In Vitro Selection of Second Site Revertants Analysis of the Hairpin Ribozyme Active Site

Received for publication, June 24, 2003, and in revised form, October 8, 2003
Published, JBC Papers in Press, October 9, 2003, DOI 10.1074/jbc.M306703200

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We have used in vitro genetics to evaluate the function and interactions of the conserved base G8 in the hairpin ribozyme catalytic RNA. Second site revertant selection for a G8X mutant, where X is any of the other three natural nucleobases, yielded a family of second site suppressors of the G8U mutant, but not of G8C or G8A, indicating that only G and U can be tolerated at position 8 of the ribozyme. This result is consistent with recent observations that point to the functional importance of G8-N1 in the chemistry of catalysis by this ribozyme reaction. Suppression of the G8U mutation was observed when changes were made directly across loop A from the mutated base at substrate position +2 or positions +2 and +3 in combination. The same changes made in the context of the natural G8 base sequence resulted in a very large drop in activity. Thus, the G8U mutation results in a change in specificity of the ribozyme from 5′-N [GUC-3′] to 5′-N [GCU-3′]. The results presented imply that G8 interacts directly with U+2 during catalysis. We propose that this interaction favors the correct positioning of the catalytic determinants of G8. The implications for the folding of the ribozyme and the catalytic mechanism are discussed.

The “hairpin” catalytic RNA is a small “self-cleaving” motif involved in resolving RNA multimers and circularizing the monomers generated by the rolling circle replication of the genome of a plant virus satellite RNA associated with satellite tobacco ringspot virus (1). It catalyzes both a phosphodiester bond breakage, yielding 5′-OH and 2′,3′-cyclic phosphate termini, and the reverse reaction, RNA ligation. A 50-nucleotide-long enzyme version has been derived that cleaves a 14-nucleotide RNA substrate (Fig. 1) (2). The cognate substrate is the mini, and the reverse reaction, RNA ligation. A 50-nucleotide-cyclic phosphate terminated molecule to be catalytically proficient (6–10). This step, known as “docking” of loops A and B, induces structural rearrangements within the two loops (3, 4, 11–14). The two domains are stitched together through a ribose zipper between nucleotides 10, 11, 24, and 25; a G+1-C25 Watson-Crick base pair; and minor groove contacts between G11 and U12 and between A22 and A23 mediated through U42 (4, 15, 16).

The unusual cation dependence of the hairpin ribozyme led to the hypothesis that the reaction was catalyzed directly by RNA functional groups. This was first postulated after the observation that Co[NH3]3+ could replace Mg2+ without loss of ribozyme activity (17–19) and was further demonstrated when it was shown that the hairpin ribozyme could function in the absence of multivalent cations (20). Finally, no divalent cations can be observed in the proximity of the cleavage site in the crystal structure (4, 21). In contrast, cations are strictly required for docking of the two domains (22).

Mutational data, the crystal structure, and nucleotide analog interference modification studies suggest that three nucleotides contained in loop A (G8, A9, and A10) and one nucleotide in loop B (A38) can be directly involved in catalysis (4, 5, 47). Three lines of evidence point to the particular importance of G8. First, its replacement by an abasic site is particularly deleterious for the ribozyme activity (23). Second, its mutation does not alter the docking of the two domains (24, 25); and third, two different models give G8 a central role in catalysis (4, 21, 23, 24). To better understand the role of and the functional interactions involving this nucleotide, we used in vitro genetics to search for second site suppressors of G8 mutations. Our results suggest that an interaction across loop A is essential to form the active molecule. A detailed analysis of the effect of U+2 and C+3 led us to propose a model in which U+2 would be required to adequately position G8 for catalysis.

MATERIALS AND METHODS

RNA Preparation—Unlabeled RNAs were transcribed from synthetic oligonucleotides essentially as described (26). Oligonucleotides (T7 promoter top strand and template; 200 nt each) were transcribed with T7 RNA polymerase at 37 °C for 3 h in the presence of 40 mM Tris-HCl (pH 8.5), 25 mM MgCl2, 2 mM NTP, 1 mM spermidine, and 5 mM dithiothreitol. The precipitate formed during transcription was removed by centrifugation, and RNAs were precipitated. Transcripts were then purified on a 7% uracil and 10–20% polyacrylamide gel, eluted by diffusion, ethanol-precipitated, and quantified using a spectrophotometer (assuming optical density of 260 = 37 μg/μl).

Prior to 5′-end labeling, RNAs were dephosphorylated for 1 h at 50 °C in the presence of calf intestinal phosphatase (1 unit/50 pmol). RNAs were ethanol-precipitated and end-labeled using 1 μCi of [α-32P]ATP (3000 Ci/mmol). Labeled RNAs were used within 3 days. Self-cleaving constructs were transcribed from linearized plasmid issued by the in vitro selection process essentially as described above, except in the presence of 6 mM MgCl2, 50 μCi of [α-32P]CTP, 0.5 mM CTP, and 1 μm other NTPs.

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Ribozyme Assays—Preliminary self-cleavage assays of activity were carried out using the hairpin selection (HS) construct (see Fig. 5). Uncleaved RNAs were kept on ice throughout the purification from transcription reactions. They were then resuspended in water, denatured for 1 min at 90 °C, renatured for 20 min on ice, and equilibrated at 37 °C for 20 min. Reactions were initiated by addition of 5× cleavage buffer (final concentrations of 12 mM MgCl₂, 40 mM Tris-HCl (pH 7.5), and 2 mM spermidine) equilibrated at 37 °C. 10-µl aliquots were removed at 0, 5, 15, 30, 60, and 120 min, and the reaction was quenched on dry ice by addition of an equal volume of loading buffer containing 10 M urea, 20% sucrose, 0.5% SDS, 0.02% xylene cyanol, 0.02% bromphenol blue, and 160 mM Tris-Cl (pH 7.5), 160 mM boric acid, and 4 mM EDTA. Single exponential cleavage rates were determined by fitting the results, plotted as ln(unreacted fraction)/time, by linear regression to the equation ln(y) = −kt + b, where y is the unreacted fraction and x is the time. The y intercept (b) was zero for all experimental curve fits. All experiments where repeated twice, yielding similar results.

For ligation assays using the HS construct, the 3′-product, a 9-nucleotide fragment corresponding to the sequence from G₁₋₁ to U₊₉, was transcribed and dephosphorylated as described above. Internally labeled cleaved molecules from self-cleavage were purified from transcribed and dephosphorylated as described above. Internally labeled cleavage buffer, denatured for 1 min at 90 °C, and then incubated at a reaction temperature of 0 °C. 10-µl aliquots were removed at 0, 5, 15, 30, 60, and 120 min, and the reaction was quenched with an equal volume of 10 M urea loading buffer. The reaction rates were calculated as described above.

Ribozyme Kinetic Analysis with the SV5 Construct—For cleavage assays, 1 µM unlabeled ribozyme strands shown in Fig. 5 and a trace amount of 5′-³²P-labeled substrate were mixed together in 50 mM HEPES/NaOH (pH 7) and incubated at 37 °C for 10 min. This mixture was allowed to equilibrate to a reaction temperature of 25 °C. Reactions were initiated by combining equal volumes of the RNA mixture with an Mg²⁺ mixture to give final reaction concentrations of 0.5 mM ribozyme, <1 mM substrate, 15 mM Mg²⁺, and 50 mM HEPES/NaOH (pH 7). This concentration of ribozyme was found to be saturating for all mutants examined. At least 10 time points were taken for each experiment by quenching aliquots on ice into 10 volumes of 15 mM EDTA, 0.02% (v/v) bromphenol blue, and 0.02% (v/v) xylene cyanol in formamide. Experiments were repeated at least three times for each mutant. The experimental variation in these experiments did not exceed 20% of the reaction rates or amplitudes.

Ligation kinetic analyses were carried out following the same general scheme using the SV5 construct shown in Fig. 5. The concentration of ribozyme and 3′-product was 12 µM. Increasing the concentrations of unlabeled RNA by 2-fold did not result in faster ligation kinetics. The 5′-³²P-labeled 5′-cleavage product was generated by ribozyme cleavage of the labeled substrate of a four-way helical junction construct called FWS (27). The 5′-cleavage product was purified on a 20% (v/v) acrylamide gel. The FWS substrate was employed specifically to improve recovery of the labeled 5′-cleavage product. The rate for ligation of the FWS 5′- and 3′-products was found to be identical, within error, to that obtained for the same ribozyme and a shortened 5′-product (data not shown).

Notice Gel Electrophoresis—The docking ability of the wild-type sequence and mutants was determined by gel shift assay as described previously (12) without modification. The affinity between the substrate and the isolated SBS was determined by gel shift assay as described previously (9, 28, 29). The substrate sequence was 5′-UGA-CAGUCUGUUGUU-3′, and the SBS sequence was 5′-AAACA-GAGAAGUCAC-3′. A constant low amount of 5′-end-labeled substrate (0.1 mM) was mixed with increasing concentrations of unlabeled SBS in cleavage buffer, denatured for 1 min at 90 °C, and incubated for 2 h at 4 °C. Incubation times up to 16 h did not significantly change the result in the case of the wild-type (WT) sequences, showing that equilibrium was reached in <2 h. Free substrate and complex were separated on a 15% nondenaturing polyacrylamide gel containing 40 mM Tris acetate (pH 7.5) and 12 mM magnesium acetate for 13 h at 4 °C. Bound and unbound species were quantified by phosphorimaging using a Bio-Rad GS-250 Molecular Imager instrument. The fraction of bound substrate was normalized to the value of maximum binding. Kᵣ values were then obtained from a nonlinear least-square fit according to an equation describing theoretical bimolecular association (30) using SigmaPlot software (Jandel Scientific). The S.E. was calculated from the fit of the observed curve to the theoretical one (reported by the SigmaPlot software). All gel shifts were repeated at least twice. It should be noted that the substrate resolved as a single species consistent with a single-stranded monomer under the conditions used.

In Vitro Selection—The second site reversion mutants were isolated using a slightly adapted protocol from the in vitro selection system described previously (31). Briefly, the template for transcription was generated by annealing and extension of two overlapping DNA oligonucleotides: a 74-mer containing the coding sequences for a T7 promoter, a primer-binding site, and part of the ribozyme sequence (5′-TAAT-ACGACTCTATAGGTACGCTCGAGCTTAAACAGACGAAATC)-AANNCACCAGAGAAACAAGATCCTTTTGTTTGGT-3′) and a 63-mer representing the antisense sequences for the rest of the ribozyme, an 8-nucleotide linker, the substrate, and a second primer-binding site (primer P3) (5′-GGCGACTGTCGATTCGCTAAACAGAGATCTGACCAAGTAGTTAAT/AATT) (overlapping sequences are in boldface; underlined nucleotides were mutagenized to frequencies of 0.88 for the wild type and 0.12 for the other 3 nucleotides). 400 pmol of double-stranded template were transcribed for 3 h. The transcription mixture was phenol/chloroform-extracted, and the RNA was precipitated. To increase the opportunity for molecules to self-cleave, the reaction was incubated for an addition hour in 1 ml of cleavage buffer at 37 °C after a denaturation step (1 min at 90 °C). Cleaved and uncleaved products were purified on a 7 M urea and 8% polyacrylamide gel. The ligation reaction

FIG. 1. Schematic representation of the trans-acting hairpin ribozyme docked and undocked forms. Substrate and ribozyme strands are labeled S and Rz, respectively. The sites of cleavage and ligation are indicated with arrows. Numbering is according to the scheme of Ref. 48, and helices 1–4 (H₁–H₄) are labeled.
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Selection of intramolecular suppressors of G8 mutants: a mutated pool of synthetic DNA used as template to generate the mutant hairpin ribozyme. The secondary structure of the ribozyme-substrate complex is shown. Nucleotides in the ribozyme are numbered from 1 to 50; nucleotides in the substrate are indicated with positive numbers (+1 to +9) on the 3′-side of the cleavage site and with negative numbers (−5 to −1) on the 5′-side. Boldface letters indicate sequence variation that was introduced into the initial pool of RNA variants as described under "Materials and Methods." Primer-binding sites P1 and P2 used for reverse transcription (RT)-PCR are indicated. The active molecules were selected on their ability to self-cleave and subsequently ligate to an RNA possessing a specific primer-binding site (P3, which replaces P2) used for reverse transcription and PCR amplification.

**Fig. 2.** In vitro selection of intramolecular suppressors of G8 mutants: a mutated pool of synthetic DNA used as template to generate the mutant hairpin ribozyme. The secondary structure of the ribozyme-substrate complex is shown. Nucleotides in the ribozyme are numbered from 1 to 50; nucleotides in the substrate are indicated with positive numbers (+1 to +9) on the 3′-side. Boldface letters indicate sequence variation that was introduced into the initial pool of RNA variants as described under "Materials and Methods." Primer-binding sites P1 and P2 used for reverse transcription (RT)-PCR are indicated. The active molecules were selected on their ability to self-cleave and subsequently ligate to an RNA possessing a specific primer-binding site (P3, which replaces P2) used for reverse transcription and PCR amplification.

was carried out by incubating at 0 °C for 30 min the active self-cleaved fraction with 3 μM DNA-RNA oligonucleotide representing the ribozyme cleavage product linked to a specific sequence complementary to primer P2 (5′-GUCGUGUUUTCTAGAGCCCTGTTG-3′). The ligated molecules were isolated on a 7 M urea and 8% polyacrylamide gel, reverse-transcribed, and amplified by PCR as described (31) using two primers: P1, 5′-TAATACGACTCACTATAGGGTACGGTCTCCAGGACTC-3′; and P2, 5′-CAGGAGCGGTCTCTAGA-3′. Inactive molecules were reverse-transcribed and amplified with primers P2 and P3 (5′-GCCGACGTGAGCTAGA-3′).

**Molecular Modeling**—Models of the ribozyme catalytic core were generated using the constraint satisfaction program MC-SYM (32, 33). The structural constraints used to generate the MC-SYM script were derived from data generated in this work, from previously published cross-linking studies (15, 24), and from crystallographic data (4). A-form RNA was assumed for all Watson-Crick helices, and the sugar puckers conformations and glycosyl angles for all nucleotides in loop A and for residues 24, 25, and 38 in loop B were assigned according to the crystallographic data (4). Solutions that were similar (root mean square deviation ≤ 2 Å) were combined by MC-SYM. Resulting structures were energy-minimized through molecular mechanics calculations performed by the molecular simulation program Sander from the Amber 7 suite of programs (34) using the Amber 2002 force field for RNA. All one to four electrostatic interactions were set to a factor of 1.2, and the distance-dependent dielectric model (ε = 4r,) for the coulombic representation of electrostatic interactions was used. As a first step, energy minimization was performed using the steepest descent for 100 steps, and then the conjugate gradient method was applied until the maximum derivative was <0.001 kcal/mol/A. Figures were prepared using MolScript (35). Root mean square deviation calculations were performed using MC-RMS from the MC-SYM package.

**RESULTS**

Identification of Second Site Suppressors of a Mutation in the Substrate-binding Strand—The SBS contains four nucleotides essential for optimum activity as defined by in vitro selection and directed mutagenesis (36–38). Substitutions at G8 result in loss of activity of 3 to 4 orders of magnitude (24, 25, 38). To define the role of this nucleotide and thus to better understand the function of the SBS, we selected for active molecules with a mutation of G8. A previously described in vitro selection method (31) was adapted to identify second site suppressors of a given mutation (8, 39). To be selected, the self-cleaving version of the ribozyme (HS construct) (Fig. 2) must self-cleave during transcription and subsequently catalyze its ligation to a specific 3′-product sequence. The 3′-product sequence is subsequently used as a reverse transcriptase primer-binding site, and PCR is employed to amplify the active molecules (Fig. 2). To prevent the appearance of revertants to the WT sequence, we carried the molecules through only one round of selection. We have therefore limited the complexity of the starting pool to recover active molecules with few changes to easily identify the suppressive mutations. The starting pool was mutagenized as follows. G8 was systematically mutated to A, U, or C; all other catalytically important nucleotides were lightly mutagenized for an average of two mutations per molecule; and the two important base pairs (G11-C-2 and U12-A-3) were randomized to maximize the potential for base pairing. Finally, because the U39C mutation has previously been identified as a general enhancer (29, 37, 40), U39 was synthesized to be equally U or C (Fig. 2). The full repertoire of this design yields 10^6 different molecules (41). We transcribed 10^14 template molecules, thus ensuring that we would screen the entire sequence space. After one round of selection, we sequenced 10 active molecules. All of these were mutated at G8 and had two mutations across loop A at C-3 and U-2 in the substrate. Two of these also had the A7U mutation. A preliminary characterization of the self-cleavage efficiency of these molecules revealed two classes of revertants (Fig. 3). The first class was 20-fold less active than the WT construct, and all molecules had the G8U mutation and two suppressive mutations: U+2C/C+3U (clones 1 and 18) or U+2A/C+3A (clones 2 and 20). The catalytic efficiency of the second class was reduced by at least 200-fold. These molecules were mutagenized at the same sites as the first class, but no obvious consensus sequence could be
observed. Clones 20 and 2 are identical, except for the A7U transversion present in the latter. They showed the same rate of cleavage, but only 10% of the molecules from clone 2 were able to self-cleave (this result was observed with two different RNA preparations). Our interpretation of this result is that the A7U mutation induces incorrect folding, resulting in a large portion of the population being inactive. Therefore, we did not further study the effect of mutations at position 7. Because the ribozymes with the triple mutation G8U/U/U were the most active, this set of mutations was chosen for the following experiments. We also sequenced 10 inactive molecules. These showed four or more mutations at the expected conserved sites, but also at U39, which was a C in half of the molecules. Nevertheless, U39C was selected in the active molecules, which raises the possibility that U39 has a more specific effect on the loop A mutants. For this reason, we quantified the effect of mutations of U39 on the rate of cleavage of molecules with the triple mutation G8U/U/U.

Because we used RNA-catalyzed ligation in the selection procedure, we performed preliminary ligation assays with the HS construct to confirm the specificity and efficiency of the suppression of the double mutation U+2C/C+3U on G8U mutants. As shown in Fig. 4, the G8U ribozyme was inactive in the presence of a WT 3'-product, but did catalyze its ligation to a U+2C/C+3U 3'-product. Conversely, the WT ribozyme was unable to utilize the U+2C/C+3U product, but self-ligated the WT product efficiently. Comparison of the ligation rates of these mutants shows that the U+2C/C+3U mutations conferred at least a 100-fold improvement in self-ligation by G8U mutant ribozymes compared with the WT substrate (data not shown). WT ribozyme self-ligation to the U+2C/C+3U product was at least a 2000-fold decreased compared with self-ligation to the WT product. The ligation rate of the G8U/U+2C/C+3U revertant was 20-fold less efficient than that of the WT molecule.
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Table I

Wild-type and mutant cleavage and ligation kinetic data for the SV5 hairpin ribozyme

| Sequence | Cleavage | Ligation |
|----------|----------|----------|
|          | $k_{obs}$ | Amplitude | Rate reduction | $k_{obs}$ | Amplitude | Rate reduction |
|          | min$^{-1}$ | -fold | | min$^{-1}$ | -fold | |
| Wild-type | 0.21 | 0.92 | 1 | 1.5 | 0.4 | 1 |
| (G8, U+2, C+3) | 0.0019 | 0.90 | 110 | 0.18 | 0.34 | 8.3 |
| G8, C+2, C+3 | 0.00017 | 0.91 | 1200 | 0.018 | 0.42 | 83 |
| G8, C+2, U+3 | 0.042 | 0.90 | 5 | 0.071 | 0.095 | 21 |
| G8, U+2, U+3 | 0.00011 | 0.90 | 1900 | 0.0023 | 0.39 | 650 |
| U8, U+2, C+3 | 0.00066 | 0.88 | 320 | 1.1 | 0.42 | 1.4 |
| U8, C+2, U+3 | 0.0012 | 0.94 | 180 | 0.14 | 0.35 | 11 |
| U8, C+2, C+3 | 0.00019 | 0.98 | 1100 | 0.0076 | 0.10 | 200 |

Combined Effect of Mutations of G8, U+2, and C+3—We further characterized the mutations and revertant constructs in a well described kinetic model, the SV5 hairpin construct, which is also resistant to many mutations that penalize docking performance (43).2 The cleavage and ligation results from this study are presented in Table I. Our results from cleavage kinetic analyses show that the G8U mutant could be rescued to some extent by the U+2C change and to a greater extent by addition of the C+3U change. In addition, we have observed that the poor cleavage activity of a ribozyme with a xanthosine base at position 8 could be rescued by these same mutations (data not shown). Because the Watson-Crick faces of U and X are identical, this result argues that the rescue is dependent upon structure formation using one or more of the functional groups along the Watson-Crick face of the base. G8A and G8C mutant ribozymes were at least 103 less active than the WT sequence in trans-cleavage assays and showed no preference for specific substrate sequences (data not shown). Using the WT ribozyme, we observed a very large 103-fold decrease in cleavage activity with the U+2C/C+3U mutations in the substrate (Table I). The ligation kinetics of this mutant fit to a double exponential equation with fast and slow phases 21- and 650-fold slower than those of the WT ribozyme and accounting for 20 and 80% of the product formation, respectively. This effect was also observed when the single mutation U+2C was tested. Overall, the ligation data, which were best fit to a double exponential equation, demonstrate that the U8 mutation could be rescued to some extent by the U+2C change. Interestingly, the U8U+2 variants exhibited unusual double exponential kinetics similar to those of the G8/C+2 mutants, where the slow phase accounted for >70% of the ligation amplitude.

The ligation data reported above for the HS construct are more pronounced than what was observed with SV5 because U8/U+2/C+3 and G8/C+2/U+3 showed no activity in the HS context. This discrepancy may be due, in part, to a difference in docking efficiency between HS and SV5 ribozymes (see “Formation of the Docked Complex” below).

Formation of the Substrate-SBS Complex—The previous results suggest a direct interaction between G8 and U+2. However, the observed co-variation effect could be indirect and reflect, for example, an interaction between these nucleotides and the loop B domain. To estimate the effect of G8, U+2, and C+3 mutations on the local structure, we studied the formation of the complex between the isolated SBS and the substrate. Using native gel shift analysis, we determined a $K_d$ for the substrate-SBS complex of 2 ± 1 nM, which is in good agreement with fluorescence resonance energy transfer data obtained previously (44).

The G8U mutation had a clear destabilizing effect on the substrate-SBS complex ($K_d = 11 ± 3$ nM), which was partially compensated by both mutations in the substrate (U+2C and C+3U), and an affinity comparable with what was observed for the WT sequence was reached when the two mutations were combined (Table II). This does not appear to be reciprocal because U+2/C+3/U mutations in the substrate did not destabilize the complex it make with the WT SBS. This is most likely due to the potential for extension of helix 1 by A7-U+3 and G8+C+2 base pairs. Taken together, these data show that an interaction between G8 and U+2 exists in the isolated substrate-SBS complex before the docking event. Furthermore, our results suggest that local structure problems are, in part, responsible for the G8U reversion and the inactivity of the WT ribozyme with respect to the U+2/C+3 substrate.

Formation of the Docked Complex—In addition to loss of local tertiary structure, we were concerned that loss of function could be due to a defect in docking of the two domains into the native tertiary structure. Native gel shift analysis was therefore employed to determine the docking efficiency of the mutant ribozymes (see Fig. 6). Partitioning of the docked and undocked forms by gel electrophoresis is dependent on the use of a form of the ribozyme in which the 3'-half of loop B is linked to the substrate through an AC linker (Fig. 5) (12). In addition, a 2'-deoxygenated substitute is used to prevent cleavage during the experiment, and a G+1A variant serves as a negative control for docking (7). We have previously demonstrated that the docked form of the ribozyme-substrate complex has greater mobility than the undocked form in this system (7). The results in Fig. 6 indicate that, in the SV5 construct, docking was not significantly perturbed for any of the mutants with the exception of U+2/C+3/U. This argues that the catalytic deficiencies of the mutants other than U+2/C+3/U are largely due to local structural defects and/or active-site defects and not to a problem with global structure formation.

The difference in ligation activity obtained with the HS construct prompted us to monitor its docking efficiency. We observed that U8/U+2/C+3 and G8/C+2/U+3 did not form

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2 R. Pinard, K. J. Hampel, J. E. Heckman, and J. M. Burke, unpublished data.
docked complexes, whereas G8U +2/C+3 and U8/C+2U+3 showed normal docking (data not shown). This explains the lack of ligation activity of U8/U+2/C+3 and shows that, in some contexts, mutations of G8 can impair docking. The enhancement in docking efficiency of the SV5 construct is likely to be due, in part, to the stabilizing influence of the U12C−A−3G base pair substitution (8). Although the SV5 construct is able to overcome some of these docking problems, it is still sensitive to G8 and reversion by U+2 substitution, although to a lesser extent than the HS construct. Furthermore, this suggests that these base changes may primarily affect the assembly and activity of the active site, which can disturb the global folding to a lesser degree.

Structural Modeling—MC-SYM was used to model the active sites of the two major revertant families, U8/C+2 and U8/A+2 (Fig. 7). The models show that general orientation of functional groups at the active site is preserved relative to the crystal structure of the WT sequence, suggesting a basis for the recovered activity of these mutants relative to the single U8 or +2 mutants. Major interdomain interactions observed biochemically and in the crystal structure, such as the G+1-C25 Watson-Crick base pair, were also preserved in the two models (data not shown). The root mean square deviations between each model and the crystal structure were calculated by comparing the backbone of A−1, G+1, N+2, N8, and A9. The values for this structural difference were 1.55 and 1.65 Å for U8/A+2 and U8/C+2/U+3 mutants, respectively. More specifically, similar distances were obtained between analogous base functional groups at the active site. For example, the U+2 O−4-G8 N−2 distance is 2.94 Å in the crystal structure, and the C+2 N−4-U8 O−2 distance is 3.16 Å in our model. Putative base-backbone interactions in the x-ray structure have also been maintained in both models. The imino proton of U8 is within H-bonding distance of the 2′-hydroxyl of A−1. In general, however, the orientation of these putative H-bonding groups is less optimal in the mutant models compared with that in the x-ray structure. In addition, the angle of the attacking nucleophile to the P−5′-O bond is less optimal than that observed in the x-ray structure. These facts may explain the loss of catalytic activity of the mutants relative to the WT sequence.

DISCUSSION

G8 and U+2 Interact within Loop A—Previous studies reported that G8 is strictly required for cleavage activity (36, 37), and it has recently been proposed that this nucleotide is a major chemical component of the active site (4, 21, 23, 24). Here, we have shown that the G8U mutation retains residual activity, which is significantly enhanced (up to 200-fold in some contexts) by mutations at substrate strand positions +2 and +3. Although the reversion is very specific for this sequence, it could potentially be due to some indirect effect, e.g. the tertiary folding of the molecule. Two lines of evidence point to a direct interaction between U+2 and G8. First, in the context of the isolated substrate-SBS duplex, the G8U mutation results in a decreased affinity of the two strands, which is rescued by the suppressive mutation. Second, docking of the two domains is not impaired in G8 or U+2 mutants as monitored by fluorescence resonance energy transfer (14, 25) and hydroxyl radical footprinting and native gel analysis (24). This suggests that most of the observed catalytic effects result from subtle changes in local structure and leads us to propose that an interaction occurs between G8 and U+2. A direct interaction across the loop is also supported by chemical probing data showing that G8 N−1 and N−2 are protected from kethoxal modification upon substrate binding (45). The U8 ribozyme shows maximum activity if nucleotide +2 is a cytosine or, to a lesser extent, an adenosine. C+2 also improves the activity of a ribozyme bearing a xanthosine at position 8. Close examination of the different catalytically active combinations at positions +8 and −2 leads us to propose that the O−4 carbonyl of U−2 interacts with the G8 exocyclic amine in the WT ribozyme. In the revertants, this interaction is replaced by a hydrogen bond between the O−2 carbonyl of U8 and the exocyclic amine of C−2 or A−2. This interaction has been observed in the X-ray structure of molecules with either a 2′-O-methyl substitution or transition state analog at the cleavage site (4, 21). Here, we have provided genetic and biochemical evidence that this interaction has a functional relevance in the native ribozyme.

Role of U+2, a Nucleotide in Dynamic Equilibrium—U+2 is clearly not a direct component of the catalytic site, as its replacement with an abasic site results in a decrease in activity of only 10-fold (14). Nevertheless, the same study and our work showed that substitution of U+2 with any of the natural bases leads to a dramatic drop in activity. Here, we have shown that the U+2C mutation, which is very detrimental to the WT ribozyme, is functional and even improves the activity in the context of a U8 ribozyme. This leads us to hypothesize that the role of U+2 is to position G8 in a suitable way for catalysis. The U+2C mutation may lead to stable alternative folding that prevents G8 from participating in catalysis. The double mutant U+2/C+3U probably exists in an alternative non-native conformation, extending helix 1 by A7-U+3 and G8-C+2 base pairs. Mutation of C+3 alone, however, does not affect the ribozyme. We conclude that the position of U+2 itself is influenced by the backbone geometry and therefore by the identity of its closest neighbors, explaining the synergistic effect of C+3 mutations. This proposal predicts that the abasic +2 substitution results in only a modest loss of activity (10-fold) because G8 is not sequestered into an alternative conformation in the absence of a mutated base at position +2. G8 is free to explore multiple conformations in the active site in the absence of a base at position +2. Indeed, an entropic penalty for the abasic mutant has been previously observed relative to the WT substrate (14).

The position of U+2 throughout the catalytic cycle is still a matter for debate. An NMR-derived model of the isolated loop A domain describes U+2 as bulging out of loop A (11). Cross-linking and circular dichroism studies suggest that U+2 is internalized upon docking (13), whereas Walter et al. (14) have shown that 2-aminopurine at this position becomes unstacked and restrained when loop A interacts with loop B. Finally, in the X-ray structure, U+2 O−6 is within H-bonding distance of the exocyclic amine of G8. Together with our results, this suggests the following sequence of events. Prior to docking, U+2 is in dynamic equilibrium between bulging out of loop A and in a position stacked within the loop, where it is probably interacting with G8. This unstable stacking in loop A favors the docked conformation and is stabilized upon interaction of the two loops, helping to correctly position G8 for the reaction.
Thus, position +2 appears to be conserved because of its role in positioning G8 during catalysis in addition to avoidance of alternative folding that would prevent docking and/or catalysis. Although it is clear that U+2 is not directly involved in the docking process, it is intuitive that the overall shape of loop A influences this step. Even minor changes in loop A and/or loop B structure can probably modify docking efficiencies, and this may lie at the root of observations that individual molecules exhibit different inherent docking rates (46). Here, we have shown that a structural defect impairs docking of a less stable construct like the HS construct, but is overcome in the optimized SV5 construct, consistent with our observations of G8U and C+2/U+3 mutants.

Implications for the Catalytic Mechanism—G8 has been extensively studied because it is believed to be of central importance to hairpin ribozyme catalysis. All of the functional groups of the Watson-Crick face have been shown to make significant contributions (24, 47). A number of different catalytic mechanisms have been proposed, including acid-base catalysis in which the 6-keto group of G8 acts as a general base and N-1 has a general acid or base (24). A recent crystal structure (solved in the presence of the transition state analog vanadate) suggests that catalysis occurs by transition state stabilization through hydrogen bonding of A38, G8 N-1 and N-2, and possibly A9 to the intermediate (21). Our work does not address the catalytic mechanism per se, but gives insight into the functional groups involved. Our results (24) and those of other investigators suggest that the keto group (guanosine O-6 or uridine O-4) is important for activity (25, 38). However, Lebruska et al. (23) showed that a ribozyme with an abasic position 8 can be rescued by cytosine, isocytosine, 2-aminopyridine, and, to a lesser extent, 2,6-diaminopurine. Indeed, Pinard et al. (24) observed a slightly better activity with a 2,6-diaminopurine than with inosine at position 8, and nucleotide analog interference modification defined the G8 exocyclic amine as an important determinant for ligation activity (47). These results suggest that the exocyclic amine of G8 is more important for catalysis than the 6-keto group. Interestingly, the NMR-derived structure of the isolated loop A shows the G8 O-6 carbonyl paired with the 2'-hydroxyl proton of G+1 (11). This suggests that the O-6 keto group could be involved in the formation of a docking-proficient structure of loop A, which would then be of no utility in the context of an abasic position 8 ribozyme. Nevertheless, the primary importance of the 6-keto group may be that its presence ensures the protonation of N-1 at neutral pH.

3 T. J. Wilson, personal communication.
because inosine and guanosine have pKa values for N-1 of >9. This is also true for uridine N-3, but not for adenosine or cytosine, for which the pKa values for N-1 and N-3 are far from neutral (3.5 and 4.1, respectively). The correlation between the presence of a protonated N-1 and the activity of the ribozyme has been illustrated by the use of non-natural bases over a range of pH values (23, 24).

How is the function of the G8 exocyclic amino group served in mutants such as uridine and xanthosine, where it is replaced by a carbonyl, or inosine, where it is absent? These mutations result in losses of activity of 2–3 orders of magnitude. This is consistent with the loss of an important catalytic determinant. We have shown that U8 ribozyme activity can be rescued by the presence of a C+2 mutation and that this co-variation is also functional with xanthosine at position 8 (data not shown). The loss of activity in I8 mutants can also be rescued by the C+2 change.4 In the course of modeling the G8U/U+2C ribozyme catalytic core, we noted that the U8 O-2/C+2 N-4 interaction places one of the two N-4 amino protons at near H-bonding distance (3.2 Å) from the Rpy-non-bridging phosphate oxygen of the scissile bond. The equivalent interaction involving G8 N-2 and the non-bridging oxygen, separated by 3.09 Å, has been predicted to promote catalysis in the native ribozyme (4). We therefore suggest that, in these mutants, the 2-amino group of C+2 may function in the same capacity as G8 N-2. In this case as in others, it may be hard to define the exact pre- and post-docking interactions within loop A because studies on single molecules have shown that slightly different structures are likely to coexist and be catalytically active to a greater or lesser degree (46).5 In summary, we have described experimental biochemical and genetic evidence in favor of an interaction between U+2 and G8 in the native hairpin ribozyme active site. This interaction is important to fine-tune the structure of the hairpin active site and directs important interactions required for the chemistry of the phosphoryl transfer reaction. Consequently, G8 mutation leads to a change in specificity of the ribozyme.

Acknowledgments—We thank Jeff McKenna for technical help, Michele Milham and Jillian Amural for T7 polymerase preparation, and David Pecchia for oligonucleotide synthesis.

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