Chimeric DNA-RNA hammerhead ribozymes have enhanced *in vitro* catalytic efficiency and increased stability *in vivo*

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ABSTRACT

Subsequent to the discovery that RNA can have site specific cleavage activity, there has been a great deal of interest in the design and testing of trans-acting catalytic RNAs as both surrogate genetic tools and as therapeutic agents. We have been developing catalytic RNAs or ribozymes with target specificity for HIV-1 RNA and have been exploring chemical synthesis as one method for their production. To this end, we have chemically synthesized and experimentally analyzed chimeric catalysts consisting of DNA in the non-enzymatic portions, and RNA in the enzymatic core of hammerhead type ribozymes. Substitutions of DNA for RNA in the various stems of a hammerhead ribozyme have been analyzed *in vitro* for kinetic efficiency. One of the chimeric ribozymes used in this study, which harbors 24 bases of DNA capable of base-pairing interactions with an HIV-1 *gag* target, but maintains RNA in the catalytic center and in stem-loop II, has a sixfold greater k_{cat} value than the all RNA counterpart. This increased activity appears to be the direct result of enhanced product dissociation. Interestingly, a chimeric ribozyme in which stem-loop II (which divides the catalytic core) is comprised of DNA, exhibited a marked reduction in cleavage activity, suggesting that DNA in this region of the ribozyme can impart a negative effect on the catalytic function of the ribozyme. DNA-RNA chimeric ribozymes transfected by cationic liposomes into human T-lymphocytes are more stable than their all-RNA counterparts. Enhanced catalytic turnover and stability in the absence of a significant effect on K_m make chimeric ribozymes favorable candidates for therapeutic agents.

INTRODUCTION

Ribozymes are RNA molecules capable of catalytically cleaving specific RNA sequences. As such, they are receiving considerable attention because of their potential utility as both genetic tools and therapeutic agents (for reviews see 1,2,3). In particular, the

'hammerhead' catalytic motif (4,5) provides a relatively simple, yet effective catalytic sequence capable of enzymatically cleaving a wide range of RNA sequences. Ribozyme catalytic centers, which are phylogenetically conserved, can be flanked by any antisense sequence, enabling specific targeting of most RNA sequences. Uhlenbeck first demonstrated that the catalytic domain of the hammerhead ribozyme could be divided into two molecules, such that a *trans*-cleavage reaction could take place (5). In such a *trans* reaction, the catalytic strand can base pair with the substrate molecule and, after cleavage and dissociation of the product, bind another substrate. Haseloff and Gerlach (6) designed *trans*-acting ribozymes in which all of the conserved bases, except a triplet at the cleavage site, were included in the catalytic strand. Thus a ribozyme can be designed to cleave virtually any RNA sequence. Optimal catalytic activity of the hammerhead ribozyme requires adherence to a consensus sequence (4). Most of the conserved bases can be replaced by other nucleotides, but this is usually accompanied by a significant drop in activity (7, 8).

In nature, all ribozymes contain ribose sugars, but ribozymes can be synthesized with modifications in the sugar moiety. For example, Perreault *et al.* (9,10), have designed ribozymes in which the ribose sugars of core nucleotides are replaced with deoxyribose sugars. Most of these substitutions lead to a modest decline (10% to 50%) in kinetic activity when compared to their ribonucleotide counterparts. Substitutions of deoxyribose for ribose at two positions in the enzymatic core lead to virtual loss of catalytic activity (G_9 and A_{13}) (10). Similar modifications are tolerated in the substrate at all positions except the actual cleavage site and the base 5' to the cleavage site. Both of these bases show an absolute requirement for a 2'-hydroxyl group (11).

While several studies have focused on modification of nucleotides in the catalytic core, no one has yet considered the effects of deoxyribose for ribose substitutions outside of the conserved core. We have been interested in synthesizing and testing chimeric DNA-RNA ribozymes in which the catalytic center remains as RNA, but the rest of the molecule is DNA. Our interest in such molecules is based upon their potential utility as anti-HIV-1 therapeutic agents. We envision several advantages

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for these chimeric ribozymes over all-RNA ribozymes, including greater ease of chemical synthesis, enhanced cellular delivery and improved *in vivo* stability. The hybrid helices formed by the interaction of a chimeric ribozyme with an RNA strand could potentially serve as a substrate for RNaseH, leading to the digestion of the RNA strand, thereby facilitating target destruction and catalytic turnover.

We have designed and synthesized model ribozymes and substrates which are identical except for their DNA/RNA composition outside of the catalytic core. We report here the kinetic characterization of these DNA-RNA catalysts. One of our chimeric ribozymes had a sixfold greater turnover rate than its RNA counterpart, due to enhanced product dissociation. When transfected into cells via cationic liposomes, this chimeric ribozyme displayed enhanced stability in comparison to the all-RNA counterpart.

MATERIALS AND METHODS

Synthesis and purification of DNA-RNA mixed polymers

The assembly of mixed oligodeoxyribo- and ribonucleotides was performed on an automated synthesizer (Applied Biosystems 380B). 5'-Dimethoxytrityl, 2'-tertbutylsilyl, and N-acyl (benzoyl for adenosine and cytosine, isobutyl for guanosine) ribonucleoside cyanoethyl phosphoramidites (American Bionetics) were used for synthesis of the RNA segments. 5'-Dimethoxytrityl, N-protected deoxynucleoside phosphoramidites (American Bionetics) and the derivatized LCAA-CPG were used for synthesis of the DNA. HPLC-grade acetonitrile was refluxed and distilled over CaH_2 . THF for the capping solution was dried over activated 4-A molecular sieves prior to use. Tetrazole (Aldrich Chemical Co.) was recrystallized from dry acetonitrile and then dried under vacuum and stored under argon.

The synthesizer was programmed such that the RNA was coupled using an RNA-synthetic cycle (720 seconds coupling time) and the DNA units were assembled by a DNA-synthetic cycle. The standard synthetic cycle (Applied Biosystem) was modified by the introduction of neutralization with 3% TEA in acetonitrile after detritylation. The average coupling yield, determined by UV quantitation of the released trityl cation (504 nm for DMT cation) was 97.5%.

Aqueous solutions used during deprotection and all HPLC buffers were sterilized by treatment with diethylpyrocarbonate (DEPC) (Aldrich Chemical Co.) followed by autoclaving for 1 h. All aqueous solutions and buffers contained 0.0001% NaN_3 to prevent bacterial growth. Only disposable, sterile plasticwares were used during the final purification. Sephadex G-25F (Pharmacia) was also sterilized. Strictly sterile conditions were maintained during purification after final deprotection.

The assembled molecule was deprotected as follows. The cyanoethyl phosphate deprotection and deacylation were provided by treatment with saturated, anhydrous ethanolic ammonia for 8 h at 55°C (12). Deprotection with anhydrous ethanolic ammonia was found to be more reproducible and reliable than treatment with 35% aq. ammonia-ethanol (3:1) (13). The products of deacylation were then treated with tetrabutylammonium fluoride (Aldrich Chemical Co.), at a 1 M concentration in THF for 30 h at room temperature to remove the 2'-OH silyl protecting groups from the RNA. The final product was then desalted on a Sephadex G-25 column, and purified by HPLC on a 4×250 mm PRP (Hamilton) column using a 0% (A) – 100% (B) linear gradient (25 minute running time) of (A) 0.005 M triethyl ammonium

acetate-water, pH 6.5 and (B) 0.005 M triethylammonium acetate-water, pH 6.5 in acetonitrile, 2:3.

An alternative method for purification of the totally deprotected oligodeoxyribo- and ribonucleotides after final treatment with tetrabutylammonium fluoride involves dialysis of the reaction mixture against water (using a 3000-molecular-weight cutoff membrane) followed by HPLC on a Vydac oligonucleotide ion-exchange column. Although this procedure is time-consuming, it is a more reliable method for purification of deprotected synthetic RNA.

For the final purification the chimeric oligonucleotides were washed twice in DEPC-treated water, concentrated by drying, and precipitated with ethanol. The samples were resuspended in DEPC-treated water and purified by electrophoresis in a 15% polyacrylamide, 7 M urea gel. The oligonucleotides were visualized by UV shadowing, excised, and eluted from the gel slice via diffusion in a solution of 0.3 M NaOAc, 0.1% SDS and 1 mM EDTA at 37°C. Crushed gel fragments were filtered in a Costar spin-X filter and the supernatant was precipitated with ethanol, lyophilized and resuspended in sterile DEPC-treated water. Concentrations were determined from the absorption at 260 nm. The DNA-RNA junctions of the oligomers were confirmed by RNAase A and T1 digestion of ^{32}P end-labeled samples.

Synthesis of RNA ribozymes and substrates and ribozyme mediated cleavage reactions

The *in vitro* transcription of rz.GH and the RNA gag-HIV target sequence (rGAG) have been described (14).

Standard cleavage reactions involved heating to 85°C for 1 min two separate tubes containing a solution of 50 mM Tris-HCl (pH 8.5) and either ribozyme (10 nM) or substrate (20–100 nM). This was followed by cooling to room temperature, addition of 20 mM MgCl_2 to each tube and incubation at 55°C for 15 min. The cleavage reactions were initiated by mixing equal volumes of the target and ribozyme mixtures for a typical final volume of 10 μl per reaction time point. In order to denature the ribozyme-product complexes, a 25% volume of 90% formamide loading solution was added and the samples were heated to 85°C for one minute, chilled on ice and electrophoresed in a 15% polyacrylamide, 7 M urea gel. Quantitations of cleavage were performed on an Ambis radioanalytic imaging system.

Temperature profile reactions did not include preincubation. A 12 μl volume containing 10 nM ribozyme and 40 nM target in 50 mM Tris-HCl (pH 8.5) was mixed with 3 μl of 100 mM MgCl_2 to start the reaction. All reactions were stopped by the addition of 3 μl of 200 mM EDTA. Some non-specific breakdown of substrate was observed at temperatures above 60°C.

For kinetic determinations, the reactions were performed as described above for standard cleavage. Substrate concentrations in 4 to 12-fold excess of the ribozyme concentration (0.05 or 0.1 pmol/10 μl reaction volume) were used and time points were selected to represent the linear burst and steady-state velocity phases of the catalyst-substrate pair being tested. Calculations of K_m , K_{mb} , k_{cat} and k_{cath} were derived from both Eadie-Hofstee (15) and direct linear plots (16).

For the sake of simplicity we use the kinetic equation:

where R equals ribozyme, S equals substrate and P equals the products of cleavage. Hence, the constants k_2 and k_3 refer to cleavage and dissociation of R from P, respectively. Using Briggs-Haldane analyses of the data, the turnover number, k_{cat} , can be determined ($k_{cat} = V_{max}/E$, where E is the enzyme concentration).

Some of the velocity profiles of the ribozymes in this study are biphasic. They show a dramatic decrease in velocity after one enzyme equivalent of substrate has been cleaved (Fig. 3, rz.GH). This phenomenon has been described previously for protein enzymes and has been labeled as burst kinetics (17). The initial burst velocity, b , can be distinguished from the steady-state velocity, v , that follows it. Bender *et al.* (18) derived the following equation for the burst velocity (where S_0 is the initial substrate concentration):

$$b = [(k_2 + k_3)S_0 + k_3 K_s] / (K_s + S_0)$$

This equation can be reduced to

$$b = [(k_2 + k_3)S_0] / (K_s + S_0)$$

when $b \gg k_3$ (19). This allows for Michaelis-Menten type kinetic analysis of b with changing initial substrate concentration, S_0 , and leads to the determination of K_s and $(k_2 + k_3)$ rather than K_m and V_{max} .

Burst kinetics are seen only when the rate-limiting step of the reaction occurs after the measured chemical step. In the case of ribozyme-mediated cleavage, burst kinetics imply that the rate of the measured step, cleavage, is fast compared to the rate-limiting step which is product dissociation.

Intracellular stability studies

rz.RLoop was kinase-labeled with ^{32}P or ^{35}S (NEN). rz.GH was labeled with $\alpha\text{-}^{32}\text{P}$ CTP during *in vitro* transcription. All labeled RNAs were gel purified prior to use.

A 0.1% solution of Lipofectin (BRL) was incubated with labeled oligomers. The mixture was vortexed gently and left at room temperature for 30 minutes, whereupon 3 ml of Opti-Mem serum-free media was added. Aliquots containing 1×10^7 H9 cells were pelleted and resuspended in the ribozyme/Lipofectin/media solutions and incubated for 14 hours. These cells were pelleted and suspended in 20 ml of CRPM1 containing 10% serum. This was considered time zero. Two hours later, each transfected cell suspension was counted, the volume of the solution that contained 10^6 cells was determined, and the cells were harvested. The same volume of cells were also harvested at 6, 12, 36 and 48 hours post-transfection. Cells were pelleted, washed twice in PBS, and then suspended in 0.8 ml of 3 M LiCl, 6 M urea. These suspensions were frozen, thawed, and then allowed to precipitate for 12 to 20 hours on ice. The precipitate was centrifuged for an hour, and the pellet was rinsed in 1 ml of 3 M LiCl, 6 M urea solution and suspended in 1 ml of 0.1 M NaOAc (pH 5.0) and 0.01% SDS at 70°C. The RNA solution was then extracted twice with phenol, once with dichloromethane, and precipitated with 1/10 volume of 3 M NaOAc and 3 volumes of ethanol. The RNA pellets were washed with 70% ethanol, dried, and suspended in 20 μl of DEPC-treated water. Total RNA was quantitated and the entire samples were electrophoresed in a 15% polyacrylamide, 7 M urea gel. Following electrophoresis, the gels were dried and radioactivity of the ribozymes quantitated on an AMBIS radioanalytic imaging system.

RESULTS

The effects of incorporating DNA within the nonconserved portion of a hammerhead ribozyme were examined by synthesizing a 47-nucleotide DNA-RNA chimeric catalyst, rz.DRD, which contained only 12 ribonucleotides, all in the highly conserved catalytic center (Fig. 1). It is important to note that stem-loop II was comprised entirely of deoxyribonucleotides. The kinetic parameters of this chimeric ribozyme were determined alongside those of an all-RNA ribozyme (rz.GH) of identical length and sequence.

Cleavage reactions were performed over a wide range of temperatures in order to assess the temperature at which the rz.DRD optimally cleaved an RNA substrate (Fig. 2). The temperature optimum for RNA cleavage by rz.DRD was *ca.* 55°C, as compared to an optimum of *ca.* 65°C for rz.GH, suggesting that the DNA-RNA hybrid helix is less stable than the RNA-RNA helix for this sequence.

The plot of time versus cleavage product formation for the ribozyme rz.GH and the RNA substrate rGAG showed a biphasic velocity (Fig. 3). This type of profile, a burst velocity followed by a slower steady-state velocity, is well documented for protein enzymes (16,18,19). In the present case, when a sixfold substrate excess was used, the 'burst' velocity for the first turnover was 3.5 times faster than the subsequent steady-state velocity (Fig. 3). In contrast, the chimeric ribozyme rz.DRD did not show a comparable burst velocity (Fig. 3). The kinetic data (Table 1) demonstrate a steady state k_{cat} for rz.DRD equivalent to that of rz.GH (0.019/min), while the K_m of rz.DRD for the RNA substrate was almost twice that of rz.GH.

The absence of a burst velocity for rz.DRD suggested that product dissociation was not rate-limiting for this ribozyme, and hence another step in the ribozyme catalysis pathway was rate limiting. To determine whether or not the hybrid helices were responsible for this altered activity, a chimeric DNA-RNA substrate, dGAGrUCA, was synthesized. In this substrate, all of the base-pairing sequences are DNA, except for the nucleotide

Figure 1. The sequences of the ribozymes and substrates utilized in this study. Capital letters represent deoxyribonucleotides, lowercase letters represent ribonucleotides in the chimeric molecules (rz.RLoop, rz.DRD, and dGAGrUCA). Boxed capital letters represent deoxyribonucleotides found in rz.DRD only.

Figure 2. Temperature profiles. Data points are expressed as a fraction of maximal product formation for the ribozyme-substrate combination considered. Due to different activity levels, the actual amount of substrate cleavage and times of incubation vary with each catalyst-substrate pair. In all cases, the open squares represent the relative activity of the ribozyme with the all-RNA substrate (rGAG), while the filled circles represent the same ribozyme's activity against the chimeric dGAGrUCA substrate. Cleavage reactions contained a fourfold excess of substrate. Ribozyme concentrations were 10 nM for rz.RLoop and 6 nM for all the other ribozymes. Total reaction times for rz.RLoop cleavage and rz.GH-dGAGrUCA were 8 minutes, while rz.DRD cleavage was monitored for 100 minutes. Data from rz.GH-rGAG was taken from previously published results (13).

at the site of cleavage and the two nucleotides flanking the cleavage site (Fig. 1). The annealing of this substrate with rz.GH reverses the positions of DNA and RNA within helices I and III, as compared to rz.DRD and the rGAG substrate.

Table 1. Kinetic parameters of chimeric ribozymes and substrates

Ribozyme	Substrate	K_{mb} (nM)	k_{catb} (/min)	K_m (nM)	k_{cat} (/min)
rz.GH	rGAG	31	0.25	38	0.019
rz.GH	dGAGrUCA			56	0.087
rz.DRD	rGAG			64	0.019
rz.DRD	dGAGrUCA			196	0.024
rz.DRD	dGAGrUCA (50°C)			48	0.012
rz.RLoop	rGAG	51	0.27	64	0.126
rz.RLoop	dGAGrUCA			91	0.108

* All measurements were taken at 55°C unless specified.

Table 2. The *in vivo* stability of chimeric (rz.RLoop) and RNA (rz.GH) ribozymes in H9 lymphocytes following Lipofectin transfection

Time following transfection (h)	rz.GH ³² P-labeled (% remaining*)	rz.RLoop ³⁵ S-labeled (% remaining*)	rz.RLoop ³² P-labeled (% remaining*)
2	100.0	100.0	+++
6	87.3	87.4	100.0
12	53.4	80.3	78.2%
24	47.9	83.7	+++
36	31.3	+++	74.7%
48	21.6	70.6	60.4%

*The percentage of the counts at first time point that remain
+++ data not available

As was observed for the chimeric ribozyme, rz.DRD, the chimeric substrate-rz.GH combination had a temperature optimum of *ca.* 55°C. Cleavage of dGAGrUCA by rz.GH did not result in a burst velocity. The steady-state velocity was intermediate to that of the rz.GH-rGAG burst and steady-state velocities. The k_{cat} determined for rz.GH cleavage of dGAGrUCA was 4.5-fold faster than cleavage of rGAG with either rz.GH or rz.DRD (Table 1). The K_m for rz.GH with the dGAGrUCA substrate was about twice that obtained with the RNA substrate rGAG. The chimeric substrate dGAGrUCA could also be cleaved by the chimeric ribozyme rz.DRD, yielding the following kinetic values: at 50°C (the optimal temperature for cleavage using the chimeric ribozyme-chimeric substrate combination) the K_m was 48 nM and the k_{cat} 0.012/min.

Since rz.DRD had reduced cleavage activity relative to rz.GH, another chimeric ribozyme (rz.RLoop) was synthesized in which only the base-pairing sequences were DNA, and the catalytic center as well as stem-loop II were RNA (Fig. 1). Like the other DNA-RNA hybrid helix-forming combinations (rz.DRD-rGAG and rz.GH-dGAGrUCA), the temperature optimum for cleavage by rz.RLoop was *ca.* 55°C. When rz.RLoop was analyzed with the rGAG substrate, a burst velocity was in fact observed (Fig. 3). A second rate change in product formation was observed for both rz.RLoop and rz.GH with rGAG when approximately one-fourth of the substrate had been cleaved, and most probably reflects product inhibition (Fig. 3).

The burst phase kinetic constants determined for the chimeric ribozyme rz.RLoop are comparable to those for the all-RNA ribozyme rz.GH (Table 1). In contrast the steady state k_{cat} for rz.RLoop is six times faster than that determined for rz.GH (0.126/min versus 0.02/min). The K_m and K_{mb} for rz.RLoop with rGAG were slightly greater than those obtained for rz.GH with rGAG. The kinetic constants determined for rz.RLoop with

Figure 3. Time-courses. The concentrations of substrate cleaved by the ribozymes (in pmol/ml) as a function of time (min) is depicted. Open squares represent cleavage of the RNA substrate (rGAG). Filled circles represent the same ribozyme's cleavage of dGAGrUCA. All reactions contained 10 nM ribozyme and 60 nM substrate.

the chimeric dGAGrUCA substrate demonstrated a slightly higher K_m value and a somewhat slower k_{cat} value than were obtained with the rGAG substrate (Table 1).

The relative stabilities of the chimeric ribozymes versus RNA ribozymes were assessed in cell culture experiments. We first incubated RNA and chimeric ribozymes with H9 lymphocytes in serum containing culture media. The RNA molecules were completely degraded within one hour of incubation, whereas the chimeric ribozymes were relatively stable for several hours, and found associated with the H9 lymphocytes, either adhered to the cell membrane, or intracellularly (data not presented). Since we were unable to stabilize the RNA in cell culture, we utilized a commercial cationic liposome preparation to deliver both the RNA and chimeric molecules to the H9 lymphocytes to evaluate the relative intracellular stabilities of the two ribozyme preparations. Total RNA (including the chimeric ribozymes) was extracted at varying times following the transfection. From the data presented in Table 2, it is suggested that the DNA in the flanking sequences provides substantial intracellular stabilization of rz.RLoop when compared to the all-RNA ribozyme rz.GH, resulting in approximately threefold greater recovery after 48 hours of incubation.

DISCUSSION

We describe the synthesis and characterization of three anti-HIV-1 *gag* ribozymes of identical base sequence, but differing in their RNA-DNA contents. The parent molecule, rz.GH, is an all-RNA ribozyme, while rz.DRD and rz.RLoop have DNA in the base-pairing flanking sequences (and in stem-loop II in the case of rz.DRD). We have demonstrated that the inclusion of DNA in these ribozymes has marked effects on ribozyme activity, and can be used to create a more catalytically active, as well as a more stable ribozyme.

Under the conditions described in these studies, the all RNA ribozyme-substrate pair (rz.GH-rGAG) gave a biphasic time-course consisting of a rapid initial burst of product formation followed by a slower steady-state velocity. This pattern is similar to the burst velocity kinetics observed for several protein enzymes such as α -chymotrypsin, carboxypeptidase Y and liver alcohol dehydrogenase (18,20,21).

Figure 4. Sample cleavage data. All cleavage reactions were incubated at 55°C. The lanes are as follows: A, 100 nM substrate and 20 mM $MgCl_2$, with no ribozyme. B, 100 nM substrate, 10 nM ribozyme and no $MgCl_2$ and C-G, 10 nM ribozyme, 100 nM substrate and 20 mM $MgCl_2$ over the course of five time points. Substrate (rGAG or dGAGrUCA) and products (P) are labeled. Samples of rz.DRD cleavage of the RNA substrate (rGAG) were taken at 15, 30, 45, 60, and 75 min, while controls were incubated for 75 min. For the cleavage of the chimeric substrate (dGAGrUCA), time points were taken at 2, 4, 6, 8, and 10 min, with controls incubated for 10 min. Samples of the cleavage of rGAG by rz.RLoop were monitored at 1, 2, 3, 4, and 5 minutes with controls incubated for 5 minutes. The products of rGAG cleavage are identical in size (14 bases each) and comigrate as a single band. The dGAGrUCA substrate was 5' end labeled with ^{32}P , and thus only one of the two cleavage products is visible.

A burst velocity is characterized by a decrease in the enzymatic-velocity at the end of the first turnover event (when one equivalent of product has been produced per equivalent of active enzyme).

Burst velocities occur when the measurement of product precedes the rate-limiting step of the reaction. In the case of the ribozymes in this study, cleaved products formed in the first turnover event are quantitated prior to dissociation from the ribozyme because the measurement technique requires denaturing polyacrylamide gel electrophoresis. Equations allowing approximations of the non-rate-limiting steps utilizing Michaelis–Menten-type analyses for initial burst velocities have been developed (17) and are applicable to the present study. It is important to note that k_{catb} is directly comparable to k_{obs} in experiments where ribozyme concentrations exceed substrate concentrations (7,22,23).

The burst and steady-state values have been obtained for the all RNA ribozyme/RNA substrate pair rz.GH-rGAG, which is our standard for comparison with the other ribozyme sequences. The values for K_m and K_{mb} are not significantly different from one another, however, the k_{cat} value is less than one-tenth that of k_{catb} . These results suggest that the steps preceding and including catalysis can occur approximately once per four minutes per ribozyme. However, when product dissociation is also considered, the turnover drops dramatically, to approximately 50 minutes for one ribozyme to fully process each substrate molecule (Table 1).

Our study is the first kinetic analysis of chimeric DNA-RNA hammerhead ribozymes in which the complementary base-pairing sequences are DNA. These chimeric molecules, when base paired with an RNA sequence, create DNA-RNA hybrid helices. The stability of hybrid helices is related to their sequence composition. Sequences with a high G + C content tend to form less stable DNA-RNA helices as compared with the corresponding RNA-RNA helices (24,25,26). In contrast, AT-rich sequences tend to form more stable hybrid helices than their all-RNA counterparts (27). The base composition of the helices in the present study is somewhat GC-rich, with 14 out of 25 base pairs being G + C. The less stable helices of the DNA-RNA pairings in this study compared to the all-RNA helix may be a consequence of this base composition.

The first chimeric ribozyme we synthesized, rz.DRD, was designed to maximize the amount of DNA in the molecule. In this molecule, deoxyribose bases were substituted for all but 12 nucleotides, leaving as RNA only those nucleotides in the catalytic center. This chimeric molecule did not display a burst velocity (Fig. 3), suggesting that product dissociation was not the rate-limiting step for this ribozyme. However, the steady-state k_{cat} value was small, suggesting an alteration in the cleavage step.

In order to determine whether or not the structural feature of hybrid DNA-RNA helices were the cause of the slower cleavage rate, we designed a substrate that contained DNA at each of the base-pairing positions except the cleavage site and the two bases flanking the cleavage site, which were left as ribonucleotides. As was observed with the chimeric ribozyme rz.DRD with the rGAG substrate, the hybrid helices of rz.GH and dGAGrUCA resulted in a 50% increase in the K_m . Also, there was no burst velocity when dGAGrUCA was cleaved, suggesting that the rate limiting step was not product dissociation, but either cleavage or an event preceding cleavage. Due to the absence of a burst velocity, we believe that the rate limiting step for the rz.GH-dGAGrUCA combination is cleavage, but the 4.5-fold increase in k_{cat} suggests enhanced product dissociation as a consequence of the hybrid helices. The mechanism by which DNA causes a reduction in the cleavage rate of dGAGrUCA is not clear. The hybrid helices could affect the conformation of the catalytic core or the structure of the DNA flanks could exert an immediate

neighbor effect on the RNA at the cleavage site. The latter seems more likely as discussed below.

In order to examine the possible negative influence of having DNA in stem and loop II, we synthesized a third ribozyme in which this region, along with the catalytic center, were comprised entirely of RNA (rz.RLoop). Unlike rz.DRD, the rz.RLoop chimera cleaved the RNA substrate with the same burst velocity (k_{catb}) as rz.GH (Fig. 3 and Table 1) suggesting their cleavage rates to be the same. A sixfold increase in the steady-state k_{cat} of rz.RLoop as compared to rz.GH was also observed (Table 1). Since k_{cat} differs from k_{catb} mainly by the inclusion of the product dissociation value, these data suggest that the hybrid helices of rz.RLoop promoted faster product dissociation, with a resultant increase in k_{cat} . Lowered stability of the hybrid helices is also indicated by a greater K_m . Even though rz.RLoop has 25 bases capable of pairing with the target RNA substrate, the k_{cat} value obtained with this ribozyme for the RNA substrate rGAG approaches values obtained with much shorter regions of base pairing (28).

The enhanced activity of rz.RLoop suggests that the poor activity of rz.DRD is not caused by DNA in stem I or stem III, but rather the DNA in stem-loop II. The extent to which DNA in stem II slowed the cleavage reaction was surprising considering that some ribo- to deoxyribo-substitutions within the conserved catalytic core region of another ribozyme had less inhibitory effects (10). DNA may not be tolerated in stem II since the C2'-endo (B form) conformation of a DNA helix in stem II could interfere with the formation of the catalytically active structure. Alternatively, the DNA in stem II may be unstable at the elevated temperatures used for cleavage, which may in turn affect the cleavage reaction itself. This latter explanation seems more likely based on unpublished observations (K.Taira) which demonstrate a chimeric ribozyme analogous to our rz.DRD but with shorter flanking sequences, is more active than an all-RNA ribozyme at 37°C.

Our experiments provide the first demonstration of site-specific ribozyme catalysis involving both chimeric ribozymes and a chimeric substrate that create DNA helices in the base-pairing stems. X-ray diffraction studies have shown the DNA conformation in RNA-DNA hybrid helices to be in the A form, similar to RNA (25,29) [although other conformations may be possible (30)]. The structure of DNA in a full DNA helix is C2'-endo (B form), yet the k_{cat} values for our chimeric ribozyme-chimeric substrate pairs (rz.DRD-dGAGrUCA and rz.RLoop-dGAGrUCA) were similar to those of rz.GH-dGAGrUCA, which has hybrid helices. Thus, structural differences in helix geometry had surprisingly little effect on the overall reaction kinetics.

It is important to consider the rate-limiting step of a reaction, as well as factors which affect it. The catalytic activity of hammerhead ribozymes with short base-pairing flanking sequences is limited by the cleavage step (28). The presence of burst kinetics in this study demonstrates that product dissociation can be the rate-limiting step for ribozymes with longer base-pairing flanks. Goodchild and Kohli (31) reported a significant decrease in ribozyme-mediated turnover of substrate when the length of base-pairing sequences was increased from 12 to 20. Our data suggests that this decrease is due to a decrease in the rate of product dissociation. Therapeutically useful ribozymes must have a minimum number of antisense base-pairs to insure target specificity in the human genome (16–17) and to facilitate rapid and stable binding (low K_m) to that target in a cellular milieu (32). The overall activity of therapeutically useful

ribozymes will, therefore, probably be limited by product dissociation. Decreasing the stability of the helices in stems I and III by incorporating DNA can facilitate product dissociation. *In vivo*, the DNA containing flanks may serve to further accelerate product dissociation since RNA in the DNA-RNA helices is a potential substrate for cellular RNaseH.

Enhancement of ribozyme stability *in vivo* is an important consideration for enhancing ribozyme function. Using cationic liposome mediated delivery of ribozymes into cells in culture, we found that the DNA flanked ribozyme, rz.RLoop, was twofold more stable than the all-RNA ribozyme of the same sequence. We attribute this increase in stability to the DNA in the flanking sequences. In support of this conclusion, we have observed that an all DNA molecule of the same sequence had an even greater stability than the chimeric molecules when it was transfected into CV1 to cells via cationic liposomes. (74% of counts detected at time zero remained after 48 h, data not presented). Recently, experiments utilizing a DNA-RNA chimeric ribozyme targeted to the CML-specific bcr-abl fusion transcript showed significant reductions in the levels of bcr-abl mRNA transcripts and the p-210 encoded protein. This chimeric ribozyme inhibited expression to a greater extent than the homologous all-RNA ribozyme (D. Snyder *et al.*, personal communication). These results demonstrate intracellular activity as well as enhanced stability of chimeric ribozymes in another cell line, thus extending the general usefulness of these molecules.

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