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Alteration of substrate specificity for the endoribonucleolytic cleavage of RNA by the *Tetrahymena* ribozyme

(active site/endoribonuclease/group I intron/RNA splicing/site-specific mutagenesis)

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ABSTRACT **A shortened form of the intervening sequence of the self-splicing RNA from *Tetrahymena thermophila* catalyzes sequence-specific cleavage of RNA. Cleavage site selection involves a base-pairing interaction between the substrate RNA and a binding site within the intervening sequence. Single-base changes in this binding site were previously shown to alter substrate specificity in a predictable manner. To examine the generality with which substrate specificity can be altered, six variant catalytic RNAs (ribozymes) have been produced with two- or three-base changes in the active site. Each ribozyme cleaves its predicted substrate. The conditions required for good reactivity and for discrimination against cleavage at mismatched sites vary and were independently determined for each ribozyme.**

The intervening sequence (IVS) of *Tetrahymena thermophila* pre-rRNA is a catalytic RNA molecule, or ribozyme, capable of self-splicing *in vitro*. Self-splicing involves two sequential transesterification reactions and requires a guanosine cofactor and a divalent metal ion, either Mg^{2+} or Mn^{2+}. Transesterification occurs in the absence of protein and is mediated by the folded structure of the IVS itself (1). The catalytic core structure of the IVS is defined by a set of base-pairing interactions that are phylogenetically conserved among group I introns (2–4).

In the first step of self-splicing, the 3′-OH of guanosine attacks the phosphorus atom at the 5′ splice site and becomes covalently attached to the 5′ end of the IVS (1). Recognition of the 5′ splice site has been shown to involve Watson–Crick base pairing between 5′ exon sequences just preceding the 5′ splice site and a sequence within the IVS termed the internal guide sequence (IGS). This base-pairing interaction was initially proposed on the basis of comparative sequence analysis of group I intervening sequences (2, 4). Though the active site duplex is phylogenetically conserved, only the G·U base pair adjacent to the site of transesterification is conserved in sequence. For the *Tetrahymena* IVS, the role of this base-pairing interaction has been demonstrated by *in vitro* mutagenesis. Single-base changes in either the IGS or the 5′ exon disrupt splicing, whereas double-base changes that maintain base pairing allow accurate and efficient splicing (5, 6).

A shortened form of the IVS, lacking both splice sites, can catalyze an intermolecular version of the first step of self-splicing when provided with an RNA substrate containing a 5′ splice site (Fig. 1). This sequence-specific RNA cleavage or endoribonuclease reaction occurs with multiple turnover, and for this reason the *Tetrahymena* IVS can be classified as an RNA enzyme (7, 8). The wild-type ribozyme cleaves long RNA molecules after the tetranucleotide consensus sequence CUCU with no sequence requirement 3′ to the cleavage site. Three single-base changes within the IGS have previously been shown to alter the sequence of the substrate molecules

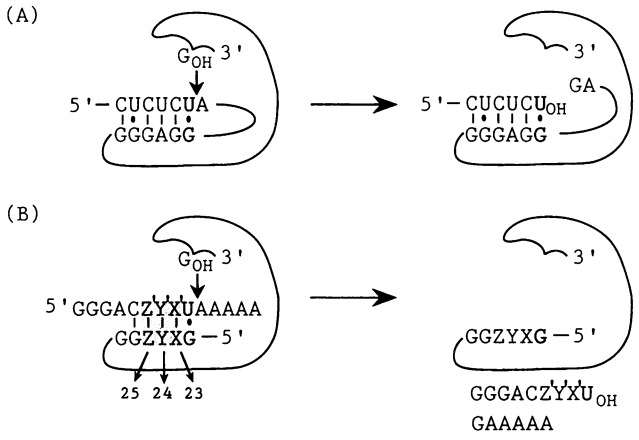

FIG. 1. Model comparing the first step of self-splicing to the endoribonuclease reaction. (*A*) In the first step of self-splicing, an exogenous guanosine (or GTP) molecule attacks the 5′ splice site and becomes covalently attached to the IVS. (*B*) In the endoribonuclease reaction, the ribozyme has had the 5′ exon and other nucleotides preceding the IGS removed. Exogenous substrate can bind to the IGS. In this case, guanosine addition cleaves the substrate into two fragments. The upper half of each duplex represents the 5′ exon or the substrate molecule, and the lower half represents the IGS of the ribozyme (5′-GGAGGG for wild type). The conserved G·U base pair is shown in boldface. The sequences varied are shaded. Positions varied by mutagenesis are indicated by X at position 23, Y at position 24, and Z at position 25 of the IVS. Numbers correspond to full-length IVS with the exogenous G added during splicing counted as nucleotide number 1.

cleaved in a manner consistent with the rules of Watson–Crick base pairing (7). This type of dissection of an RNA that undergoes a self-catalyzed reaction into enzyme and substrate components has subsequently been applied successfully to the hammerhead ribozyme and the minus strand of the satellite RNA of tobacco ringspot virus (9–11).

The IGS of the *Tetrahymena* ribozyme is protected from cleavage by a solvent-based probe, which suggests that it is buried within the catalytic core of the molecule (12). Tertiary contacts between the active site duplex and the core of the IVS have been postulated previously (5, 6, 13). Since such tertiary contacts might be directly or indirectly affected by nucleotide sequence, it was not obvious that all sequence variants of the IGS would be active. We now assess the generality with which we can alter the sequence of the IGS to create a set of designer ribozymes.

MATERIALS AND METHODS

Mutagenesis. Oligonucleotide-directed mutagenesis of pBGST7 (5) was done as described (5, 14) except that the oligonucleotide containing mismatches was added immedi-

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Abbreviations: IVS, intervening sequence; IGS, internal guide sequence.

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ately after denaturation at 95°C and the first hybridization step was done at 19°C instead of 37°C. The mismatched deoxyoligonucleotide GCCTGATAACTTTTCCNNNCAA-AGGTAAAT was synthesized on an Applied Biosystems 380A DNA synthesizer with an equal mixture of all four phosphoramidite bases at positions N. Plasmid DNA from splicing-defective mutants was isolated (15) and sequenced using dideoxynucleotides (16), 5′ end-labeled primer, and avian myeloblastosis virus reverse transcriptase. Plasmids with unique sequences were recloned and purified using ethidium bromide/CsCl density gradient centrifugation.

Preparation of RNA. Plasmids encoding variant RNAs were linearized with *Sca* I, which cleaves 5 nucleotides preceding the 3′ end of the IVS, and transcribed using T7 RNA polymerase as described (17). RNA was ethanol precipitated and subsequently incubated at 42°C for 1 hr under site-specific hydrolysis conditions [200 mM NaCl/50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid, pH 9.0/10 mM $MgCl_2$/0.5 mM GTP] to truncate the 5′ end of the IVS. RNA was purified by PAGE and gel filtration as described (17). For the data presented in Figs. 4, 6, and 7, ribozymes were heated at 95°C for 2 min and then cooled to room temperature in the presence of 20 mM $MgCl_2$ prior to reaction. Heat–cool treatment has been shown to increase activity of the wild-type IVS in some cases, presumably by increasing the population of ribozymes in an active conformation (S. Walstrum and O. C. Uhlenbeck, unpublished data). Oligoribonucleotide substrates were synthesized by transcription of synthetic DNA templates using phage T7 RNA polymerase (18). The RNA was gel purified and ethanol precipitated prior to use. Substrates were either labeled with α-^{32}P-labeled NTPs during transcription or 5′ end-labeled using calf intestinal phosphatase (New England Nuclear), T4 polynucleotide kinase (United States Biochemical), and [γ-^{32}P]ATP.

Endoribonuclease Reactions. Except as noted, cleavage of oligoribonucleotide substrates was done using 0.1 μM substrate, 0.01 μM ribozyme, 20 mM $MgCl_2$, 50 mM Tris·HCl (pH 7.5), and 0.5 mM GTP at 50°C for the times indicated. Portions (2 μl) were stopped on ice by addition to 10 μl of 95% formamide usually containing 0.1× TBE (1× TBE = 0.1 M Tris base/0.083 M boric acid/0.001 M Na_2EDTA), 5 mM Na_2EDTA (pH 8.0), and dyes. Samples were electrophoresed in 20% polyacrylamide/7 M urea gels, and bands were either cut out and assayed for radioactivity in scintillation fluid or scanned on a gel scanner (model 15060-1, Ambis Systems, San Diego, CA). Reaction rates were found to vary by as much as a factor of 2 between independent experiments.

RESULTS

Construction of Variant Ribozymes and Substrates. Oligonucleotide-directed mutagenesis was used to introduce random base changes at positions 23, 24, and 25 of the IVS adjacent to the conserved guanine at position 22. Splicing-defective mutants were selected using plasmid pBGST7. This plasmid has the IVS inserted into the polylinker of a pUC18 derivative containing a phage T7 promoter. Accurate splicing restores the open reading frame encoding the amino-terminal fragment of β-galactosidase and yields a blue colony phenotype in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside. Disruption of splicing results in a white or light blue colony phenotype (5). From the set of variant ribozymes obtained, we chose to study six with two- and three-base changes, which represented the greatest base composition divergence from the wild-type sequence. These variants have a white colony phenotype and are listed in Fig. 2. They are named by specifying all three bases varied in the 5′ → 3′ direction in the ribozyme and are also assigned a number according to the matrix in Fig. 3.

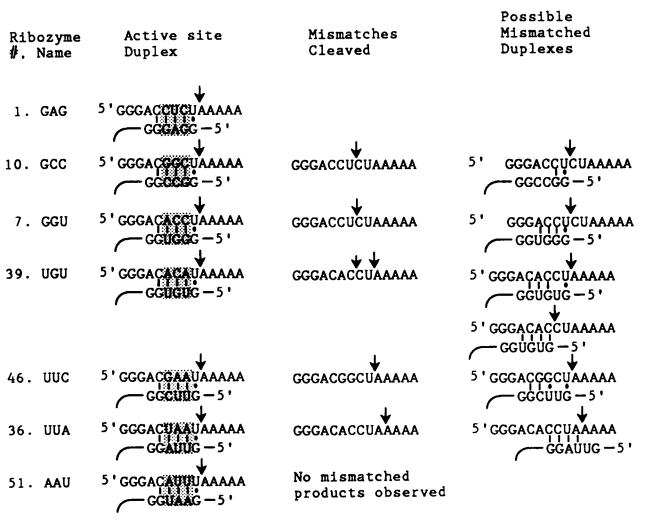

FIG. 2. Sites of cleavage of matched and mismatched substrates by wild-type and variant ribozymes. Ribozymes are named by specifying the three bases varied in the 5′ → 3′ direction beginning at position 23. Each ribozyme has also been assigned a number according to the matrix in Fig. 3. Active site duplexes showing the expected base pairing between each ribozyme and its matched substrate are shown in column 2. Mismatches observed to cleave are shown in column 3. Possible duplexes that may form between the mismatched substrate and the active site are shown in column 4.

Plasmids encoding these variants were cleaved at a *Sca* I site and transcribed using T7 RNA polymerase. The resulting RNAs have a 274-base 5′ exon and are missing 5 nucleotides from the 3′ end of the IVS. RNA was then incubated under site-specific hydrolysis conditions (see *Materials and Methods*). This initiates a cascade of reactions that, for the wild-type ribozyme, result in elimination of 15 and finally 19 nucleotides from the 5′ end of full-length (L) IVS (19). The 5′ ends of variant RNAs were mapped by primer extention with reverse transcriptase (data not shown). They have heterogeneous 5′ ends in the range of the L-15 and L-19 length with the exception of ribozyme GCC, which ends at about L-8. Ribozymes retaining the natural 3′ end were found to be less active than *Sca* I runoff transcripts, which lack 5 nucleotides from the 3′ end (data not shown). This may be due to competition of the 3′ terminal G of the IVS with free GTP for the guanosine-binding site.

Position 23	Position 24: A	G	C	U	Position 25
G	1. GAG 2. GAC 3. GAU 4. GAA	5. GGG 6. GGC 7. GGU 8. GGA	9. GCG 10. GCC 11. GCU 12. GCA	13. GUG 14. GUC 15. GUU 16. GUA	G C U A
C	17. CAG 18. CAC 19. CAU 20. CAA	21. CGG 22. CGC 23. CGU 24. CGA	25. CCG 26. CCC 27. CCU 28. CCA	29. CUG 30. CUC 31. CUU 32. CUA	G C U A
U	33. UAG 34. UAC 35. UAU 36. UAA	37. UGG 38. UGC 39. UGU 40. UGA	41. UCG 42. UCC 43. UCU 44. UCA	45. UUG 46. UUC 47. UUU 48. UUA	G C U A
A	49. AAG 50. AAC 51. AAU 52. AAA	53. AGG 54. AGC 55. AGU 56. AGA	57. ACG 58. ACC 59. ACU 60. ACA	61. AUG 62. AUC 63. AUU 64. AUA	G C U A

FIG. 3. Numerical designation of ribozymes. Each possible permutation of the IGS at positions 23, 24, and 25 is assigned a number according to this matrix. The wild-type ribozyme is assigned number 1. Gray shading highlights ribozymes that have been tested previously (7). Black background highlights ribozymes in this study.

A short ribonucleotide substrate was made for each variant ribozyme by T7 RNA polymerase transcription. These substrates are of the sequence GGGACZ'Y'X'UAAAAA, where X', Y', and Z' have been varied so each substrate could form five base pairs with its cognate ribozyme. The duplexes designed to form are shown in column 2 of Fig. 2.

Activity and Fidelity. Under standard reaction conditions (defined in *Materials and Methods*), each variant ribozyme cleaved its matched substrate as predicted. Cleavage occurred at the correct site as judged by the mobility of the product of cleavage of 5' end-labeled substrate relative to products of a partial digestion with S1 nuclease (data not shown). S1 nuclease and the ribozyme-catalyzed reaction leave 5'-phosphate and 3'-OH ends.

With the exception of AAU, however, all ribozymes were also observed to cleave mismatched substrates under these conditions. Of the set of substrates tested, the mismatched sequence most persistently cleaved by each ribozyme was mapped relative to an S1 ladder (column 3 of Fig. 2). Several of these cleavage sites were also verified by sequencing. In addition to cleavage of mismatched substrates, we observed cleavage after each of the first three Gs of the substrate as mapped by electrophoretic mobility relative to an S1 ladder using 5' end-labeled substrate (data not shown). This reaction liberating pG, pGpG, and pGpGpG decreased either when the 5'-phosphate was removed (B. Flanegan and T.R.C., unpublished) or when urea was included in the reaction (data not shown; see *Discussion*).

A useful site-specific endoribonuclease must not only cleave at the correct site, but it must also discriminate against similar sites. Zaug *et al.* (7) have previously shown that 2.5 M urea or low Mg^{2+} concentrations allowed the wild-type ribozyme and two single-base variants to discriminate against substrates that form single-base mismatches with the ribozyme. For the wild-type ribozyme, each substrate tested had a unique urea profile (20), such that conditions could be chosen to optimize fidelity. We define fidelity as the ratio of the observed rate of cleavage of the matched substrate to the observed rate of cleavage of the mismatched substrate.

Good fidelity of cleavage was also observed for the variants GCC and GGU at low Mg^{2+} concentrations or in the presence of 2.5 M urea (Fig. 4 *B* and *C* and data not shown). In the experiment shown in Fig. 4, the ribozyme was held constant and was incubated with each of the seven different substrates. Cleavage of the substrate results in two product molecules with different intensities because they are internally labeled with [α-^{32}P]ATP. Ribozyme GGU and substrate 2 can form four base pairs, and the product of this association was also observed (column 4 of Fig. 2).

The remaining variant ribozymes, however, exhibited low activity in the presence of 2.5 M urea. Activity profiles as a function of Mg^{2+} and urea concentration were examined in order to obtain fidelity while maintaining good activity. These ribozymes were all found to have a Mg^{2+} requirement greater than that of wild type, which has a Mg^{2+} optimum of about 2 mM (21), or GCC, which has similar activity between 2 mM and 25 mM (data not shown). For variant ribozymes UUC and UUA, the rates of cleavage of the matched and the mismatched substrates increased with increasing Mg^{2+} concentration. For this same set of ribozymes, cleavage activity of a matched and a mismatched substrate decreased with increasing urea concentration. Some enhancement of selection for the matched substrate was obtained at low Mg^{2+} or high urea concentrations. Representative graphs are shown in Fig. 5.

Various solvent and salt conditions were tested in an attempt to find a set of conditions that would enhance activity of variant ribozymes on their matched substrates. Increasing the ionic strength of the medium did not enhance activity but did increase fidelity. This effect was observed with several salts, but their efficacy varied. For example, at a given ionic strength in the 0.1–1.0 M range, we observed suppression of cleavage of the mismatched substrate by ribozyme UUC in the order LiCl > NaCl > KCl (data not shown). The best fidelity was obtained with LiCl and with NH_4OAc. For ribozyme UGU, the rate of cleavage varied by a factor of <2 as NH_4OAc was increased from 0 to 0.6 M. However, the cleavage rate of a mismatched substrate decreased 20-fold. The ratio of the rates of cleavage of the matched to the

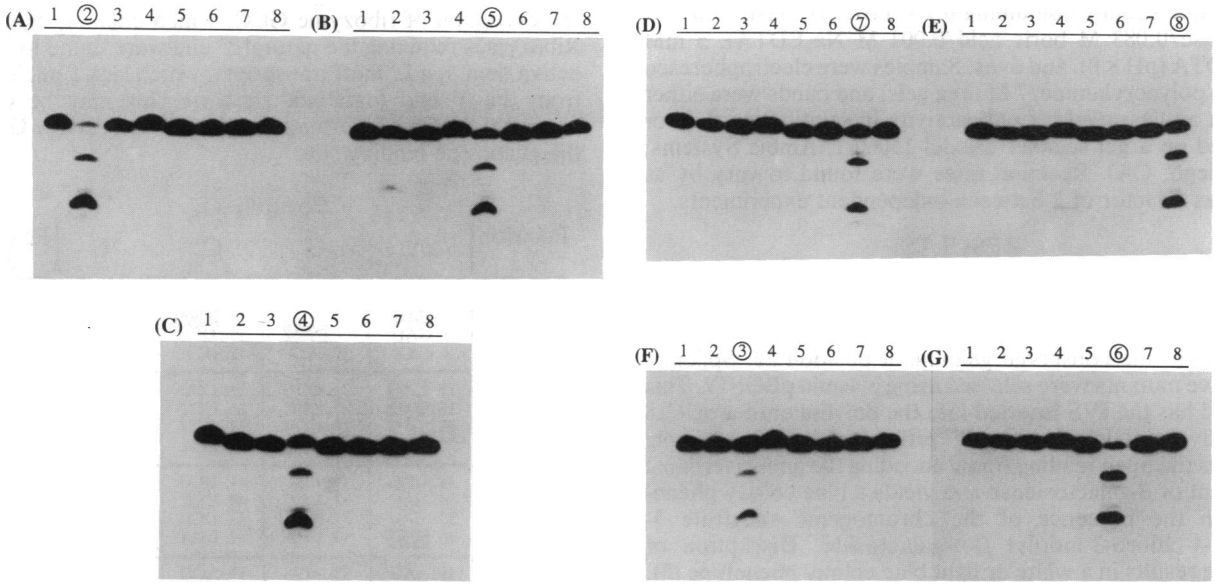

FIG. 4. Conditions giving high fidelity of cleavage can be obtained for each variant ribozyme. In each panel, the ribozyme was held constant and was challenged with each substrate molecule. Ribozymes are GAG (*A*), GGU (*B*), GCC (*C*), UUA (*D*), UUC (*E*), AAU (*F*), and UGU (*G*). Standard reaction conditions were modified as follows: 2.5 M urea was included in *A–C*; 0.1 M NH_4OAc was included in *D*; 0.6 M NH_4OAc was included in *E* and *G*; *F* was not modified. The first lane in each set of eight is the substrate, which matches the ribozyme, incubated alone. In lanes 2–8 the ribozyme was challenged with each substrate molecule of the sequence GGGACZ'Y'X'UAAAAA, where Z'Y'X' equals CUC (lanes 2), AUU (lanes 3), GGC (lanes 4), ACC (lanes 5), ACA (lanes 6), UAA (lanes 7), and GAA (lanes 8). The number of the lane containing a complementary ribozyme–substrate complex is circled. Substrates were internally labeled with [α-^{32}P]ATP during transcription. Reaction was at 50°C for 60 min.

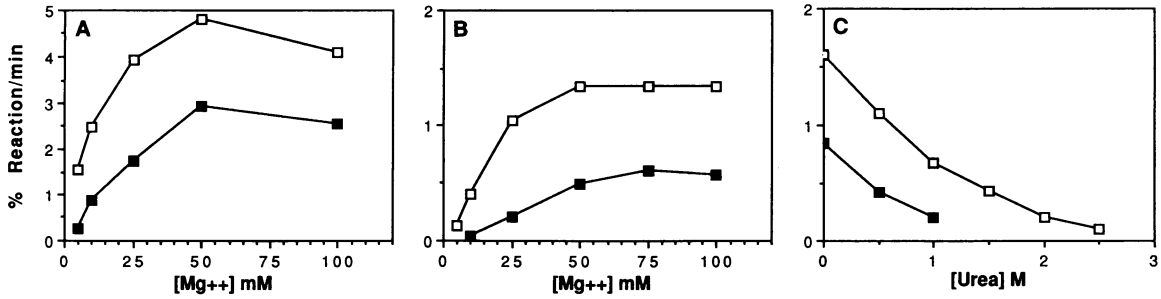

FIG. 5. Effect of Mg^{2+} and urea on activity of ribozymes UUA and UUC. Reaction rate as a function of Mg^{2+} concentration for ribozymes UUC (*A*) and UUA (*B*). (*C*) Reaction rate as a function of urea concentration for ribozyme UUA. Matched substrate (□) is compared to the most problematic mismatched substrate identified in Fig. 2 (■). Reaction conditions are as summarized in the text except that the urea curve was done at 75 mM Mg^{2+}. Quantitation was done using unlabeled substrate and a small amount of 5′ end-labeled substrate totaling 0.1 μM. Three time points were taken in the linear range of the reaction at each Mg^{2+} or urea concentration. The slope of the linear least squares line is plotted as a function of concentration. Lines are drawn to facilitate visualization of the data.

mismatched substrates increased from 4.5 in the absence of NH_4OAc to 80 in its presence (Fig. 6).

For ribozymes UUC and UUA, the rate of reaction of the matched substrate decreased in the presence of NH_4OAc, but the ratio of the matched to mismatched rates increased (data not shown). For ribozyme UUC, the rate of the reaction of the matched substrate decreased 3-fold as NH_4OAc was increased from 0 to 0.6 M, whereas the rate of reaction of the mismatched substrate decreased 20-fold. Thus the fidelity increased by a factor of 6.5. For ribozyme UUA, the ratio of the rates of the matched to the mismatched substrate increased from 6 in the absence of NH_4OAc to 30 in the presence of 0.1 M NH_4OAc, thereby gaining a factor of 5.

The fidelity of cleavage in the presence of NH_4OAc was tested for ribozymes UGU, UUC, and UUA using all seven substrates (Fig. 4 *D–F*). In each case, the substrate that forms a matched five-base duplex is selected. Reaction rates seen under conditions chosen to enhance fidelity in Fig. 4 are shown in Fig. 7. For UUA and UUC, we observed enhanced fidelity only at the expense of decreased rate. It is worth noting that we have observed good activity for UUA in the presence of urea at lower temperature, but these conditions have not been tested for fidelity.

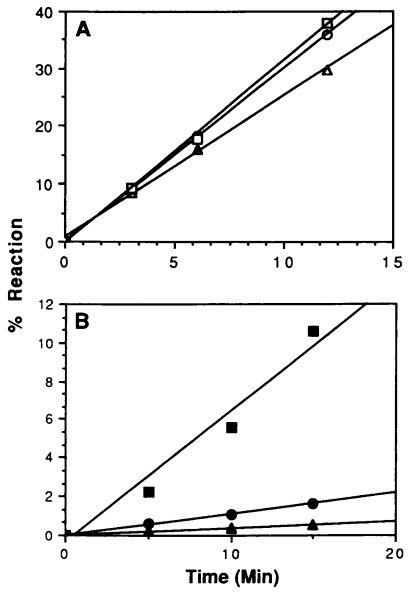

FIG. 6. Effect of NH_4OAc on activity of ribozyme UGU with matched substrate (*A*) and mismatched substrate (*B*) (products of cleavage at the two sites shown in Fig. 2 are added). NH_4OAc concentration was 0 (□, ■), 0.3 M (○, ●), and 0.6 M (△, ▲). Label present as free Gs became significant in 0 M NH_4OAc. These were counted as products so this rate may be overestimated. Regardless of whether these are included as products or not, data points at all concentrations of NH_4OAc in *A* are within a factor of 2. A mixture of unlabeled substrate and a small amount of 5′ ^{32}P-labeled substrate totaling 0.1 μM was used.

DISCUSSION

We have extended the set of variants in the IGS to include six ribozymes with a greater sequence divergence from wild type than has previously been examined. Each of the variant ribozymes cleaves its complementary RNA substrate as predicted.

Variants forming active site duplexes rich in A·U base pairs have an increased Mg^{2+} dependence. This suggests that Mg^{2+} may act by facilitating substrate–ribozyme complex formation. Stabilization of bound substrate could explain the increased Mg^{2+} requirement for weaker duplexes and mismatches relative to strong duplexes. Though Mg^{2+} may act to stabilize the substrate–ribozyme complex, urea may serve to destabilize it.

Changes in the base composition of the active site duplex could disrupt tertiary interactions that enhance activity. Disruption of such tertiary interactions could also result in an increased Mg^{2+} requirement. Although base-specific tertiary interactions may exist and facilitate the reaction they must not be required, since all three positions have been varied with maintenance of activity.

The effect of NH_4OAc on the fidelity of the reaction may be mainly one of ionic strength, since there is similar activity with several ions. The increased ionic strength may shield charges on phosphates and decrease the affinity of one or more magnesium ions for the structure, resulting in destabilization of the active site duplex (22). Furthermore, there

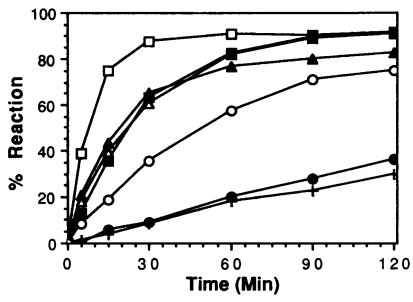

FIG. 7. Reaction rates for ribozymes GAG (□), UGU (■), GCC (△), GGU (▲), UUC (○), UUA (●), and AAU (+) under the conditions summarized in the legend to Fig. 4. Substrates were internally labeled during transcription with [α-^{32}P]GTP and treated with calf intestinal phosphatase to remove the 5′-phosphate. Substrate was present at 0.08 μM; ribozyme was at 0.01 μM.

could be a critical magnesium ion near the duplex (13) that is held more loosely in the mismatched structure due to the different geometry of a matched versus a mismatched duplex. In addition to an ionic strength effect, there appears to be a specific ion effect. Thus, binding of salts to the nucleic acid may also contribute, possibly by competing directly for a Mg^{2+} binding site. The order in which Li^+, Na^+, and K^+ affect the reaction parallels the strength of association of these ions to adenine nucleotides (23). Unlike the alkaline metals, NH_4^+ may exert its effect by forming specific hydrogen bonds to the nucleic acid.

We expect that cleavage of mismatched duplexes also requires base pairing to the IGS. Duplexes that may form between mismatched substrates and the IGS are shown in column 4 of Fig. 2. Two duplexes containing single-base mismatches are postulated. Cleavage of similar single-base mismatches has been described previously for the wild-type ribozyme (7, 20). The mismatched site cleaved by ribozyme GCC is unusual in that it may involve only a two-base-pair interaction with the IGS. Precedence for as few as two nucleotides prior to the site of cleavage acting as substrate is found in another enzymatic reaction of the IVS (24). It is possible that the binding of the natural substrate CU is specifically stabilized by tertiary interactions in addition to binding to the IGS, as suggested by Sugimoto *et al.* (13, 25).

The site of mismatched cleavage of ribozyme UUA is unusual in that cleavage occurs after A and the postulated duplex involves an U·A base pair instead of the conserved G·U base pair. Cleavage after A has not been observed previously in the endoribonuclease reaction nor are any group I IVSs known in which A replaces the conserved U at position −1 preceding the 5′ splice site. However, a low level of activity has been observed in self-splicing of the *Tetrahymena* RNA *in vitro* when U at −1 was replaced with A by site-specific mutagenesis (26). The duplex postulated for pairing between ribozyme UUA and its mismatched substrate (Fig. 2) shows pairing to a different portion of the IGS. Such sliding of the active site has also been postulated to explain the cyclization reaction of the wild-type IVS (27, 28).

Cleavage by ribozyme UGU after C was also observed. Cleavage after C is precedented by the polymerization reaction catalyzed by the wild-type ribozyme (8) and by phylogeny. Among group I IVSs, two cases are known in which the U preceding the 5′ splice site is not conserved, and in both cases it has been replaced with a C (3).

The reaction liberating free Gs from the 5′ end of the substrate may result from G exchange (29) or from a reaction resembling the second step of self-splicing in which the 3′-OH of a product molecule attacks the substrate after G (24). We have observed products corresponding to such a ligation reaction in the absence of GTP (unpublished data).

In summary, variants of the *Tetrahymena* ribozyme containing 10 of the 64 possible permutations of the IGS at positions 23, 24, and 25 have now been tested. They each cleave their matched substrate. Conditions for optimal reactivity and fidelity vary among the different ribozymes. As previously demonstrated for the wild-type ribozyme, good fidelity can be observed in the presence of urea for some variants, including ribozymes GCC and GGU and those with single-base changes previously characterized (7). Others, such as ribozymes UUA and UUC, which form weaker duplexes than wild type, require higher Mg^{2+} concentrations for activity and lose activity in the presence of urea. Inclusion of salt can be of practical value since enhanced fidelity is observed in its presence, although this can be accompanied by some loss in reaction rate. We expect all permutations at these three positions will be active, but ribozymes that form very weak duplexes or duplexes that are inactive at low Mg^{2+} concentrations may be more difficult to use in practice. Perhaps some of these variants would benefit from increasing the length of the complementarity between the IGS of the ribozyme and the substrate. As this and other ribozyme systems continue to be engineered, they may become useful tools for site-specific cleavage of RNA both *in vitro* and *in vivo*.

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