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A ribozyme with DNA in the hybridising arms displays enhanced cleavage ability

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ABSTRACT

Hammerhead ribozymes cleave RNA substrates containing the UX sequence, where X = U, C or A, embedded within sequences which are complementary to the hybridising 'arms' of the ribozyme. In this study we have replaced the RNA in the hybridising arms of the ribozyme with DNA, and the resulting ribozyme is many times more active than its precursor. In turnover-kinetics experiments with a 13-mer RNA substrate, the k_{cat}/K_m ratios are 10 and 150 $\mu M^{-1}min^{-1}$ for the RNA- and DNA-armed ribozymes, respectively. The effect is due mainly to differences in k_{cat}. In independent experiments where the cleavage step is rate-limiting, the DNA-armed ribozyme cleaves the substrate with a rate constant more than 3 times greater than the all-RNA ribozyme. DNA substrates containing a ribocytidine at the cleavage site have been shown to be cleaved less efficiently than their all-RNA analogues; again however, the DNA-armed ribozyme is more effective than the all-RNA ribozyme against such DNA substrates. These results demonstrate that there are no 2'-hydroxyl groups in the arms of the ribozyme that are required for cleavage; and that the structure of the complex formed by the DNA-armed ribozyme with its substrate is more favourable for cleavage than that formed by the all-RNA ribozyme and its substrate.

INTRODUCTION

Ribozymes are RNA molecules that can cut or ligate other nucleic-acid molecules (usually RNA) in a catalytic fashion (1, 2). The hammerhead ribozyme is one of the best-known ribozymes. It has been studied extensively in isolated chemical systems (3–7), and used in gene-control studies in living cells (8–14). A hammerhead ribozyme as defined by Haseloff and Gerlach (5) is shown in Figure 1. It contains two stretches of conserved nucleotides (boxed), a stem-loop structure (bases 18–29) containing helix II, and flanking nucleotides which form double-helices I and III in combination with the substrate.

The instability of ribozymes in living cells is a major concern. One approach taken to protect transcribed ribozymes from nuclease attack in cells has been to embed the ribozyme in a larger, folded structure. Thus, hammerhead ribozymes have been placed next to the anti-codon loop in t-RNA^{met} (8), in the 3' untranslated region of the luciferase gene (9), and in a molecule with a bacteriophage T7 transcription terminator at its 3' end (13). These ribozymes appeared to be more stable than the corresponding, unprotected ribozymes; however, in the only comparative study, the stabilized ribozyme did not cleave more target RNA than the shorter-lived ribozyme, indicating that the protecting structure may have decreased the specific activity of that ribozyme (13).

An alternative approach has been to chemically synthesize ribozymes with ribonucleotides modified at the 2'-position. The modified nucleotides have included 2'-deoxy-, 2'-fluoro-, 2'-amino-, 2'-O-allyl- and 2'-O-methyl-ribonucleotides (15–20). A ribozyme consisting predominantly of 2'-O-allyl ribonucleotides displayed greatly improved stability compared to an unmodified ribozyme in the presence of bovine serum (20). Modifications to nucleotides in the hybridizing arms and/or in helix II of the ribozyme have little effect on catalytic efficiency (17–20); for example, substitution of the 2'-hydroxyl groups with 2'-O-allyl groups in all non-conserved nucleotides of a hammerhead ribozyme resulted in full retention of activity (20). On the other hand, changing the 2'-substituent in any of the conserved nucleotides of the ribozyme resulted in a decrease in catalytic activity, the magnitude of which varied greatly depending on the number of changes, the nature of the change, and the particular nucleotides modified (15–20).

Ideally, any ribozyme which has been stabilized against intracellular degradation should also be reasonably active. In the course of our work on reducing the size and RNA-content of the hammerhead ribozyme, we produced a minimized ribozyme in which the stem-loop of helix II was replaced by four ribonucleotides (21). This 'minizyme' was less active, at cleaving a synthetic substrate of 21 nucleotides, than its parent ribozyme which contained a helix II. However, the cleavage activity of the minizyme increased unexpectedly, when the RNA nucleotides in the hybridizing arms of the minizyme were replaced by DNA (21). Here we investigate whether a similar effect might be obtained for an all-RNA hammerhead ribozyme, when the RNA nucleotides in its hybridizing arms are also replaced with DNA.

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MATERIALS AND METHODS

All oligonucleotides were prepared by solid-phase methods using 2′-silyl-protected phosphoramidites (Milligen) for RNA (benzoyl-protected A, G and C) and phosphoramidite monomers for DNA (Applied Biosystems). The oligonucleotides were deprotected as described earlier (21), and purified by electrophoresis on 10–20% polyacrylamide gels (depending on the length of the oligonucleotide) containing 7M urea, also as described (21). The purity of each oligonucleotide was checked by labelling its 5′-terminus with ^{32}P phosphate using T4 polynucleotide kinase and γ-^{32}P ATP, separating the molecules on a 10–15% polyacrylamide gel containing 7M urea, and visualizing the molecules by autoradiography. The concentrations of the purified oligonucleotides were determined by UV spectroscopy using the following molar extinction coefficients for the various nucleotides at 260 nm: A, 15.4×10^3; G, 11.7×10^3; C, 7.3×10^3; T, 8.8×10^3; U, 10.0×10^3. All oligonucleotides were stored in either water or 10 mM Tris.Cl, pH 8.0, 0.2 mM EDTA at −20°C.

Enzyme kinetic experiments were conducted in 50 mM Tris.Cl, pH 8.0, 10 mM $MgCl_2$, at 30°C. The concentration of the all-RNA substrate (S13, Figure 1) ranged from 10 to 200 nM, and the concentration of both ribozyme 1 and ribozyme 2 (Figure 1) was 0.77 nM. Initial-rate measurements were made up to 15% cleavage of the substrate. Neither ribozyme nor substrate were heat-treated before initiating the reaction by adding the substrate. Reactions in 20–40 μL volumes were performed in 96-well polypropylene tissue-culture trays, as these were found to adsorb less ribozyme or substrate than either siliconized or autoclaved Eppendorf tubes. Samples of 2–3 μL were removed at given times and quenched with 2 volumes of 80% formamide containing 20 mM EDTA and dye. Samples were analysed by electrophoresis on 15% denaturing, polyacrylamide gels, followed by autoradiography and excision of the bands corresponding to the substrate and 5′ cleavage product. The amounts of radioactivity in the bands were quantitated by Cerenkov counting. Enzyme kinetic data were analysed by Eadie–Hofstee plots to yield K_m and V_{max}. It was found that K_m and V_{max} from individual experiments varied by up to a factor of 2, and therefore the initial rates at each concentration, from at least 4 independent experiments, were averaged and plotted in the form of rate *versus* rate/[substrate] to yield the kinetic parameters in Table I.

Experiments with ribozyme in excess of the substrate S13 were also conducted in 50 mM Tris.Cl, 10 mM $MgCl_2$, at 30°C; the concentrations of the ribozymes were 1.5 μM and the substrate was 500 nM. The ribozyme and substrate were heated together to 75°C for 3 minutes, then allowed to cool to 30°C for 1–2 minutes before initiating the reaction by adding the magnesium-containing buffer. The kinetic parameters were obtained by fitting the data for percentage of product formed *versus* time to the equation:

$$P_t = P_\infty - (\exp(-kt)P_\Delta)$$

where P_t is the percentage of product at any given time, P_∞ is the percentage of product at $t = \infty$, k is the first-order rate constant for the reaction, t is the time, and P_D is the difference between the percentage of product at $t = \infty$ and $t = 0$. This is a conventional first-order kinetic equation from which k, P_∞ and P_Δ are determined by least-squares fitting of the data. The quoted rate constants and P_∞ values in Table II are the mean (± SD) for at least 2 independent experiments. In the time-scale of these experiments, the reactions do not proceed to 100%.

Kinetics of cleavage of the DNA substrate containing a ribocytidine at the cleavage site (S21D, Figure 1) were conducted under conditions identical to those described above for the experiments with S13, except that the concentrations of the ribozymes were 1.5 μM and the substrate was 200 nM.

Stability of the ribozymes in serum was determined by incubating the ribozymes, labelled at the 5′ end with ^{32}P phosphate, in various concentrations (5 to 0.01%) of foetal calf serum (Cytosystems). Samples were removed at various times and added to 2 volumes of 80% formamide containing 20 mM EDTA and dye, and then quickly frozen in dry ice. The solutions were thawed immediately prior to loading on to a 10% denaturing (7 M urea) polyacrylamide gel for analysis. Percentage ribozyme remaining at these times was determined by excising gel fragments corresponding to the position of the full-length ribozyme and quantitating by Cerenkov counting.

RESULTS AND DISCUSSION

Cleavage of the RNA substrate

Ribozyme 1 is an all-RNA hammerhead ribozyme with the sequence shown in Figure 1. Ribozyme 2 is an analogous ribozyme in which the non-conserved nucleotides in the hybridizing arms, underlined in Figure 1, are DNA, and all other nucleotides are RNA. In previous work, we have observed that when helix II was removed from ribozyme 1 and replaced by a 4-nucleotide linker, a very large proportion of the resultant loss in activity could be regained by placing DNA in the hybridising arms of the minimized ribozyme (21). Therefore, we synthesized ribozyme 2 in order to see if an increase in activity also could be achieved by placing DNA in the hybridizing arms of a normal hammerhead ribozyme.

The cleavage reactions were analysed according to Scheme 1, using Michaelis-Menton equations. In the scheme, S is substrate, R is ribozyme, and P_1 and P_2 are the cleavage products. The scheme is a simplification of the real situation since (i) it does not allow for alternate conformations of any of the participants and (ii) the dissociation of the products of the reaction is undoubtedly a multistep process. However, it is acceptable to approximate the product dissociation to a single step since one of the products, by virtue of its G/C content, is expected to dissociate from the ribozyme very much more slowly than the other; and the question of alternate conformations will be addressed as it arises.

$$S + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_1P_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} R + P_1 + P_2$$

Scheme 1

Table I shows the values of the catalytic constant, k_{cat}, ($k_{cat} = V_{max}/[\text{ribozyme}]$) and the Michaelis constant, K_m, for ribozymes 1 and 2 in reactions with an RNA substrate of 13 nucleotides at 30°C. k_{cat} for ribozyme 2, 8.9 min^{-1}, is 20-fold higher than for ribozyme 1. The K_m's for the two ribozymes differ slightly, with ribozyme 1 having the lower value, 38 nM. The ratio k_{cat}/K_m, which is often used as a measure of the relative efficiency of enzymatic activity, is 10 $min^{-1}\mu M^{-1}$ for ribozyme 1 and 150 $min^{-1}\mu M^{-1}$ for ribozyme 2. The catalytic constant

of 8.9 min^{-1} for the DNA-armed ribozyme in reaction with an RNA substrate is, to our knowledge, the highest yet observed for any ribozyme with Mg^{2+} as the activating ion. A value of k_{cat} of 41 min^{-1} has been reported by Olsen *et al.* (17) for a hammerhead ribozyme activated with Mn^{2+}, but the same ribozyme activated by Mg^{2+} has a k_{cat} of only 2.1 min^{-1} at 25°C.

Experiments to measure the first-order rate constant for cleavage of the substrate (data in Table II) were conducted under conditions where all the available substrate was expected to be bound to ribozyme. The substrate concentration of 500 nM was about 10-fold greater than the K_m for the reactions, and the ribozyme was present in 3-fold excess over the substrate. In addition, the ribozyme and substrate were heated together in the absence of Mg^{2+} and then cooled together to the reaction temperature, thus assisting the formation of ribozyme-substrate complexes. The reaction was initiated by the addition of the Mg^{2+}-containing buffer. Under these conditions the rate-limiting step is almost certainly the cleavage step, since the ribozyme-substrate complex should be fully formed, the addition of Mg^{2+} is expected to be rapid, and the product dissociation is irrelevant in these conditions. Hence, the observed rate constant equals k_2 in scheme 1 and should equal k_{cat} for the turnover reaction, if in the turnover reactions the cleavage step is rate-determining.

The rate constant, k_2, observed for the cleavage of S13 by ribozyme 2, 5 min^{-1}, is slightly less than the k_{cat} of 8.9 min^{-1} observed for the reaction under similar conditions. This difference is not significant, given the following experimental limitations. In the turnover experiments to determine k_{cat}, absolute concentrations of both the ribozyme and the substrate are important in determining the kinetic parameters. Additionally, the trace amounts of ribozyme used make the results of the experiments subject to error due to adsorption of the ribozyme on to the surface of the reaction vessels. In the experiments to independently determine k_2, the kinetic data are independent of absolute concentration of both ribozyme and substrate; however, in this case, the rates of the reactions ($t^1/_2$ ~ 8 seconds for ribozyme 2) make it difficult to determine the rate constants accurately. Therefore, it is reasonable to assume that the limiting step in the turnover reaction for ribozyme 2 is the cleavage step.

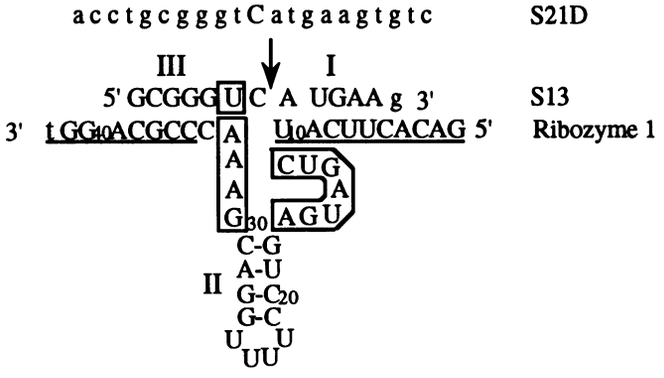

Figure 1. Sequences of the ribozymes and substrates used in this study. Lower case letters are deoxyribonucleotides, upper case letters are ribonucleotides, and boxed letters are conserved nucleotides. Ribozyme 2 has the same sequence as ribozyme 1, except that the underlined letters are all deoxyribonucleotides in ribozyme 2 with U_6, U_7 and U_{10} being replaced by T.Roman numerals label double helices. All oligonucleotides used in this study have a 3'-deoxy-ribonucleotide.

On the other hand, the observed rate constant of 1.6 min^{-1} for the cleavage of S13 by ribozyme 1 is 4-fold greater than the k_{cat} of 0.4 min^{-1} for the turnover reaction. This discrepancy is too large to be accounted for by experimental error, and it suggests that for ribozyme 1 the cleavage step is not rate-determining. In the cleavage of S13 under conditions where the ribozymes were in excess, less than 100% of the substrate was cleaved; this is due to the formation of inactive ribozyme-substrate complexes (21). Heat-pulsing the reaction mixture to 75°C for 3 minutes, followed by incubation at 30°C, allows the reaction to proceed further (data not shown). This observation suggests that the measured values of k_{cat} underestimate the activity of the ribozymes in the active complex. The error is likely to be more significant for ribozyme 1 than for ribozyme 2, since in the experiments with ribozyme in excess only 50% of S13 was cleaved by ribozyme 1 but 62% was cleaved by ribozyme 2 (Table II). Assuming this to be the case for the turnover experiments as well, then the measured k_{cat} of 0.4 min^{-1} for ribozyme 1 (the average for both the inactive and active complexes) should be increased to ~0.8 min^{-1} for the active complex alone. This value is closer to the independently-measured k_2, but still differs from it by a factor of 2, and so alternatives to the cleavage reaction being rate-determining must be considered. If the dissociation of one or both products from the ribozyme were rate limiting, a burst of product would be observed at the beginning of the reaction; this was not observed for either ribozyme. If the rate of formation of complex were rate limiting for ribozyme 1, as may be the case, then the likelihood is that ribozyme 1, when uncomplexed, adopts a conformation which is not readily able to bind the substrate. This conformation must be stabilized, relative to ribozyme 2, by the all-RNA hybridising arms. The rate-limiting step then becomes the rate of unfolding of this ribozyme into a form capable of binding the substrate, and this unfolding rate may be exacerbated by longer-than-necessary hybridising arms. Further experiments are necessary to unambiguously determine the rate-limiting step for ribozyme 1.

The K_m's for DNA and RNA substrates with all-RNA ribozymes have been found to differ by 6- to 16-fold in one study (22) and were estimated to be ~100-fold greater for the DNA substrate in another (23). In contrast, in our study where the DNA is introduced into the ribozyme, the K_m for ribozyme 2 with the RNA substrate is only 1.5-fold greater than found for ribozyme

Table I. Results of turnover experiments with substrate S13

Ribozyme	k_{cat} (min^{-1})	K_m(nM)	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
ribozyme 1	0.4	38	10
ribozyme 2	8.9	59	150

Conditions: 30°C, pH 8.0, 10 mM $MgCl_2$.

Table II. Rate constants and % product formed at infinite time (calculated) for the cleavage of substrate S13 (all RNA) and substrate S21D (DNA with ribo C at cleavage site) by ribozymes 1 and 2 when in excess over substrate

Ribozyme	k_2 (min^{-1})	% P_∞
ribozyme 1 + S13	1.6±0.6	49±3
ribozyme 2 + S13	5.0±1.0	61.5±2
ribozyme 1 + S21D	0.044±0.004	50±1
ribozyme 2 + S21D	0.12±0.01	70±1

Conditions as for Table I.

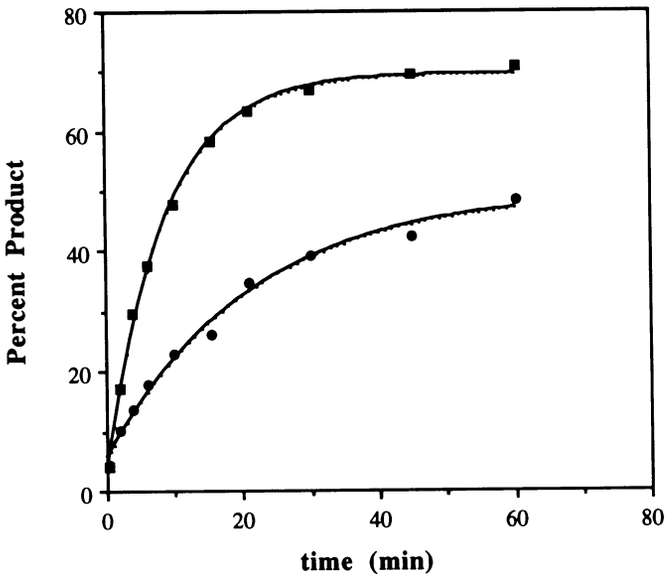

Figure 2. Percent product versus time for reaction of ribozyme 1, ●, and ribozyme 2, ■, with substrate S21D at 30°C, 50 mM Tris.Cl, pH 8.0 and 10 mM $MgCl_2$; concentration of ribozyme was 1.5 μM and substrate, 200 nM. Solid lines are lines of best fit to the data, using the first-order kinetic equation given in the Methods section.

1. In Michaelis–Menton kinetics, $K_m = (k_{-1} + k_2)/k_1$. k_2 has been measured for both ribozymes and is 3-fold greater for ribozyme 2, and k_{-1} is expected to be greater for ribozyme 2 than ribozyme 1 because of the expected, relatively weaker binding in DNA/RNA duplexes. It follows therefore that k_1 for ribozyme 2 must also be greater than for ribozyme 1 in order for the difference in K_m's to be as small as observed. This reduction in apparent k_1 for ribozyme 1 relative to ribozyme 2 supports the above speculation about an alternate conformation for ribozyme 1 which limits the rate of association with substrate.

Cleavage of the DNA substrate

The cleavage of the DNA substrate, S21D, by the two ribozymes was performed under conditions where the ribozyme-substrate complex was expected to be fully formed prior to addition of Mg^{2+} to initiate the reaction. The substrate in this case was chosen to be 21 nucleotides to compensate for the expected weaker binding of ribozymes to a DNA substrate compared with an RNA substrate. Use of the longer substrate should not result in serious discrepancies in comparing data, since the rate of cleavage of the complex is not expected to depend strongly on the length of the substrate. Figure 2 shows an example of the data used to determine the rate constants. The data fit the first-order model well. The observed rate constants for cleavage of the DNA substrate are given in the lower half of Table II. k_2 for ribozyme 2 (0.12 min^{-1}) is 3 times greater than for ribozyme 1, the same ratio as observed for cleavage of the all-RNA substrate by these ribozymes. The absolute values for rates of cleavage of the DNA substrate are approximately 40-fold less than observed for the all-RNA substrate, and are of the order observed in earlier studies (22,23). Why should DNA in the substrate decrease the rate of cleavage, but DNA in the arms of the ribozyme increase the rate? It is known that the 2′-hydroxyl of the uridine immediately 5′ to the cleavage site on the substrate is involved in binding the Mg^{2+} ion (22). However, even if both that important uracil and the cytosine at the cleavage site are ribonucleotides, and the remainder are deoxyribonucleotides, such a substrate is still not cleaved as efficiently as an all-RNA substrate (22). Therefore, either there are other 2′-hydroxyl groups in the substrate that are directly involved in stabilizing the active complex, or the structure of the complex formed by the ribozyme and the predominantly-DNA substrate is sufficiently different from that formed by the ribozyme with the all-RNA substrate to result in a slightly different arrangement of the crucial groups involved in the reaction. Our observation that activity does not decrease when DNA is substituted into the arms of the ribozyme indicates there are no crucial 2′-hydroxyl groups in the arms, at least in positions 1–10 and 35–42 (Figure 1). This result is consistent with the observations of Paolella *et al.* (20) which showed complete retention of activity by a ribozyme in which all 2′-hydroxyl groups in the hybridizing arms had been replaced by 2′-O-allyl. The fact that the rate of cleavage *increases* with DNA in the arms of the ribozyme suggests that most likely there is a subtle change in the conformation of the resulting double-helix allowing a more favourable positioning of the critical groups involved in the cleavage reaction. Thus, it may be expected that differences between the effects of all-RNA ribozymes and analogous DNA-armed ribozymes against specific targets will vary with the sequence of the target, as the local structure and flexibility of the helices formed will depend also on the sequence.

Stability of ribozymes in serum

Ribozymes 1 and 2 were subjected to degradation in foetal calf serum in order to investigate their relative stabilities. At all concentrations of serum between 5% and 0.01%, no significant differences were observed. At a serum concentration of 0.1%, the half-life of both ribozymes was around 1–2 minutes. Ribozyme 1 was cleaved into small fragments with no preferred cleavage sites. Ribozyme 2, on the other hand, was initially cleaved between G_{13} and A_{14} and, subsequently, after T_{10} to yield a 10-mer of DNA which was relatively stable and constituted >90% of the 5′ end-labelled material observed on the gel after 20 minutes incubation in 0.1% serum. At higher serum concentrations and longer times the 10-mer product was degraded to a 9-mer, but no shorter fragments appeared even after 60 minutes in 5% serum. Clearly the DNA portion of ribozyme 2 is several orders of magnitude more stable than the RNA. The observation that both ribozyme 1 and ribozyme 2 are degraded in serum at the same rate, without significant degradation of the DNA portion of ribozyme 2, implies that RNA endonucleases are predominantly responsible for the degradation in this medium. In contrast, the DNA portion appears to be very slowly degraded, largely, if not exclusively, by 3′ exonucleases.

CONCLUSIONS

The ribozymes described here differ in their efficiency, as measured by k_{cat}/K_m, by a factor of 15. The directly-measured first-order rate constants for the cleavage reactions differ only 3-fold. The discrepancy probably arises largely from different rate-determining steps for the two reactions. Under the conditions used in this study, the rate-determining step for the all-RNA ribozyme may be association with substrate, whereas for the DNA-armed ribozyme it is the cleavage reaction. The question of the rate-determining step for ribozyme 1, whilst interesting, does not affect the conclusions of this communication which are

(i) that a DNA-armed ribozyme is an order of magnitude more active than its all-RNA analogue and (ii) that this difference originates, in part, in the cleavage step. If the observation holds generally, or even for a subset of ribozymes, it means that DNA-armed ribozymes will be very useful as starting molecules for the introduction of further modifications designed to protect the conserved nucleotides against nuclease attack: they possess enhanced cleavage activity and nuclease-resistant hybridising arms.

Since the submission of this paper, Taylor *et al.* (24) have reported data on a DNA-armed ribozyme that has 6-fold greater catalytic activity than an analogous all-RNA ribozyme, when targeted against a 28-mer RNA substrate at 55°C. They attributed the difference in effect to the faster rate of dissociation of the cleavage products from the DNA-armed ribozyme. They also investigated the stability of the ribozymes introduced into cells with Lipofectin, and found that the DNA-armed ribozyme survived longer in cells than the all-RNA ribozyme. Our observations, that a DNA-armed ribozyme displays faster rates of cleavage and faster turnover at 30°C compared to an all-RNA ribozyme, and the observations of Taylor *et al.* (24), of increased turnover rates on a different sequence, together imply a general usefulness for these types of molecules.

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