Optimizing the Substrate Specificity of a Group I Intron Ribozyme†

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ABSTRACT: Group I ribozymes can repair mutant RNAs via *trans*-splicing. Unfortunately, substrate specificity is quite low for the *trans*-splicing reaction catalyzed by the group I ribozyme from *Tetrahymena thermophila*. We have used a systematic approach based on biochemical knowledge of the function of the *Tetrahymena* ribozyme to optimize its ability to discriminate against nonspecific substrates in vitro. Ribozyme derivatives that combine a mutation which indirectly slows down the rate of the chemical cleavage step by weakening guanosine binding with additional mutations that weaken substrate binding have greatly enhanced specificity with short oligonucleotide substrates and an mRNA fragment derived from the *p53* gene. Moreover, compared to the wild-type ribozyme, reaction of a more specific ribozyme with targeted substrates is much less sensitive to the presence of nonspecific RNA competitors. These results demonstrate how a detailed understanding of the biochemistry of a catalytic RNA can facilitate the design of customized ribozymes with improved properties for therapeutic applications.

Repair of defective RNAs represents a potential new approach to genetic therapy (*1*). Group I ribozymes can accomplish RNA repair through a two-step *trans*-splicing reaction closely related to their natural self-splicing activity (Figure 1). To repair a mutant RNA, the ribozyme first cleaves the defective transcript upstream of the mutated site and then ligates a wild-type version of the sequence onto the upstream cleavage product. By use of model substrates and the group I ribozyme from *Tetrahymena thermophila*, the feasibility of RNA repair by *trans*-splicing has been demonstrated in vitro as well as in *Escherichia coli* and in mammalian cells (*2*−*4*). More recently, mutant transcripts associated with common genetic diseases (*5, 6*) have been repaired in clinically relevant cells (*6*).

Despite these successes, as is the case for all novel therapeutic agents, significant obstacles must be overcome before ribozyme-mediated RNA repair can become an effective therapeutic strategy (*7*). Although *trans*-splicing of up to 50% of a simple model substrate has been observed in mammalian cells (*4*), repair of similar percentages of clinically important transcripts in therapeutically appropriate settings may be difficult to achieve (*6*). Improvements in efficiency will therefore likely be required to induce desired phenotypic changes in treated cells. A second, and related, problem is the low substrate specificity of *trans*-splicing. In vivo, the *Tetrahymena* ribozyme reacts with numerous cellular transcripts in addition to its intended target (*3*). Low specificity may result in unwanted effects on the cell, as well as limiting the efficiency of repair of the targeted transcript, as each ribozyme molecule can deliver only a single wild-type corrective sequence. If this sequence is spliced onto a nontargeted RNA it will not be available for repair of the intended target.

Two possible reasons exist for the low in vivo specificity of *trans*-splicing (*8*). One is the limited extent of base pairing that occurs between ribozyme and substrate. The *Tetrahymena* ribozyme recognizes and binds its substrates through base pairing to a six-nucleotide internal guide sequence (IGS)[1] at the 5′ end of the ribozyme (Figure 2) (*9, 10*). While different sequences may be targeted by altering the IGS (*9, 11*), any six-nucleotide sequence is most likely present in a variety of cellular RNAs. The second possible reason is that the ribozyme may react at sites that are not fully complementary to the IGS. In vitro experiments have shown that the ribozyme does not discriminate well against such mismatched substrates (*12, 13*). This also appears to be the case in mammalian cells, where reaction at sites that form mismatches with the guide sequence can be observed (P.P.Z. and B.A.S., unpublished results). Significant improvements of in vivo specificity may therefore be achieved if group I ribozyme variants can be developed that do not react at mismatched sites.

While several mutations have been described that improve the ability of the *Tetrahymena* ribozyme to discriminate against mismatched substrates (*14*), no concerted effort has yet been made to optimize specificity. Guided by the increasingly detailed understanding of the structure and activity of this group I intron (*15*−*19*), we have taken a systematic approach to begin to solve the problem of low specificity of *trans*-splicing/RNA repair. A ribozyme deriva-

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[1] Abbreviations: IGS, internal guide sequence; pG, guanosine 5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid.

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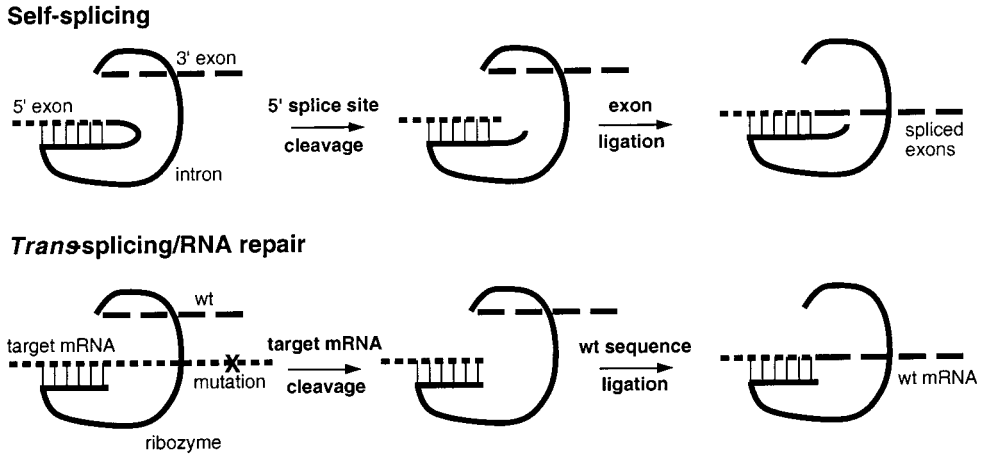

FIGURE 1: RNA repair through group I intron *trans*-splicing. The *trans*-splicing/repair reaction is analogous to the natural self-splicing reaction, except that prior to the cleavage step the target mRNA is not covalently attached to the ribozyme.

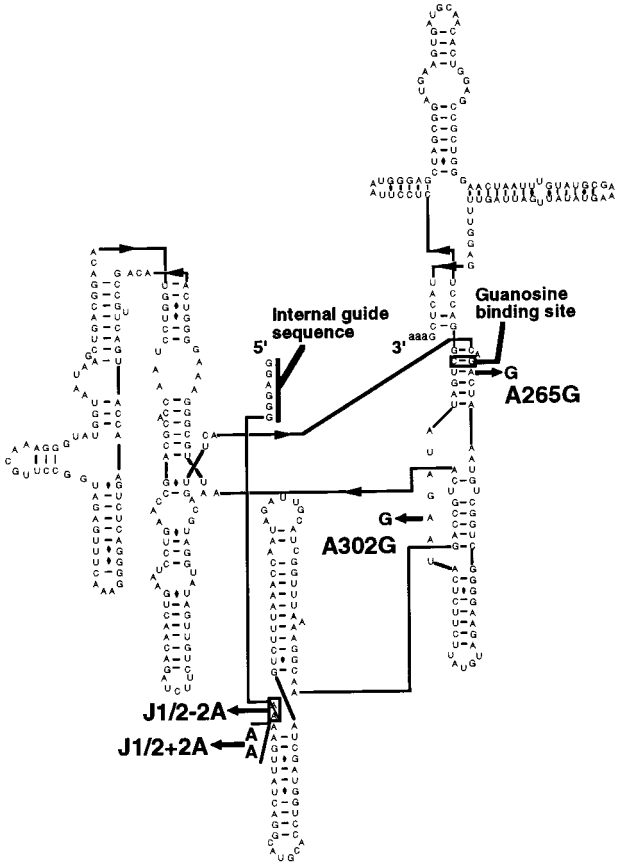

FIGURE 2: Secondary structure of the L-21A_3 version of the *Tetrahymena* ribozyme. The three adenosines of the 3′ exon are in lowercase letters. The internal guide sequence is indicated, and the base pair proposed to interact with guanosine is boxed. Mutations tested for increased specificity are shown.

tive with two mutations, each affecting catalysis in a different way, was found to discriminate between cleavage of matched and mismatched substrates as much as 150-fold better than the wild-type ribozyme. We also show that, in contrast to the wild-type ribozyme, the efficiency of reaction with this more specific ribozyme is not affected when a matched target RNA has to compete with mismatched substrates for ribozyme binding, as is the case in vivo. Moreover, the more specific ribozyme forms fewer undesired products when reacting with a fragment of the p53 mRNA, a therapeutically relevant transcript. The design approach we have employed contrasts with in vitro selection strategies commonly used to alter ribozyme activities (*20*, *21*) and demonstrates how the available biochemical knowledge of ribozyme activity may be exploited to further the development of therapeutically useful ribozymes.

MATERIALS AND METHODS

Ribozymes and Substrates. The L-21A_3 ribozyme was transcribed as described previously (*22*), using a template generated from plasmid pT7L-21 (*13*) by PCR with *Pfu* polymerase (Stratagene), which does not add nontemplated nucleotides and thus minimizes 3′ end heterogeneity in the final transcript. The 5′ PCR primer (5′-ATT TCA CAC AGG AAA CAG CTA TG-3′) hybridizes upstream of the T7 promoter and was used as supplied by the manufacturer. The 3′ primer (5′-TTT CGA GTA CTC CAA AAC TAA TC-3′) is complementary to the last 20 nucleotides of the intron, followed by an overhang to encode the three adenosine residues that constitute the 3′ exon, and was purified on a 20% polyacrylamide gel prior to use to further limit length heterogeneity of the transcription template. Mutations were introduced into pT7L-21 by standard PCR methods, and transcription templates were then produced as for the wild-type ribozyme. Transcription conditions were 40 mM Tris (pH 7.5), 5 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 4 mM each nucleoside triphosphate, 500 units of T7 RNA polymerase, and 0.5−1.5 μg of PCR product per 100 μL volume. $MgCl_2$ concentrations were kept low to avoid 3′ exon hydrolysis during transcription (*23*). Ribozymes were purified on denaturing 6% polyacrylamide gels and ethanol-precipitated, and concentrations were determined by measuring the OD_{260}, assuming 1 OD_{260} = 40 μg/mL.

The matched (CCCUCUAAAAA), −3G (CCCGCUAAAAA), and −3C−2U (CCCCUUAAAAA) substrates were purchased from Dharmacon Research Inc. (Boulder, CO), deprotected according to the manufacturer's instructions, and purified on denaturing 20% polyacrylamide gels. Concentrations were determined by measuring the OD_{260}, assuming 1 OD_{260} = 20 μg/mL. They were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol) and gel-purified, and concentrations were estimated by comparing the counts per unit volume to known standards of [γ-^{32}P]-ATP with a Storm Imager (Molecular Dynamics).

The p53 substrate was transcribed from a PCR product obtained by amplifying the desired region of the p53 coding sequence from the cDNA cloned into the pBluescript SK vector (Stratagene), using a 5′ PCR primer that contained a T7 RNA polymerase promoter (TAA TAC GAC TCA CTA TAG GGC GAT GGA GGA GCC GCA GTC AGA) and an internal 3′ primer (TAG CTG CCC TGG TAG GTT TT). Transcription conditions were as above, except that nucleotide concentrations were 1 mM each ATP, GTP, and UTP, 0.1 mM CTP, and 150 μCi of [α-^{32}P]CTP (3000 Ci/mmol). Transcripts were purified on denaturing 6% polyacrylamide gels, and concentrations were estimated as described for radiolabeled oligonucleotide substrates.

Kinetics. All reactions were single-turnover, with trace concentrations of radiolabeled substrate (final concentrations were ~0.2 nM for the oligonucleotide and ~1 nM for the p53 substrates) and ribozyme excess. Ribozyme in 20 μL buffer (50 mM Tris, pH 7.5) was heated to 95 °C for 1 min, followed by addition of 20 μL of ice-cold buffer (50 mM Tris, pH 7.5, and 20 mM $MgCl_2$) containing enough magnesium to give a final concentration of 10 mM. The ribozyme was then allowed to fold for 3 min at 37 °C before the reaction was initiated by adding substrate and guanosine 5′ monophosphate (pG), preequilibrated for 4 min at 37 °C, in 40 μL of buffer (50 mM Tris, pH 7.5, and 10 mM $MgCl_2$). Reactions were performed at 37 °C, and at least eight time points were taken at regular intervals by removing 5 μL aliquots from the reactions and adding them to 5 μL of stop solution (90 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol in 88% formamide). Reactions with the p53 substrate were the same, except that this longer substrate was denatured at 95 °C in the absence of $MgCl_2$, as described for the ribozyme, before addition of $MgCl_2$ and pG and was then equilibrated at 37 °C for 7 min before addition to the ribozyme. Products were separated on denaturing polyacrylamide gels and quantitated with a Storm Imager (Molecular Dynamics). Rate constants were obtained either by fitting the data to single exponentials or, for slow reactions, by fitting a plot of the natural logarithm of the fraction substrate remaining (corrected for an end point that was assumed to be the same as that observed in faster reactions with the same preparation of substrate) vs time to a straight line, where k_{obs} is the slope of the line. $(k_{cat}/K_m)^{S'}$ values were obtained in one of two ways. In most cases, at least four experiments were performed in the range where k_{obs} varies linearly with the ribozyme concentration. A plot of k_{obs} vs ribozyme concentration gives a straight line, and the slope is equal to $(k_{cat}/K_m)^{S'}$. For ribozyme/substrate combinations where the $K_{1/2}$ was very low, k_{obs} was measured over a broader range of ribozyme concentrations and the data were fit to the equation $k_{obs} = (k_{cat}[E]/(K_{1/2} + [E]))$, where [E] is the ribozyme concentration. $(k_{cat}/K_m)^{S'}$ corresponds to the initial slope of the plot and was determined from k_{cat} and $K_{1/2}$.

RESULTS

Modeling RNA Repair. The ultimate goal of this project is to develop group I ribozyme derivatives that are highly specific in vivo. A necessary first step toward this goal is to identify *trans*-splicing ribozymes that can discriminate between matched and mismatched substrates in a simplified in vitro model system for RNA repair. We recently developed such a model system (*22*), derived from earlier systems used to study group I ribozyme catalysis (*13*, *24*−*26*). In the model reaction a version of the *Tetrahymena* group I ribozyme, called L-21A_3, which lacks the first 21 nucleotides of the intron and contains a short 3′ exon of three adenosine residues (Figure 2), reacts with oligoribonucleotide substrates supplied in trans. During the first step of *trans*-splicing the ribozyme (Eaaa) cleaves the substrate (CCCUCUAAAAA) to produce a six nucleotide intermediate (CCCUCU), using guanosine or guanosine 5′-monophosphate [pG] as the nucleophile. In the second step of splicing the three-nucleotide 3′ exon is then ligated onto the upstream cleavage product:

$$\text{CCCUCUAAAAA} + \text{pG} \rightarrow \text{CCCUCU} + \text{pGAAAAA} \quad \text{(step 1)}$$

$$\text{CCCUCU} + \text{Eaaa} \rightarrow \text{CCCUCUaaa} + \text{E} \quad \text{(step 2)}$$

Lowercase letters indicate the adenosines derived from the 3′ exon of the ribozyme, which model the wild-type sequence used to replace the mutated fragment of an mRNA in a repair reaction (see Figure 1). Although repair of a mutant mRNA requires successful completion of both splicing steps, undesired effects may result from miscleavage, even without ligation, of nontargeted transcripts. We therefore focused on improving the specificity of the cleavage step and the results described here refer to the first step only, even though all experiments were performed with the two-step *trans*-splicing system.

Measuring Specificity. Kinetically, specificity is defined as the ratio of second-order rate constants k_{cat}/K_m for reaction with matched (M) and mismatched (MM) substrates [$(k_{cat}/K_m)^M/(k_{cat}/K_m)^{MM}$] (*14*, *27*). This essentially corresponds to the relative rates of reaction at low ribozyme concentrations. For the *Tetrahymena* ribozyme $(k_{cat}/K_m)^S$ reflects reaction of the ribozyme·pG complex with free substrate (E·pG + S), measured at saturating concentrations of pG (*14*, *24*). Because we are interested in specificity at varying pG concentrations (see below), we use the term $(k_{cat}/K_m)^{S'}$ to indicate that pG concentrations were not always saturating.

To avoid development of ribozymes that are very good at discriminating against only a particular mismatch, two different mismatched substrates were used to determine specificity. The first substrate forms a G·A mismatch with the IGS three nucleotides upstream of the initial cleavage site (CCCGCUAAAAA) and has been used previously to study reaction of the ribozyme with mismatched substrates (*12*−*14*). The second substrate forms a C·A mismatch at the same position, as well as a G·U wobble pair one nucleotide closer to the cleavage site (CCCCUUAAAAA). For simplicity we will call the two mismatched substrates −3G and −3C−2U, respectively. The −3C−2U substrate was chosen because a number of *trans*-splicing events to this sequence were observed in mammalian cells (P.P.Z. and B.A.S., unpublished results).

Substrate Binding by the Tetrahymena Ribozyme. To understand and enhance specificity, it is important to know how the ribozyme recognizes and binds its substrates. Substrate binding by the *Tetrahymena* ribozyme has been the subject of intense investigation (*15*, *19*) and is known to occur in two steps (Figure 3) (*28*, *29*). First the substrate base pairs to the IGS, forming a short helix called P1 in an

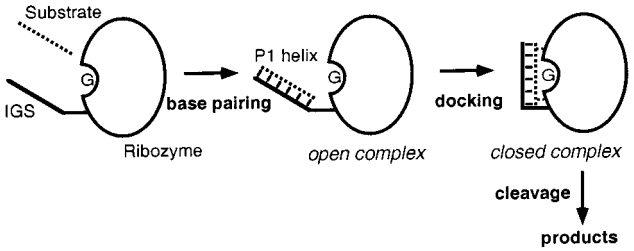

FIGURE 3: Substrate binding by the *Tetrahymena* ribozyme. Guanosine is shown bound in the ribozyme core and the substrate is shown as a dashed line.

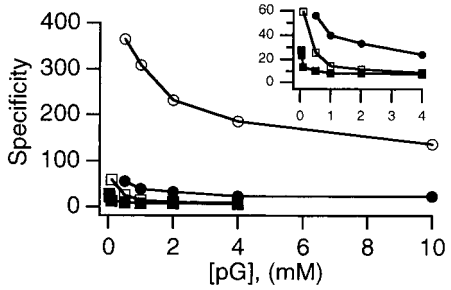

FIGURE 4: Specificity varies with pG concentration. Specificity is the ratio of $(k_{cat}/K_m)^{S'}$ for reaction with matched and the respective mismatched substrates $[(k_{cat}/K_m)^{S'M}/(k_{cat}/K_m)^{S'MM}]$. The mismatched substrates were −3G for the wild type (■) and the A265G (●) ribozymes and −3C−2U for the wild-type (□) and the A265G (○) ribozymes. The inset shows the region at lower specificity in greater detail. The specificities at 1 mM pG are given in Table 1.

open complex. P1 then docks into the active site to form a closed complex. Docking is mediated in part by a series of tertiary interactions between 2′ OHs on both strands of P1 and the ribozyme core (*30*−*32*). Due to the significant binding energy contributed by these tertiary interactions, substrates that form mismatches with the IGS can still bind relatively tightly. Since cleavage takes place rapidly after docking, even mismatched substrates react before they can dissociate (*12*, *14*). Two possible approaches to allow the ribozyme to detect and reject mismatched substrates have been suggested (*8*). Either the rate of the chemical cleavage step could be decreased or the rate of substrate dissociation could be increased, in both cases making it more likely that mismatched substrates dissociate before cleavage. We decided to pursue both strategies.

Slowing Down the Chemical Step. Group I ribozymes require guanosine (or pG) to act as a nucleophile during the cleavage step. Guanosine binds in a specific site with moderate affinity (*33*) [for the L-21A_3 ribozyme, $K_{d\ app}$ = 0.56 ± 0.05 mM (*22*)], and at pG concentrations below the $K_{d\ app}$ the binding site is occupied only a fraction of the time in any one ribozyme molecule (because guanosine binding is in rapid equilibrium) (*33*). If a substrate binds the ribozyme while no pG is bound, reaction is delayed until pG is present. This in effect slows down the cleavage step, and specificity should therefore increase at low pG concentrations. Indeed, this has been observed when the guanosine concentration was lowered from 800 μM to 1 μM (*12*). To explore more thoroughly how specificity depends on pG concentration we evaluated discrimination against mismatched substrates at a series of pG concentrations (Figure 4, inset). While little change is apparent between 4 and 1 mM pG, specificity increases below 1 mM pG. The effect is more pronounced for the −3C−2U substrate. It is also apparent that the increase in specificity is augmented as the pG concentration is decreased below the $K_{d\ app}$. As would be expected, specificity therefore likely depends not on the absolute pG concentration but on the pG concentration relative to its dissociation constant.

It will not be possible to increase in vivo specificity by manipulating the guanosine concentration inside a cell. However, weakening pG binding to the ribozyme, and thus increasing the dissociation constant, should result in enhanced specificity at higher guanosine concentrations. Guanosine binds by interacting with a base pair in the ribozyme core (Figure 2) (*34*, *35*), as well as through a second contact with an adjacent base (A265) (*35*, *36*). Mutation of the second contact (A265G, see Figure 2) weakens guanosine binding about 100-fold (*22*, *35*). With the A265G mutant ribozyme, specificity indeed begins to increase at much higher pG concentrations than with the wild-type ribozyme and is improved at all pG concentrations tested for both mismatched substrates (Figure 4). At 1 mM pG, specificity is enhanced almost 5-fold for the −3G substrate and over 20-fold for the −3C−2U substrate, compared to the wild-type ribozyme (Table 1). Mutating the guanosine binding site therefore is a practical alternative to decreasing the concentration of pG and improves specificity by indirectly decreasing the rate of the chemical cleavage step at potentially physiologically relevant guanosine concentrations.

Increasing the Rate of Substrate Dissociation. Interfering with docking of the P1 helix should weaken substrate binding and accelerate dissociation. The IGS is connected to the ribozyme core by three adenosine residues (called J1/2 since they join the IGS, which forms the P1 helix, to the P2 helix) which allow P1 to move from the open to the closed conformation (*29*). Inserting two additional adenosine residues or deleting two of the three present in the wild-type sequence hinders P1 helix docking and these mutations (J1/2+2A and J1/2−2A, see Figure 2) have been shown to increase discrimination against mismatched substrates (*14*). Under our conditions, improvements in substrate specificity with these mutants are more modest than previously described (*14*), most likely due to the lower temperature used (37 °C compared to 50 °C). At 1 mM pG the J1/2+2A mutant shows only a minimal enhancement of specificity with the −3G substrate and actually discriminates less well against the −3C−2U substrate than the wild-type ribozyme (mainly due to miscleavage at sites other than the intended cleavage site; data not shown) (Table 1). The J1/2−2A mutant, however, does increase specificity 35-fold with the −3G substrate and 3-fold with the −3C−2U substrate, compared to the wild-type ribozyme (Table 1).

A second approach to weaken docking of P1 is to prevent formation of specific tertiary contacts. One of the 2′ OHs of the substrate interacts with the adenosine at position 302 in the ribozyme, and mutating A302 to G (A302G, Figure 2) has been shown to eliminate this interaction (*32*). While no significant change in specificity was observed with the A302G mutant ribozyme for the −3G substrate, a noticeable increase is apparent with the −3C−2U substrate (Table 1).

Together, these results confirm that slowing down the chemical step or weakening substrate binding by any one of several means can increase the ability of the *Tetrahymena* ribozyme to discriminate against mismatched substrates. It is also clear, however, that while some of the single

Table 1. Specificity of Ribozyme Variants[a]

ribozyme	CCCUCUA$_5$ $(k_{cat}/K_m)^{S'}$ (M^{-1} min^{-1})	CCC**G**CUA$_5$ $(k_{cat}/K_m)^{S'}$ (M^{-1} min^{-1})	S^b	S_{rel}^c	CCC**CU**UA$_5$ $(k_{cat}/K_m)^{S'}$ (M^{-1} min^{-1})	S^b	S_{rel}^c
wt	$(0.90 \pm 0.06) \times 10^8$ [d]	$(1.0 \pm 0.3) \times 10^7$ [d]	9.0	**1**	$(0.62 \pm 0.03) \times 10^7$ [d]	14.5	**1**
A265G	$(0.80 \pm 0.18) \times 10^8$ [d]	$(0.20 \pm 0.02) \times 10^7$ [d]	40	**4.4**	$(0.26 \pm 0.05) \times 10^6$ [d]	308	**21**
J1/2+2A	$(0.59 \pm 0.05) \times 10^8$ [d]	$(0.34 \pm 0.09) \times 10^7$ [d]	17	**1.9**	$(0.36 \pm 0.03) \times 10^8$ [d]	1.6	**0.1**
J1/2−2A	$(0.37 \pm 0.13) \times 10^8$ [d]	$(0.12 \pm 0.02) \times 10^6$ [d]	308	**35**	$(0.83 \pm 0.16) \times 10^6$ [d]	45	**3.1**
A302G	$(0.30 \pm 0.14) \times 10^8$ [d]	$(0.43 \pm 0.07) \times 10^7$ [d]	7.0	**0.8**	$(0.41 \pm 0.06) \times 10^6$ [d]	73	**5.0**
A265G/A302G	$(0.12 \pm 0.02) \times 10^8$ [e]	$(0.96 \pm 0.04) \times 10^5$ [d]	125	**14**	$(0.18 \pm 0.07) \times 10^5$ [e]	667	**46**
J1/2−2A/A302G	$(0.10 \pm 0.03) \times 10^7$ [e]	$(0.59 \pm 0.25) \times 10^4$ [d]	169	**19**	$(0.48 \pm 0.04) \times 10^4$ [e]	208	**14**
J1/2−2A/A265G	$(0.52 \pm 0.16) \times 10^7$ [e]	$(0.37 \pm 0.19) \times 10^4$ [d]	1405	**156**	$(0.93 \pm 0.27) \times 10^4$ [d]	559	**39**

[a] Measured at 1 mM pG, pH 7.5 and 37 °C, as described in Materials and Methods. [b] Specificity: the ratio of $(k_{cat}/K_m)^{S'}$ for the matched substrate (CCCUCUA$_5$) and the respective mismatched substrate. [c] Relative specificity: the ratio of the specificities for the mutant and wild-type ribozymes. [d] Determined at subsaturating ribozyme concentrations, as described in Materials and Methods. The error represents the range of values obtained when k_{obs} was divided by the ribozyme concentration for each concentration used. [e] Calculated from k_{cat} and $K_{1/2}$ obtained as described in Materials and Methods. The error represents the maximum range of $(k_{cat}/K_m)^{S'}$ calculated from the standard errors for k_{cat} and $K_{1/2}$ from the fit of the data.

mutations tested significantly improve discrimination against one or the other of the two mismatched substrates, none of them is very effective against both. Since they act in different ways, we reasoned that it should be possible to enhance specificity further by combining mutations. We therefore focused on the three most promising mutations (A265G, A302G, and J1/2−2A) and constructed all three possible double mutants, as well as the triple mutant (which reacted too slowly with even the matched substrate to be useful and therefore will not be further discussed).

Specificity for the double mutant ribozymes is increased compared to the single mutants, although the effects in some cases appear to be less than multiplicative (Table 1). Moreover, in contrast to the single mutants, each of the double mutant ribozymes discriminates well against both −3G and −3C−2U. The best results were observed with a ribozyme containing the J1/2−2A and A265G mutations together. Compared to the wild-type ribozyme, the J1/2−2A/A265G mutant discriminates more than 150-fold better against the −3G substrate, corresponding to a greater than 1400-fold rate discrimination between matched and mismatched substrates, and almost 40-fold better against the −3C−2U substrate, corresponding to a greater than 500-fold rate discrimination (Table 1). The J1/2−2A/A265G ribozyme not only was significantly more specific than the wild type during the cleavage step but also was able to catalyze the ligation step of the *trans*-splicing reaction, as evidenced by the appearance of ligated product at the expected rate (data not shown). We therefore decided to characterize the activity of this ribozyme variant further.

Specificity Affects Efficiency. In the experiments described so far, specificity was determined by measuring rate constants for reaction with matched and mismatched substrates in separate experiments. In the cell, however, a ribozyme must react with its intended target in the presence of a large number of potential nonspecific substrates. To mimic this situation in vitro, we measured the observed rate constant of reaction with radiolabeled matched substrate in the presence of increasing concentrations of unlabeled −3G (Figure 5). For the wild-type ribozyme, k_{obs} decreases rapidly with addition of the −3G competitor, while reaction of the J1/2−2A/A265G ribozyme is largely unaffected by −3G concentrations as high as 5 μM. Although k_{obs} for reaction of the J1/2−2A/A265G ribozyme with the matched substrate in the absence of competitor is 25-fold lower than for the wild-type ribozyme (see legend for Figure 5), this difference is decreased to only slightly more than 2-fold in the presence of 5 μM −3G. This result is anticipated because if a ribozyme binds to and reacts with mismatched substrates, then reaction with the intended target will be inhibited. Therefore, by increasing substrate specificity one can affect the efficiency of reaction with a targeted substrate in the presence of competing RNAs.

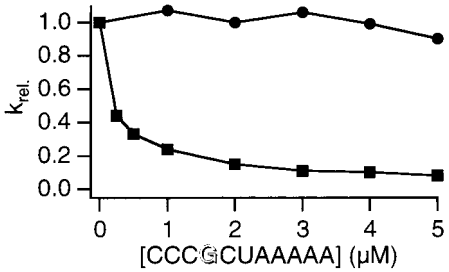

FIGURE 5: Competition between matched and mismatched substrates. k_{obs} for reaction with trace concentrations of the matched substrate was measured as described in Materials and Methods (final concentration of ribozyme 2.5 nM) at 1 mM pG and increasing concentrations of the −3G substrate for the wild-type (■) and J1/2−2A/A265G (●) ribozymes. $k_{rel} = k_{obs}^{-3G}/k_{obs}$, where k_{obs}^{-3G} and k_{obs} are the observed rate constants in the presence and absence of competitor, respectively. k_{obs} is 0.17 min^{-1} for the wild-type and 0.0070 min^{-1} for the J1/2−2A/A265G ribozyme at 0 μM −3G and 0.014 min^{-1} for the wild-type and 0.0063 min^{-1} for the J1/2−2A/A265G ribozyme at 5 μM −3G.

Reaction with an mRNA Substrate. To be specific, ribozymes will have to avoid reaction not only with nontargeted transcripts but also at unintended sites within the target RNA. To evaluate the effect of specificity-enhancing mutations on reaction with more realistic substrates, we examined reactivity with a fragment of p53 mRNA consisting of the first 320 nucleotides of the coding sequence (Figure 6). p53 is an important tumor suppressor gene, and a potential therapeutic target for RNA repair. Embedded within the 320 nt is the six-nucleotide recognition sequence of the *Tetrahymena* ribozyme (CCCUCU), as well as several sites of partial complementarity to the IGS.

Reaction of the wild-type ribozyme with this partial p53 mRNA at 1 mM pG results in a prominent product whose size is consistent with reaction at the matched target site (Figure 6, closed arrow). More importantly, a number of additional reaction products are generated that must be due to reaction at mismatched sites (Figure 6, open arrows). In

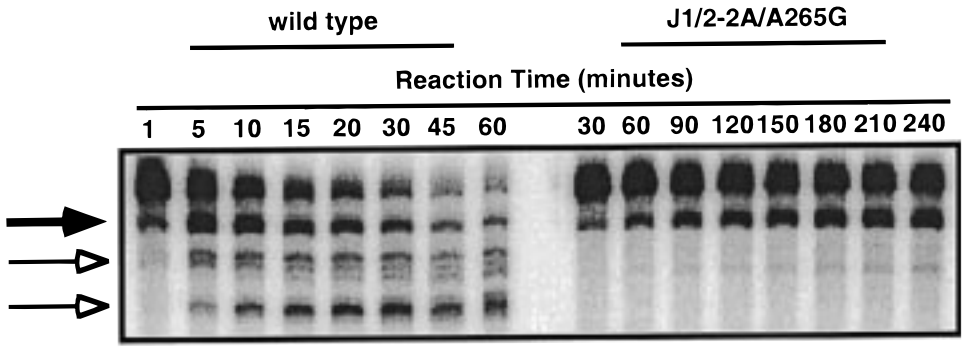

FIGURE 6: Reaction with a fragment of p53 mRNA reveals formation of many nonspecific products (open arrows) with the wild-type, but not the J1/2−2A/A265G ribozyme. The final concentrations of ribozyme and pG were 75 nM and 1 mM, respectively. The substrate is 325 nt long (320 nt p53 coding sequence, starting with the AUG initiator codon, preceded by GGGCG to allow transcription by T7 RNA polymerase), and cleavage at the matched site would result in products of 46 and 279 nt. Although exact cleavage sites were not mapped, the size of the largest product (closed arrow) is consistent with reaction at the matched site within the substrate RNA. While only the upper part of the gel is shown, the pattern in the lower region is similar, with numerous bands apparent in the wild-type reaction but only a few additional weak bands with the J1/2−2A/A265G ribozyme. The L-21A_3 *trans*-splicing ribozyme was used in this reaction, but due to the short length of the 3′ exon (3 nt), cleavage and ligation products (see Figure 1) cannot be resolved.

contrast, the J1/2−2A/A265G ribozyme reacts almost exclusively at a single site. Most of the additional products observed with the wild-type ribozyme are not observed with the mutant, and those that are seen are present in much lower amounts (Figure 6, lower and upper open arrows, respectively). An intermediate pattern results from reaction with the A265G single mutant ribozyme (data not shown). Although the slower reaction of the J1/2−2A/A265G ribozyme, expected from the lower rate constants measured with short substrates (see legend for Figure 5 and Table 1), complicates a direct comparison, the difference between the J1/2−2A/A265G and wild-type ribozymes is quite clear when time points at which similar amounts of the major product are present are compared (5 min reaction time for the wild type vs 240 min for the mutant).

The enhanced specificity of the J1/2−2A/A265G ribozyme determined with simple model substrates is thus also apparent with more realistic targets and shows that the mutations do not prevent its ability to react with complex, presumably structured, RNAs. These results again underscore the link between specificity and efficiency. In the reaction with the wild-type ribozyme, the initial p53 product rapidly disappears, likely due to additional cleavages, whereas the lack of nonspecific cleavage by the J1/2−2A/A265G ribozyme allows the wanted product to accumulate despite the slower reaction rate.

DISCUSSION

One of the major obstacles to the use of the *Tetrahymena* group I ribozyme as an RNA repair agent is its low sequence specificity. The propensity of this ribozyme to react at sites that form mismatches with the internal guide sequence has previously been observed with substrates as varied as short oligonucleotides (*12*, *13*), a fragment of mouse β-globin pre-mRNA (*37*), and ribosomal RNAs (*38*, *39*). We find that mutations that either decrease the rate of the chemical step by weakening guanosine binding or weaken substrate binding by interfering with formation of tertiary interactions do increase specificity, as previously predicted (*8*). However, the improvements are modest and depend on the nature of the mismatch between substrate and ribozyme. Ribozymes with greatly enhanced ability to discriminate between matched and the two mismatched substrates used here can be produced by combining mutations that act through the two different mechanisms. We also demonstrate that for a ribozyme with enhanced specificity the efficiency of reaction with a targeted site is much less affected by competition with nonspecific sites, present on other transcripts or even within the same mRNA, as compared to the wild-type ribozyme.

The effects of ribozyme kinetics on substrate specificity have been discussed (*8*) and have previously been demonstrated with group I (*14*), hammerhead (*40*), and group II ribozymes (*41*). The present study builds upon and extends these earlier observations by illustrating how knowledge of ribozyme biochemistry may be used to deliberately and logically alter the kinetic properties of the ribozyme to enhance its substrate specificity. The rational design approach described here represents an alternative to in vitro selection strategies commonly used to modify the activities of ribozymes (*20*, *21*) and also differs fundamentally from previous design strategies based on secondary structure changes (*21*, *42*).

It will be very interesting to determine whether the enhanced in vitro specificity of the ribozyme variants described here will be maintained in mammalian cells, and what effect the slower in vitro reaction rate of the J1/2−2A/A265G ribozyme will have on in vivo RNA repair. Comparing the in vivo activity of a series of ribozymes with different levels of sequence specificity and defined in vitro behavior will also augment our understanding of how group I ribozyme catalysis and RNA repair is affected by the cellular environment. In summary, we show that detailed biochemical knowledge of ribozyme activity can facilitate the design of ribozyme derivatives with desired properties that are valuable for therapeutic applications.

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