcat versus ribozyme concentration plot.

Ribozyme Stability Assay—Five hundred pmol of gel-purified 5'-end-labeled or internally labeled ribozymes were ethanol-precipitated and then resuspended in 20 μl of appropriate fluid (human serum, human plasma, human synovial fluid, or fetal calf serum) by vortexing for 20 s at room temperature. Samples were placed at 37°C, and 2 μl aliquots were withdrawn after the times indicated in the figures (30 s to 72 h). Aliquots were quenched by the addition of 20 μl of 95% formamide, 0.5 × TBE (50 mM Tris, 50 mM borate, 1 mM EDTA) and were frozen prior to gel loading. Ribozymes were size-fractionated by electrophoresis in 20% acrylamide, 8 μM urea gels. Gels were imaged on a Molecular Dynamics PhosphorImager, and the stability half-life (t1/2) for each ribozyme was calculated from exponential fits of plots of the percentage of intact ribozyme versus the time of incubation.

RESULTS AND DISCUSSION

Modification and Testing Strategy—We focused our efforts on substitutions of the 2'-hydroxyl group since these modifications were considered least likely to perturb the overall structure of the hammerhead ribozyme and were more easily introduced than backbone modifications. Ribozymes were chemically synthesized and gel-purified, and the nucleotide

\[ \text{Compounds 1–8 were replaced by the eight 2'-substituted nucleotides shown in Fig. 2 (compounds 1–8).} \]

**FIG. 1. Sequences of ribozyme and substrate used in this study.** Conserved nucleotides within the central core are numbered according to Hertel et al. (1993). Lowercase letters represent sites that were substituted with 2'-O-methyl nucleotides in the final, nuclease resistant motif. Underlined letters at U4 and U7 indicate positions that were replaced by the eight 2'-substituted nucleotides shown in Fig. 2 (compounds 1–8). Uppercase letters represent ribonucleotides; five positions (G5, A6, G8, G12, and A15.1) within the nuclease-resistant ribozyme were kept as ribonucleotides to maintain catalytic activity. X represents the 3'-3' linked (inverted) T residue (Fig. 2, compound 9) that was added to the 3'-end of Rzs 29 and 30. Arrow indicates the site of substrate cleavage.

**FIG. 2. Structures of the 2'-modified nucleotides used in this study.** 1, 2'-O-Me-U; 2, 2'-amino-U; 3, 2' C-allyl-U; 4, 2'-arabino-fluoro-U; 5, 2'-fluoro-U; 6, 2'-deoxy-U; 7, 2'-methylene-U; 8, 2'-difluoromethylene-U; 9, 3'-3' inverted T.

1 Beigelman, L., Karpeisky, A., Matulic-Adamic, J., Haeberli, P., Sweetser, D., and Wincott, F. (1995) Nucleic Acids Res. 23, in press.

2 A. D. DiRienzo, K. Levy, P. Haeberli, S. Grimm, J. Shaffer, N. Usman, and F. Wincott, manuscript in preparation.
The sensitivity of human serum and plasma, and least in human synovial fluid, exonuclease activity was greatest in fetal calf serum, less in others. Over time, all of the fragments were cleaved at their 3'-termini to generate smaller fragments. The amount of 3'-exonuclease activity was greatest in fetal calf serum, less in human serum and plasma, and least in human synovial fluid. The sensitivity of the 2'-O-Me fragments to cleavage by the ribozymes was then assayed for in vitro cleavage activity, and for nuclease resistance in a range of biological fluids. Activity measurements were made in enzyme excess at concentrations (40 nM ribozyme, ~1 nM substrate) that approach saturating conditions for the all-RNA control ribozyme. Ribozyme activity is reported in Tables I and II as the activity half-time ($t_{1/2}$) at 40 nM ribozyme; a larger number represents a slower cleavage rate and is less desirable. The stability of ribozymes to nuclease digestion was assessed in fetal calf serum, human serum, human plasma, and human synovial fluid using 5'-28-end-labeled ribozymes. Ribozyme stability is reported in Tables I and II as the stability half-life in human serum ($t_{1/2}$); a larger number represents a slower degradation rate. To compare one ribozyme's activity and stability to another, we have defined a parameter, $\beta$, which is the ratio of the stability and activity half-times compared to a reference, Rz 1 (Table I). Thus, in Tables I and II,

$$\beta(Rz \, n) = \frac{t_{1/2}(Rz \, n)}{t_{1/2}(Rz \, 1)}$$

(Eq. 1)

Larger $\beta$ values represent an improvement in ribozyme activity and/or stability relative to Rz 1.

5'- and 3'-Modifed Ribozymes Are Catalytically Active but Not Stable in Biological Fluids—To establish a base line for ribozyme catalytic activity and stability in biological fluids, ribozymes were synthesized containing RNA only (Rz 1, Table I), or RNA at all positions except in the substrate-binding arms (Stem I, positions 2.2–2.6; and Stem III, positions 15.3–15.7, Fig. 1). Table I shows that 2'-O-Me sugar, or phosphorothioate backbone modifications in the substrate-binding arms (Rz 2 and 3, respectively) had minimal effects on catalytic activity. However, ribozyme stability in human serum also remained unchanged with these modifications, and all three ribozymes were rapidly degraded (Fig. 3). No full-length ribozymes were present after 30 s in any of the biological fluids tested; however, stable fragments were observed in ribozymes containing 2'-O-Me modifications (Fig. 3). Modification of the Stem I and III backbones with phosphorothioate substitutions did not increase the nuclease resistance of the ribozymes or result in the generation of stable ribozyme fragments (Fig. 3).

The profile of stable fragments generated with the 2'-O-Me modified ribozymes varied with the medium and, to a lesser degree, with the base sequence of ribozyme stems (data not shown). At the earliest times, modified ribozymes were digested to fragments between 6 and 10 nucleotides in length whose relative abundance varied somewhat between experiments. Over time, all of the fragments were cleaved at their 3'-termini to generate smaller fragments. The amount of 3'-exonuclease activity was greatest in fetal calf serum, less in human serum and plasma, and least in human synovial fluid. The sensitivity of the 2'-O-Me fragments to cleavage by the ribozymes was then assayed for in vitro cleavage activity, and for nuclease resistance in a range of biological fluids. Activity measurements were made in enzyme excess at concentrations (40 nM ribozyme, ~1 nM substrate) that approach saturating conditions for the all-RNA control ribozyme. Ribozyme activity is reported in Tables I and II as the activity half-time ($t_{1/2}$) at 40 nM ribozyme; a larger number represents a slower cleavage rate and is less desirable. The stability of ribozymes to nuclease digestion was assessed in fetal calf serum, human serum, human plasma, and human synovial fluid using 5'-28-end-labeled ribozymes. Ribozyme stability is reported in Tables I and II as the stability half-life in human serum ($t_{1/2}$); a larger number represents a slower degradation rate. To compare one ribozyme's activity and stability to another, we have defined a parameter, $\beta$, which is the ratio of the stability and activity half-times compared to a reference, Rz 1 (Table I). Thus, in Tables I and II,

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### Table I

| Rz | Modification | Activity ($t_{1/2}$) | Stability ($t_{1/2}$) | Relative stability/activity $\beta(Rz \, n)$ |
|----|--------------|----------------------|----------------------|------------------------------------------|
| 1  | All RNA: UCUCCAU CUGAUGGGCGAAGGCCGAA AAGCCCU | 1.0 | 0.1 | 1 |
| 2  | 2'-O-Me arms: UCUCCAU CUGAUGGGCGAAGGCCGAA AAGCCCU | 1.0 | 0.1 | 1 |
| 3  | 5'5+5 P=5 arms: UCUCCAU CUGAUGGGCGAAGGCCGAA AAGCCCU | 3.0 | 0.1 | 0.3 |
| 4  | 2'-C-Allyl: UCUCCAU CUGAUGGGCGAAGGCCGAA AAGCCCU | 13.0 | 120 | 92 |
| 5  | 2'-Fluoro-Pyr: UCUCCAU CUGAUGGGCGAAGGCCGAA AAGCCCU | 30.0 | 15 | 5 |

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### Table II

| Rz | 2'-Modification (U4/U7) | Activity ($t_{1/2}$) | Stability ($t_{1/2}$) | Relative stability/activity $\beta(Rz \, n)$ |
|----|------------------------|----------------------|----------------------|------------------------------------------|
| 6  | OH/O-Me | 1.0 | 0.1 | 1 |
| 7  | O-Me/O-Me | 4.0 | 260 | 650 |
| 8  | =CH2/O-Me | 6.5 | 250 | 380 |
| 9  | O-Me=CH2 | 8.0 | 320 | 400 |
| 10 | =CH2=CH2 | 8.5 | 250 | 300 |
| 11 | =CF3/O-Me | 4.5 | 400 | 900 |
| 12 | O-Me=CF3 | 5.5 | 250 | 220 |
| 13 | =CF3=CF2 | >15 | 380 | 250 |
| 14 | F/O-Me | 3.0 | 300 | 1000 |
| 15 | O-MeF | 8.0 | 300 | 375 |
| 16 | F/F | 3.5 | 300 | 850 |
| 17 | H/O-Me | 5.5 | 250 | 450 |
| 18 | O-MeH | >10 | 250 | <250 |
| 19 | H/H | 4.0 | 280 | 700 |
| 20 | araF/O-Me | 5.5 | 500 | 900 |
| 21 | O-Me/araF | 4.0 | 350 | 675 |
| 22 | araF/araF | >15 | 500 | <330 |
| 23 | NH2/O-Me | 10.0 | 500 | 500 |
| 24 | O-Me/NH2 | 5.5 | 500 | 900 |
| 25 | NH2/NH2 | 2.0 | 300 | 1500 |
| 26 | C-Allyl/O-Me | 3.0 | >500 | >1700 |
| 27 | O-Me-C-Allyl | 3.0 | 300 | 1000 |
| 28 | C-Allyl/C-Allyl | 3.0 | 300 | 1000 |
| 29 | C-Allyl/O-Me+1 | 3.0 | 16,000 | 53,000 |
| 30 | NH2/NH2+IT | 2.0 | 16,000 | 80,000 |

a) Modifications follow the numbering scheme shown in Fig. 1.

b) Ribozyme activity expressed as cleavage half-time against the substrate shown in Fig. 1.

c) Ribozyme stability expressed as half-life of ribozyme in human serum. Times <1 min are estimated and may be shorter.
reduced activity by 25–50-fold (Peiken et al., 1991; Heidenreich et al., 1994). We chose to test ribozymes containing uniform 2'-C-allyl and 2'-F pyrimidine substitutions. The choice of the 2'-C-allyl modification was based on the observation that 2'-O-allyl substitutions in hammerhead ribozymes improve stability but cannot be introduced at positions U4 and U7 without a significant detrimental effect on catalysis (Paolella et al., 1992). The 2'-C-allyl group should be less bulky than the 2'-O-allyl group near the sites required for catalysis, but may still provide sterically and conformationally based nuclease protection.

The uniformly substituted 2'-C-allyl-pyrimidine ribozyme showed no activity in the cleavage assay (data not shown), which was likely due to the inability of Stem II to form (De Mesmaeker et al., 1993). Thus, another ribozyme was synthesized that lacked the 2'-C-allyl-pyrimidine substitutions in Stem II (Rz 4). Ribozyme 4, showed a 13-fold reduction in cleavage activity relative to Rz 1 (t50 = 13 min), but also exhibited enhanced nuclease resistance in all sera (t50 = 120 min in human serum). A significant amount of full-length ribozyme was present after 4 h (Fig. 4 and Table I). Incubation of Rz 4 in serum resulted in the slow formation of stable oligonucleotide fragments of ~16 nucleotides in length (Fig. 4). This digestion pattern suggested that Stem-Loop II was a primary site of nuclease activity in these ribozymes. Our data and the observations of Eckstein and colleagues (indicating that pyrimidines are the primary sites of endonuclease cleavage in hammerhead ribozymes; Heidenreich et al. (1993)) suggested that modification of the pyrimidines in Stem-Loop II might afford even greater nuclease protection.

The 3'-exonuclease degradation of the C-allyl modified ribozyme was minimal over the time period. In contrast, the 2'-F-pyrimidine modified Rz 5 showed better protection against endonuclease attack, but gave less protection from 3'-exonuclease activity than the C-allyl modifications. The cleavage activity of Rz 5 was reduced 30-fold (t50 = 30 min) relative to Rz 1. Since the 3'-exonuclease degradation of Rz 5 was much more pronounced than the Stem II endonuclease degradation of Rz 4, the overall stability of Rz 5 was ~8-fold lower than Rz 4 (Table I).

It has been shown that 2'-O-Me modifications stabilize RNA-RNA duplexes (Inoue et al., 1987) and do not have detrimental effects on the catalytic properties of hammerhead ribozymes when incorporated into the binding arms (Goodchild, 1992). We confirmed this latter observation by comparing the activity of Rz 1 with that of Rz 2. The effect of 2'-O-Me substitutions in the catalytic core on catalysis is less predictable (Paolella et al., 1992; Yang et al., 1992) but may be beneficial for stability considering the nuclease resistance of the 2'-O-Me fragments generated from Rz 2 (see below).

Selective Ribozyme Modifications Maintain Catalytic Activity and Enhance Nuclease Resistance—We considered two models of essential hydroxyl groups for the hammerhead ribozyme catalytic core in the development of our consensus, nuclease-resistant motif. Yang et al. (1992) showed that hammerhead ribozymes containing 2'-O-Me nucleosides at all positions except (ribonucleotides) G5, G8, A9, A15.1, and G15.2 resulted in a ribozyme with significantly reduced activity, but with a 104-fold increase in nuclease resistance in yeast extracts. Paolella et al. (1992) placed 2'-O-Me nucleosides at all positions except U4, G5, A6, G8, G12, and A15.1 and saw better activity (20% of wild type), while maintaining reasonable nuclease resistance (RNase A resistance increased by a factor of 102 and t50 in bovine serum increased to ~1 h). These results indicated that a modicum of ribonucleotide positions were required within the ribozyme core to maintain catalytic activity.

Based on the above data, we postulated a consensus motif (Fig. 1) that focused on positions U4 and U7 as pyrimidines within the core that might be 2'-modified without a drastic loss in catalytic activity. To test the importance of the U4 modification, Rz 6 was synthesized using a substitution pattern identical to the one reported by Paolella et al. (1992), except that 2'-O-Me was used instead of 2'-O-allyl at nonessential positions. The choice of 2'-O-Me substitutions was based on reports that this 2'-modification (i) confers stability to the hammerhead ribozyme (Yang et al., 1992), (ii) is more stable to nuclease.
Bulky 2'-O-Me or 2'-NH₂ analogs (Kawasaki et al., 1993), (iii) is naturally occurring, thereby reducing the possibility of toxicity in vivo, and (iv) is relatively easily synthesized and incorporated. The resulting catalytic activity of Rz 6 was the same as the all-RNA Rz 1 (t₅₀ = 1 min). Unfortunately, Rz 6 showed no improvement in nuclease resistance. In human serum Rz 6 was rapidly cleaved to give smaller fragments that were ~8 nucleotides in length (Fig. 5). The generation of 8-mer cleavage fragments from the 5'-end of Rz 6 suggested that the U4 site (the only unmodified pyrimidine residue within Rz 6) remained hypersensitive to nuclease. The different stability of Rz 6 compared to the reported 2'-O-allyl analog (Paolilla et al., 1992) could reflect a different accessibility of position U4 in a more sterically hindered 2'-O-allyl core compared to the less bulky 2'-O-Me core of Rz 6 and/or different nuclease compositions of bovine and human sera. The stability over time of the intact ribozyme fragment from Rz 6 suggested that the 2'-O-Me modification may be as good as the C-allyl modification at providing nuclease resistance. Thus, another 2'-O-Me substituted ribozyme was made and tested (Rz 7) that contained the same substitutions as Rz 6 with an additional 2'-O-Me substitution at the U4 position. Ribozyme 7 showed a 4-fold reduction in catalytic activity (t₅₀ = 4 min) but also gave a dramatic improvement in the nuclease resistance of the ribozyme (t₅₀ = 260 min, Fig. 6), so that the overall stability/activity ratio, β, improved 650-fold for Rz 7 compared to the all-RNA Rz 1.

To further elaborate on this model, the seven 2'-modified-uridine nucleotides shown in Fig. 2 were introduced into positions U4 and U7 (ribozymes 8-28). These modifications were chosen for a variety of reasons. 2'-Fluoro- and 2'-NH₂-U modifications have been successfully applied by Eckstein's group (Heidenreich et al., 1993) but have not been used in a highly 2'-O-methylated motif. The 2'-ara-F-U modification was introduced to probe the influence of configuration of the fluoro substituent on activity and stability. 2'-Deoxy-2'-methylene and difluoromethylene nucleotides were introduced under the assumption that imposing conformational restrictions on ribose sugar puckering of these monomers could provide increased nuclease resistance without reducing catalytic activity. Yamagata et al. (1992) showed by x-ray analyses that the C1', C2', and C3' carbons in 2'-deoxy-2'-methylene pyrimidine nucleosides are nearly coplanar. Finally, 2'-dU was introduced to probe the effect of removing substituents from the 2'-position. In the case of single U4 or U7 substitutions, the other uridine site contained a 2'-O-Me uridine.

The cleavage activity (t₅₀), human serum half-lives (t₅₀), and overall stability/activity ratios (β) for Rzs 8-28 are shown in Table II. All modifications to U4 and/or U7 gave significant increases in nuclease resistance for these ribozymes, while varying levels of ribozyme activity were observed. The most dramatic increases in nuclease resistance were seen in Rzs 20, 22-24, and 26, where stability times of greater than 500 min were observed (equivalent to >5000-fold stability increase relative to Rz 1). Ribozyme 25 gave a less dramatic increase in stability (t₅₀ = 300 min); however, its catalytic activity (t₅₀ = 2 min) made it attractive for further investigation. All of the ribozymes containing U4/U7 modifications were active to some degree, and the majority had activity decreases of less than 5-fold relative to Rz 1. The best overall ribozymes in terms of combined stability and activity were ribozymes 25 and 26 with β values of 1500–1700.

Certain trends that correlated with the type of 2'-modification and catalytic activity were noted. Modifications that distorted the normal ribose ring pucker resulted in ribozymes with reduced activity; examples included Rzs 8-10 (2'-methylene) and 11-13 (2'-difluoromethylene). Double modification of both U4 and U7 with these nucleotides had an even more pronounced negative effect (Rzs 10 and 13). 2'-Fluoro substitutions at U4 and U7 were less detrimental to catalysis than the related 2'-arabino-F-substitutions (Rzs 14-16 versus Rz 20-21).
Modified Hammerhead Ribozyme Activity and Nuclease Resistance

An especially striking difference was observed for the F/F-modified Rz 22 when compared to araF/araF-modified Rz 22. Our observations with the F/F-modified Rz 16 are consistent with the recently published observation by Heidenreich et al., (1993) for a hydrogen bonding network, which includes the 2'-hydroxyl of U4 and U7 and is relatively undisturbed by 2'-F substitutions due to their hydrogen acceptor properties. The greater reduction in activity observed for the araF/araF-modified Rz 22 could then be explained as a significant disruption of these hydrogen bonds due to the altered configuration at the 2'-position. However, this model would suggest that all modifications that remove or shift the position of the 2'-hydroxyl at U4 and U7 should significantly reduce ribozyme activity. In fact, only moderate (4-fold) reductions in activity are observed for H/H-modified Rz 19, and for a recently tested araF/araF-modified ribozyme (data not shown).

The high activity of Rz 25 (U4/U7 = 2'-NH2-U) is in agreement with the recently published observation that incorporation of 2'-NH2-U into both the U4 and U7 positions rescues the activity of uniformly 2'-F-substituted ribozymes at pyrimidine sites (Heidenreich et al., 1994). Interestingly, the combination of 2'-NH2-U and 2'-O-Me substitution at positions U4 and U7 yielded Rz 24 (O-MeNH2) with moderate and Rz 23 (NH2/O-Me) with low catalytic activity. Only the double modification (NH2/2-NH2) provided a highly active ribozyme. The intrinsic dual role of the amino group as a potential hydrogen bond donor and acceptor could be responsible for the observed effect if both 2'-NH2 groups are the partners in a hydrogen bonding network. In contrast, the relatively high catalytic activity of the 2'-C-allyl modified Rzs 26–28 is not consistent with the hydrogen bonding network proposed by Heidenreich et al., (1993) since it is unclear how the 2'-C-allyl group could participate in the normal hydrogen bonding or Mg2+-coordination networks that create the active catalytic conformation.

Having identified two ribozymes with substantially increased stability (Rzs 25 and 26), we wanted to confirm that the activity screens were correctly representing the activity of these ribozymes. Thus, more complete activity profiles were determined for Rzs 25 and 26 and were compared to the kinetic parameters of the control Rzs 1 and 2. Table III shows that Rzs 1, 2, and 25 all have similar kinetic behavior. These ribozymes show little difference in the values of the specificity constant, kcat/S/KmS, while the less certain estimates of kcat/S and KmS vary by only 2-fold. In contrast to these three ribozymes, Rz 26 shows a ∼10-fold reduction in kcat/S/KmS, which is almost completely due to reductions in kcat/S.

We have attempted to compare our findings with the interactions seen in two recently published and very similar crystal structures (Pley et al., 1994; Scott et al., 1995). However, it is difficult to compare our results to these crystal structures for two reasons. First, most of our substitutions are conservative 2'-O-Me sugar substitutions, which should cause a minimum of steric clash with neighboring groups and which can still act as H-bond acceptors, while the remaining, extensive substitutions have focused on the 2'-positions only at U4 and U7. Second, the crystal structures appear to represent a ground-state structure that is fairly distant from the transition state. Nevertheless, McKay and colleagues described three positions (U4, G5, and G8) at which H-bond contacts are made with the 2'-hydroxyl. The H-bond contacts at G5 and G8 are in agreement with the observations that these hydroxyl groups cannot be substituted without substantial loss of activity. However, the data for position U4 would suggest that H-bond interactions with this 2'-hydroxyl are not essential for cleavage activity, since substitutions that abolish H-bonds (→CF2 in Rz 11, and C-allyl in Rz 26) show the same moderate reductions in activity as do substitutions that maintain H-bonds (F in Rz 14).

3'-Modifications Maintain Catalytic Activity and Extend Ribozyme Serum Half-life at Nanomolar Concentrations—Ribozymes 7–28 all showed dramatic improvements in nuclease resistance compared to the all-RNA Rz 1, or even compared to the highly modified Rz 6, which still contains a ribonucleotide at U4. However, all of these ribozymes still exhibit slow degradation at the 3'-end (cf. Fig. 6). Addition of a 3'-3' linked, inverted T (iT) residue at the 3'-end of DNA oligonucleotides has been reported to inhibit the digestion of DNA by 3'-exonucleases (Ortigao et al., 1992). We therefore added an iT to Rzs 25 and 26 to give Rzs 29 and 30. The iT residue protected the 3'-terminus of the ribozymes for at least 48 h, when present at 25 nM in either human serum or fetal calf serum (data not shown). We viewed this as a conservative estimate of nuclease resistance due to a low level of phosphatase activity present in both sera.

To eliminate the effect of 5'-phosphatase activity on ribozyme stability measurements, the stability of Rz 26 was

| Rz | kcat/S | KmS | kcat/S/KmS |
|----|-------|-----|-----------|
| 1  | 0.14  | 56  | 0.3       |
| 2  | 0.17  | 25  | 0.7       |
| 24 | 1.2   | 20  | 0.6       |
| 25 | 1     | 20  | 0.5       |
| 26 | 0.14  | 56  | 0.3       |
evaluated using ribozymes that contained an internal $^{32}$P label (see “Experimental Procedures”). Fig. 7 shows that >75% of internally labeled Rz 30 remained intact after a 72 h incubation in human serum ($t_{50} = 16,000$ min). In contrast, the all-RNA Rz 1 was degraded to small fragments within the 30 s that it took to add ribozyme to serum, mix, and quench the reaction (time 0 h, Fig. 1). During the incubation of Rz 30, a small number of minor bands appeared that have mobilities consistent with digestion at the five remaining ribose sites within the ribozyme. Thus, even greater stabilization of the ribozymes is likely to require substitution of the 5 remaining ribose residues.

To verify that the 3’-exonuclease activity in serum was not significantly diminished during the 72 h assay, Rzs 1 and 2 were added to a sample of the serum after the 72 h incubation period. These nuclease-sensitive ribozymes were degraded immediately (data not shown).

The presence of the inverted T residue at the 3’-end of Rzs 29 and 30 has no effect on catalytic activity. Their activity half-times were identical to the equivalent Rzs 26 and 25, respectively, which lack the inverted T (Table II). Thus, Rzs 29 and 30 show an overall 50,000–80,000-fold increase in the relative ribozyme stability/activity compared to the all-RNA ribozyme.

Incubation of Nuclease-resistant Ribozymes in Human Serum Does Not Alter Catalytic Activity—To assess the effect on catalytic activity of prolonged incubation of Rzs 29 and 30 in human serum, samples of the Rzs were removed from the serum after 72 h and assayed for activity. To inhibit nuclease digestion of the substrate in serum, yeast tRNA was added to each sample. No diminution in ribozyme catalytic activity was noted in this assay (data not shown).

Conclusions—We have systematically investigated the influence of certain 2’-modifications on hammerhead ribozymes, with the goal of conferring high cleavage activity and increased nuclease resistance in biological sera. We have identified a consensus motif of 2’-hydroxyl groups required to maintain catalytic activity in the context of a persubstituted 2’-O-Me hammerhead ribozyme. In this motif, the 5 purine ribonucleotides G5, A6, G8, G12, and A15.1 remain unmodified. Selective modifications, at positions U4 and U7 in the “5-ribose” hammerhead ribozyme, maintain catalytic activity while dramatically increasing the nuclease resistance of the ribozymes in biological sera. The best U4 and U7 modifications for stability and activity were U4A7 2’-NH$_2$ and U4 2’-C-allyl/ U7 2’-O-Me, which provided more than a 1500-fold increase in stability/activity ratios ($\beta$) over the unmodified all-RNA ribozyme. An additional increase in $\beta$ values to 53,000–80,000 was achieved by introducing a 3’-3’-linked thymidine to the 3’-end of these ribozymes.

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