Analysis of the functional role of a G·A sheared base pair by

*in vitro genetics*†

Bruno Sargueil1,3, Jeffrey McKenna2, and John M. Burke2

1 Centre de génétique moléculaire CNRS avenue de la terrasse 91190 Gif sur Yvette France
2 Markey Center for Molecular Genetics Department of Microbiology and Molecular Genetics
The University of Vermont Burlington, Vermont 05405 USA

Running Title: selection of suppressor mutations in the hairpin ribozyme

3*Correspondence to:*
Bruno SARGUEIL
Centre de Génétique Moléculaire
CNRS
avenue de la terrasse
91190 Gif sur Yvette
France
Phone: 00-33-1-69-82-31-54
Fax: 00-33-1-69-82-43-86
email: sargueil@smigiris.cgm.cnrs-gif.fr

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A classical genetic strategy has been combined with an in vitro selection method to search for functional interactions between the two domains of the hairpin ribozyme. G21 is located within internal loop B, it is proposed to form a sheared base pair with A43 across loop B and to bind a Mg$^{2+}$ ion. Both nucleotides are important for ribozyme function, and G•A sheared base pairs are a very widespread motif in structured RNA. We took advantage of its presence in the hairpin ribozyme to study its functional role. Pseudorevertants, in which the loss of G21 was compensated by mutations at other positions, were isolated by in vitro selection. The vast majority of G21 revertants contained substitutions within domain A, pointing to functional communication between specific sites within the two domains of the hairpin ribozyme. The possibility of a direct or redundant contacts is supported by electrophoretic mobility shift studies showing that a complex formed between domain B of the ribozyme and the substrate was disrupted and restored by base substitutions that have analogous effects on catalytic activity. The functional significance of this complex, the role of the nucleotides involved and the basis for magnesium ion requirement is discussed.

Keywords:
Combinatorial selection, compensatory mutations, UV cross-linking, catalytic RNA, G•A sheared base pair, ion binding site.
Introduction

Four natural small catalytic RNA motifs are known to mediate cleavage and ligation, reactions central to RNA processing events associated with replication of the genomes of certain RNA viruses or virus-like RNA replicons. These include the hairpin (1-3) hammerhead (4,5), hepatitis delta (6), and Varkud satellite (7) ribozymes (also see (8)). Divalent cations including Mg$^{2+}$ are known to support catalysis. However, recent evidence indicates that divalent metal ions function to facilitate ribozyme folding, but do not play a direct obligatory role in catalysis for the hairpin, hammerhead, and VS ribozymes (9-12). For each of these three ribozymes, it appears that catalytic function is resident in the folded structure of the RNA itself.

The 21 kDa complex between the 50 nt hairpin ribozyme and its 14 nt substrate consists of four short helical elements and two internal loops (Fig. 1). The complex is organized into two domains. Domain A consists of the substrate and substrate-binding sequence, forming helix 1, internal loop A, and helix 2. Domain B is located entirely within the ribozyme, and consists of helix 3, internal loop B, and helix 4. The two domains of the ribozyme-substrate complex interact through tertiary contacts to generate a complex necessary for catalytic activity. This was initially established through linker insertion studies (13,14), and domain separation and reconstitution experiments (15). Covalent crosslinking studies were used to develop a preliminary tertiary structure model for the ribozyme-substrate complex (16). Subsequently, fluorescence resonance energy transfer (FRET) analysis (17,18), and hydroxyl radical protection experiments (19) were used to establish the requirements for docking of the two domains, and to map the interface between the domains in the docked complex.

G21 is located at a particularly interesting location within the hairpin ribozyme. Ultraviolet irradiation at low intensities induces a specific photo-crosslink between G21 and
U42 (20). Although the crosslinked RNA has little or no catalytic activity (20), the crosslinkable conformation is likely to represent a ground state structure of the active ribozyme species because, (1) 90% crosslinking can be achieved with molecules that fold homogeneously, and (2) domain B modifications that increase crosslinking yield concomitantly increased catalytic activity ((21) and B. Sargueil, S. Butcher, and J. Burke, unpublished results). The region of loop B in which G21 is embedded represents a common folding motif, also found within loop E of eukaryotic 5S rRNA, the conserved central domain of viroids and the sarcin-ricin loop of large rRNA (20,22-24). Within this motif, a local reversal of strand direction results in cross-strand stacking of the two crosslinkable bases. In the context of the hairpin ribozyme, G21 forms a sheared base pair with A43, and stacks on U42 (Figure 2). A remarkable feature of this structure is that both the major and minor grooves are wide and the Watson-Crick base-pairing faces of some of the bases are left unpaired, and are projected into the grooves. This structural model has recently been confirmed by the NMR derived structure of the isolated loop B (22). It has been proposed that this motif is well suited for specific binding of protein or RNA species (25). Particularly noteworthy is the observation that N7, N1 and N2 of G21 are protected from chemical modification upon Mg\(^{2+}\) addition (22). This could be related to the recent finding that a Mn\(^{2+}\) ion can bind in between A20 and G21 (26).

The identification of specific tertiary contacts between the two domains presents a very challenging problem. Several biochemical and biophysical strategies have been used successfully to probe the tertiary structure of small ribozymes. Recently a interaction between the nucleotide G+1 of the substrate and C25 has been biochemically identified (27).

Here, we describe a fundamentally different strategy, based on a classical genetic approach, to search for functional interactions between the two domains of the hairpin ribozyme. The method is \textit{in vitro} selection of pseudorevertants of loss of function mutations. In genetically tractable biological systems, the selection and analysis of second site revertants has been widely applied to examine functional interactions between molecules and...
within different parts of the same molecules. Advantages of the *in vitro* adaptation of this strategy to RNA structure are, first, that the investigator has complete control over the location, distribution, and frequency of mutations and, second, that the selection can be performed with very many more variants than can be accommodated in an *in vivo* selection. Our results clearly demonstrate functional compensation between specific sites within the two domains of the hairpin ribozyme. A detailed study allowed us to assess the function of the G•A sheared base pair and to understand the role of one of the magnesium ions required for activity.
Materials and methods

(a) RNA preparation

RNA was transcribed with T7 RNA polymerase using synthetic oligonucleotide templates (28). Partially double-stranded oligonucleotides (200 pmol) were transcribed by T7 RNA polymerase at 37°C for 3 hours in 1 ml reactions containing 40 mM Tris-HCl (pH 8), 25 mM MgCl₂, 2 mM NTPs, 1 mM spermidine, and 5 mM DTT. Transcripts were purified by electrophoresis through 20% polyacrylamide/7M urea gels, eluted by diffusion, precipitated, and quantitated by UV absorbance. Body-labeled substrate RNA was prepared as described above, except that 100 pmol template was used in a 100 µl reaction containing 50 pmol of α³²P-CTP and 0.5 mM unlabeled CTP. Following gel purification, substrates were quantified by measuring radioisotope incorporation.

Mixed RNA-DNA oligonucleotides were synthesized by solid-phase synthesis as described (29). End-labeling of RNA molecules was accomplished by dephosphorylation of 5’ ends using calf intestinal alkaline phosphatase (1 unit/50 pmol RNA) for 1 h at 50°C, followed by phosphorylation with T4 polynucleotide kinase (2 units/10 pmol of RNA) using 25 pmol of γ³²P-ATP, and purified as described above.

(b) Activity assays

Cleavage assays were conducted using hairpin ribozymes synthesized in two segments, as described (30). The 5’ and 3’ ribozyme segments were mixed at a concentration of 100 nM, denatured for 1 min at 90°C, then renatured for 20 min on ice in cleavage buffer (5 mM or 12 mM MgCl₂ (as indicated), 40 mM Tris-HCl (pH 7.5)). To assay a large number of mutants under standardized conditions, we examined cleavage time courses under the following multiple turnover conditions which allows us to have a reasonable estimate of the cleavage efficiency over a wide range of activity. In these experiments, 20 nM ribozyme and 200 nM substrate were incubated at 37°C in cleavage buffer (5 mM or 12 mM MgCl₂ (as indicated), 40 mM Tris-HCl (pH 7.5), reactions were initiated by mixing an equal volume of ribozyme and
substrate, and aliquots were removed and quenched at 0, 5, 10, 15, 30 and 60 min. Reaction products were separated using a 20% denaturing polyacrylamide gel, and dried gels were quantified using a Bio-Rad GS-250 Molecular Imager. To determine initial reaction rates, results were fitted by linear regression to the equation \( \ln F = -kt + b \), where \( F \) is the unreacted fraction and \( t \) is the reaction time. Each mutant was assayed side by side with the wild type construct and relative initial rate constants \( (k_{\text{rel}} = k_{\text{mutant}}/k_{\text{wt}}) \) were calculated. Each construct was assayed at least twice. Absolute initial rates varied by up to 30% from experiment to experiment, but the reported relative values were reproducible to within 10% error, usually less. Mg\(^{2+}\) dependence was determined using the cleavage protocol described above, but using single turn-over conditions (200 nM ribozyme, 20 nM substrate), the Mg\(^{2+}\) concentrations used were 2,5,10,20,50,100 mM and depending on the efficiency of the considered ribozyme, aliquots were taken at 0, 2, 5, 10, 15 minutes, or 0, 5, 10,15, 30, 60 minutes.

(c) Photo-cross-linking

RNA cross-linking was performed as described in (20). Gel-purified radiolabeled RNA was denatured at 70°C for 2 minutes and then renatured for 20 minutes on ice in cleavage buffer (12 mM MgCl\(_2\)). Twenty microliters aliquots were irradiated at a distance of 1 cm with a 254 nm hand-held ultraviolet light source. Irradiated samples were then analyzed by electrophoresis through 15% denaturing polyacrylamide-urea gels and reaction products were quantitated as described above. Cross-linked nucleotides were mapped using end-labeled RNA and partial alkaline hydrolysis, and identified by electrophoresis alongside enzymatic RNA sequencing ladders (20).

(d) In vitro selection

The general method was carried out as described (31,32). The transcriptional template was generated by annealing and extension of two overlapping oligonucleotides, a 74 nt segment containing the coding sequences for a T7 promoter, a primer binding site, part of the
ribozyme sequences (5'- T A A T A C G A C T C A C T A T A G G A C G C A C G T G A G C
T C G A G T A A C A G    A G A A N C A C C C A G A (ATC) A A A C A C A G T T G T
G G T -3'), and a 63 nt segment representing the antisense sequences for the remainder of
the ribozyme, a linker, the substrate and a second primer binding site (5'- C G A C G T C G G
C T C T A G A G A A A C A G G A C T N C A G A G A T C T G A C C A G G T T A A T A
A C C A C A A C G T G 3'). Overlapping sequences are in bold. Underlined nucleotides
were mutagenized to a total frequency of 12% (i.e. each mutant base was present at a
frequency of 4%). The double-stranded template (400 pmol) was transcribed with T7 RNA
polymerase for 3 hours; these conditions are permissive for self-cleavage of the wild type
molecule and active variants. Cleaved (active) and uncleaved (inactive) molecules were
separated on a 8% polyacrylamide/7M urea gel and eluted. For the active pool, ligation
reactions were carried out by incubating the active fraction with 200 pmol of a substrate for
ribozyme ligation in cleavage buffer (12 mM MgCl₂) at 0°C for 30 minutes. The
oligonucleotide substrate for ligation was a DNA-RNA hybrid containing a binding site for
primer 2 (P2, 5'- G U C C U G U U U T C T A G A G A C G C T G C T G -3'; ribonucleotides
in bold). The ligation product was isolated using a 8% polyacrylamide/7M urea gel, reverse-
transcribed, and amplified by PCR using the primers P1 (5'- C A C G A G G C T C T C T A
G A -3') and P2 (5'- T A A T A C G A C T C A C T A G G A C G C A C G T G A C T C G -3').
Inactive molecules were reverse transcribed and amplified using primers P2 and P3 (5'- C G
A C G T C G G C T C T A G A G -3').

e) Gel shift assay
Substrate and loop B domain interactions were analyzed using a gel shift assay essentially as
previously described (33). Trace amounts of 5' radiolabeled substrate (<1 nM) was incubated
in siliconized tubes with increasing amounts of unlabeled loop B domain (0, 0.1, 0.5, 1, 2, 5,
10, 20 µM). The mix was incubated for 20 minutes at 37°C, and binding was allowed to
proceed for 2 hours on ice. The 37°C-20 min incubation allows the loop B domain to reach
the same folding state as a “standard” denaturation renaturation (90°C for 1 min followed by 20 min on ice), but avoids dimer formation as monitored with a cross-link assay ((20) and S. Butcher, personal communication). After addition of 5% glycerol, samples were run on a 15% non-denaturing polyacrylamide gel containing 25 mM magnesium acetate and 40 mM Tris-acetate pH 7 for 16 hours at 11 watts and 4°C. The results were quantitated using a Bio-Rad GS 250 molecular imager. Proportion of bound substrate as a function of concentration of loop B domain was plotted on a semilogarithmic scale, the $K_D$ value was estimated as the observed concentration corresponding to half of the binding at saturation.
Results

(a) G21 mutants strongly inhibit cleavage

*In vitro* selection experiments showed that G at ribozyme position 21 is important for cleavage (34). However, selection experiments generally are of limited utility in understanding defects associated with specific mutations. Typically, only a small fraction of inactive clones are recovered and inactive variants usually contain multiple base substitutions. To begin analysis of the functional importance of G21, we synthesized ribozymes containing each of the four possible bases at position 21, and conducted parallel *trans*-cleavage assays. G21 substitutions reduced cleavage efficiency by 10- to 100-fold in a cleavage buffer containing 5 mM Mg$^{2+}$, with U being the most highly inhibitory (Table 1; see also (35,36)). Partial suppression of the cleavage deficiencies was observed at 12 mM Mg$^{2+}$ for all three mutants. To better evaluate the effect of ions, we carried out a study to compare the magnesium dependence of cleavage for the G21U mutant and the WT. The apparent $K_{Mg^{2+}}$ for the WT ribozyme is 15 mM, and the saturation is obtained by 50 mM. These values are in good agreement with the values found in other studies using the same sequences (17,37,38). The requirement is clearly increased for the G21U mutant for which the $K_{Mg^{2+}}$ is around 30 mM, although a level of activity comparable to the WT molecule is reached over 100 mM.

When analyzed on a non denaturing polyacrylamide gel, the WT ribozyme-substrate complex run as two bands. The fastest migrating band corresponds to the active “docked” ribozyme, while the low mobility complex was shown to be an inactive complex in which domain A is stacked over domain B (39,40). When conducted with the G21U ribozyme, the gel shift mobility assay in presence of 5 mM Mg$^{2+}$ showed that most of the complex is undocked (Data not shown).

Mutations at A43, the pairing partner of G21 in the loop B structure, indicated that a purine is required at this position. A43G shows only a two to three -fold reduction in cleavage activity.
However, no cleavage was detectable during analysis of the A43C and A43U mutation (see also (31,35-37)).

It is striking that the most inhibitory substitutions at positions 21 and 43 are those with the potential to generate canonical base pairs (G21U, A43C and A43U). These results could reflect that the formation of a 21• 43 a Watson-Crick base pair induces an inactive conformation, possibly by collapsing the helix 3 proximal segment of internal loop B into an A-form extension of helix 3.

(b) *In vitro selection of second-site revertants of G21 mutations*

*In vitro* selection was used to search for second site revertants that could compensate for mutation of G21 to A, U, or C (Fig. 1). The synthetic DNA template encoded a complex population of sequence variants containing equimolar quantities of A, U and C at position 21, while G was absent. In addition, we mutagenized the nineteen functionally important bases within internal loops A and B to a frequency of two additional mutations per molecule, and completely randomized the two base pairs of helix 2 that are proximal to internal loop A (see Materials and Methods). The mutation U39C had previously been isolated as a nonspecific suppressor of several ribozyme mutations (31,34,41). To avoid the potential recovery of this previously characterized suppressor, we did not mutagenize U39 into the population before initiating selection. This design yields a pool of variant molecules with a complexity of approximately $10^6$ unique sequences (42). To prevent recovery of a true revertant (G21) due to polymerase misincorporation, a single round of selection was performed. Previous experience shows that a single round selection for active variants provides a very high level of enrichment for DNA encoding active ribozymes, and that the selected population consists of both highly active and a variety of suboptimal species (B. Sargueil, A. Berzal-Herranz, and J. Burke, unpublished results). It should be noted that a round of selection provides two selective steps for RNA catalysis, cleavage followed by ligation.
The DNA template pool was transcribed for 3 h under conditions favorable for self-cleavage; then cleaved (active) and uncleaved (inactive) species were separated by preparative gel electrophoresis. To empirically assess the sites and frequencies of sequence variation in the pool, cDNA from inactive transcripts was synthesized, cloned, and sequenced. The frequency and position of mutations were consistent with those predicted by design of the variant pool. To recover the active populations, molecules that had undergone cleavage were allowed to carry out RNA-catalyzed ligation, then cDNA copies of active ribozymes were synthesized, amplified, cloned, and sequenced.

Cloned cDNA molecules from the active pool were characterized by sequencing, and by monitoring the amount of self-cleavage during in vitro transcription (reaction including 20 mM MgCl₂ and 2 mM spermidine). Representative clones derived from the active pool are shown in Table 2. As expected, all recovered clones contained G21 substitutions. A small number of clones containing only G21 mutations were identified, and showed the very low levels of self-cleavage activity expected from the trans-cleavage assays described above. A number of pseudorevertants were identified which displayed self-cleavage activities much higher than those with G21 mutations alone. Most of these clones had dual base substitutions at the -3•12 base pair within helix 2, previously shown to be essential for substrate binding and cleavage (43). In these clones, G21 mutations to U, C, and A were accompanied by changes of the a-3•U12 base pair to g•C, c•G, and u•G. In addition, a single pseudorevertant was identified which contained a transversion of the base immediately 5’ of G21, A20C.

(c) Second-site mutations in a helix 2 base pair (-3•12) rescue G21 mutations
The functional compensation of G21 mutants by substitutions at the -3•12 base pair is particularly interesting because the compensatory mutations lie in the loop A domain of the ribozyme, while the original mutation is in the loop B domain. Trans-cleavage assays using synthetic ribozymes and oligonucleotide substrates were carried out, in order to examine
more closely the efficiency and specificity of this compensatory effect. Using the two piece ribozyme construct previously described and characterized (30,37), we generated ribozymes and substrates to test all possible combinations of bases at position 21 and base-pairs at -3•12. Results are shown in Fig. 3 and table 3 (please note that helix 4 sequence is 5’-C_{27}ACC - 3’ and 5’ - GGUG_{35} - 3’ except for the simple G21 mutants that have a 2 G•C bp extended helix 4 which slightly enhance their activity (21) and therefore slightly undermine their mutant phenotype).

Because it is the most strongly inhibited, we will first discuss the G21U mutant. The autoradiograms of Fig. 3 indicate that changing the a-3•U12 base pair to g•C dramatically increases the activity of the G21U mutant. The increase in the initial cleavage rate is over 100-fold in presence of 5 mM MgCl$_2$ (Table 3). This same base-pair substitution also increases the initial cleavage rate of the G21 ribozyme, but by a factor of 3 only. Interestingly, all base pair substitutions at -3•12 enhance the activity of the G21U mutant ribozyme. Rate enhancement varies from seven-fold to 110-fold. A similar pattern is observed when G21 is changed to A or to C. For each G21 mutation, the naturally occurring base pair (a-3•U12) is the least favorable for cleavage. For G21A and G21C, base pair substitutions at -3•12 increase initial cleavage rates by factors of 2 to 16 relative to a-3•U12. In every cases, g-3•C12 has the most significant effect.

Regardless of the identity of the base at position 21, cleavage is strongly inhibited by mutational disruption of the -3•12 base pair (data not shown). This result is consistent with the selection experiment described above, in which all active variants recovered could form -3•12 base pairs, and are also fully consistent with previous selection and mutational studies (43,44).

To screen for potential backbone interactions involving nucleotides G21, a-3 and U12, the effects of substitutions with 2’ deoxyribonucleotides at these three sites were examined. None had a strong effect, deoxyribose substitution at positions G21, a-3 and U12 reduced initial cleavage rates by two-fold, no reduction, and four fold, respectively (Data not shown,
and (45,46)). We conclude that only U12 2’OH could potentially be involved in the rescue phenomenon observed.

Another suppressor of G21 mutations has previously been isolated (31,34,41). To analyze the possibility of an interaction between U39 and the -3•12 base pair, we combined mutations at these positions together with the G21U mutation. U39C appeared to have a very marginal effect compared to g-3•C12. We concluded that these two suppressors act through different ways.

Finally, the magnesium requirement for cleavage was determined for the U12C and G21U:U12C ribozymes (figure 4). The $K_{\text{Mg}^2+}$ apparent for the U12C construct is 3-4 mM, and the saturation is obtained around 10 mM. Once more, this is in good agreement with the data obtained previously by others using the U12C variant of the WT ribozyme (47,48). The revertant shows a similar behavior except that the $K_{\text{Mg}^2+}$ apparent is slightly higher (around 6 mM).

(d) Partial suppression of G21U by A20C

Independent of the -3•12 substitutions, the mutation A20C was recovered as a second-site revertant of the G21U mutation, and showed a significant increase in self-cleavage activity relative to G21U. Cleavage assays using trans-acting ribozymes were conducted and confirmed that A20C is indeed a second-site suppressor of G21U, increasing activity by a factor of approximately 15 (Table 4A).

To explore the specificity of this suppression, we synthesized and analyzed a panel of ribozymes with mutations within loop B at positions 20 and 21. In the context of the wild type ribozyme, all mutations of A20 are slightly inhibitory (Table 4A). In the G21U background, the only significant compensatory effect observed was that of the original isolate, G21U:A20C.

To investigate the possibility of a direct contact between A20 and the -3•12 base pair, we examined the effects of combinations of A20 and helix2 mutants on the suppression of G21U mutations (Table 4B). The g-3•C12 substitution strongly enhanced the activity of the
G21U:A20C and G21U:A20U ribozymes, but had little effect on the inactive G21U:A20G ribozyme. The effect of the -3•12 base pair mutation is independant of the nucleotide present at position 20, except for the inactive A20G mutation (see below). The rescue by a-3•U12 substitution may in some cases obscure the A20 effect.

Two further results point to the likely importance of non-canonical base-pairing between A20•C44 and G21•A43. First, mutations that would be expected to extend Watson-Crick base pairing of helix3 into loop B are severely inhibitory (Table 4A). Second, the loss of activity induced by the A20G transition can be partially rescued by the C44U mutation (data not shown). A G20•U44 wobble pairing is nearly isosteric to the one seen for A20•C44 in the NMR derived structure (22).

The suppressive effect of A20C is likely to be a consequence of a local conformational effect within loop B, this could for example reflect the presence of a Mg$^{2+}$ binding site in between A20 and G21 (26).

(e) Effects of A43 mutations on the suppression of G21 mutants

In the model of loop B structure, G21 forms a sheared base pair with A43 (16,20,22,49). Therefore, the functional effects of A43 mutations were examined in the wild type molecule (Table 5). Since G21 is also linked genetically to the -3•12 base pair, we also examined the effects of base-pair changes at -3•12 on the A43 mutants. The simultaneous mutation of G21 and A43 is very detrimental in any context (table 5). The effect of the mutations of the -3•12 base pair is only seen with G43, and is really significant for the g-3•C12 substitution which partially restores activity in the G21U:A43G and G21C:A43G contexts (at least a 40 fold improvement). Together with the data on G21, this strongly suggests that the purine requirement at position 43 is not only constrained by the G•A sheared base pair.

(f) UV-cross-linking assays to monitor folding of loop B

The structure-induced photo-cross-linking of G21 to U42 (20) can be used as an activity-independent assay to monitor folding of the RNA within loop B. We examined the time course
and reaction sites of photo-cross-linking for several of the mutants that are under investigation in this study.

Interestingly, all variants at position 21 retain their ability to form a UV-induced cross-link (Fig. 5A), although the yield of cross-linked product is significantly reduced relative to the wild type molecule (Fig. 5B). Mapping of the 5' cross-linking site for the G21U mutant shows that the cross-link occurs at or in the immediate vicinity of U21 (data not shown). The 3' cross-linking site maps unambiguously to U42, the same site used by the wild type molecule (note that in (20) the 3' cross-linking site was incorrectly interpreted as U41). Together, these results indicate that we observe the same cross link reflecting the same local conformation present in all the molecules tested, although a smaller proportion of the mutant molecules are folded into the photoreactive structure.

In contrast, A43 mutants show a slightly different pattern. Cross-links to the correct sites are observed for each mutant, but with different efficiencies (Fig. 5B). A43G and A43U mutants retain a significant amount of crosslinking, while A43C almost abolish the photoreactivity.

Although the helix 2 base pair substitution g-3•C12 rescues the cleavage deficiency of the G21U mutant, it has no effect on the reduction in cross-linking efficiency induced by the same mutation. As expected, the a-3g•U12C substitution has no effect on the cross-linking rate of the wild type molecules.

These results indicate that the photoreactive structure containing cross-strand stacking is robust enough to withstand base substitutions that result in severe loss of catalytic activity. The observation that the g-3•C12 substitution partially rescues activity without increasing the cross-linking efficiency confirms that the helix 2 substitution does not affect folding of loop B.

\[(g)\text{ A complex between the substrate and domain B}\]
Gel mobility-shift assays were used to examine possible interactions between the substrate (S: from u-5 to u+9), substrate-binding strand (SBS: from A1 to A13), and domain B (from A15 to A50). When labeled SBS was used, the addition of substrate caused a decrease in mobility consistent with formation of the substrate-SBS duplex (Fig. 6A). However, no such shift was observed in the presence of domain B, whether or not the substrate was present. These results are consistent with previously published observations, which demonstrate formation of a stable S•SBS duplex (40,50,51). In contrast, a complex was observed when labeled substrate was incubated with domain B (Fig. 6B). Two bands with mobilities lower than that of unbound substrate were observed, with the lower mobility species predominating at higher loop B domain concentrations. These two species may reflect differences in conformation, or differences in composition. The titration experiment indicates that the substrate-domain B complex has an apparent KD of approximately 0.6 µM (Fig. 6B). To better evaluate the significance of the complex, we repeated the experiment with the “docking” mutant g+1a substrate. Our results are fully consistent with what has been observed with the complete ribozyme using FRET experiments: the g+1a substrate shows a decreased affinity (apparent KD of 3.5 µM) for the loop B (18).

To examine the possibility of fortuitous base pairing between the substrate and the loop B domain, we used a DNA analog of the substrate as a control, and found that no complex could be detected (data not shown). We tested the effect of combinations of mutations at ribozyme position 21 and substrate position -3. Strikingly, the G21U substitution in domain B eliminated the formation of the substrate-domain B complex, while the a-3g substrate substitution restored complex formation (Fig. 6C et D). The apparent dissociation constant for the complex between the mutant substrate and mutant domain B is 0.1 µM, slightly lower than that of the wild type molecules (fig 6C et 6D). It also runs more homogeneously than the wild type, consistent with the slight increase of activity (fig 6C). Disruption and restoration of the substrate-domain B interaction by the same mutations that inhibit and rescue catalytic activity suggests that this complex could be relevant to normal ribozyme function. However, it
is important to note that we have made numerous attempts to identify catalytic activity in the substrate-domain B complex under a variety of conditions, and no evidence for catalytic function has been obtained.
Discussion

(a) Isolation of suppressor mutations by in vitro selection

In vitro selection technology has provided new tools for investigating the structure-function relationships of biologically important nucleic acids, and for the isolation of RNA molecules with novel properties from random sequence pools. Here, we report an in vitro selection strategy to search for second-site revertants of strongly inactivating point mutations. This strategy has been adapted from organismal genetics, where both intragenic and extragenic suppressors have long been invaluable for identifying functional interactions between sites within and between gene products. Our results show clearly that the same strategy can be successfully conducted entirely in vitro. Biochemical studies of synthetic trans-acting ribozymes confirm that the base substitutions isolated in the selection experiments do, indeed, result in the restoration of RNA cleavage activity to the G21 mutant ribozymes. While the reversion of the G21 mutants phenotype is clearly established, these studies do not themselves tell us the exact nature of the functional compensation of G21 mutations (discussed below).

(b) Structural importance of the G21•A43 sheared base pair

Because mutations at G21 retain some activity, the presence of the G21•A43 sheared base pair in the catalytic structure is still a matter of discussion (37,45). The following chapter discuss the possibility for the local geometry to withstand G21 mutations. The G21•A43 sheared base pair exposes N7 and O6 of G21 on the outside of the helix (22-24,52-54). Stacking of G21 and U42 is likely to be responsible for the observed photocrosslinking reaction (22). In the phylogenetically and NMR derived model, this base-pairing appears as a keystone of the peculiar loop B structure. Strikingly, our findings show that some G21 and A43 mutations retain a significant crosslinking activity and, even more surprising, that the hairpin
ribozyme can recover a full activity despite the presence of this expected important structural
defect.

Several lines of evidence suggest that the overall geometry of a G•A sheared base pair may
be retained in the presence of mutations. First, the G•A pair contains two hydrogen bonds to
the backbone (23,24), and the geometry of the pair may be maintained with only one base-
specific hydrogen bond when a third strand interacts with the sheared pair (53,54). Second,
the base-pairs that flank G•A pairs are known to constrain backbone orientation and stacking
(55). Finally, hydrogen bonds are formed with adjacent backbone (54,56). Congruent with
our finding that some mutations can be tolerated, functional group substitutions of a G•A pair
in two different sequence contexts can be introduced without dramatic loss of stability (52,57).
Similarly, the two A's participating in the sheared base pairs of the hammerhead ribozyme
can also be substituted with ribopurine without dramatic activity loss (58,59). These data
together with the retention of a substantial photosensitivity suggest that a significant fraction of
the mutants molecules still adopt the correct overall geometry. A recent study combining
phylogenetic comparison and molecular modeling of the 5S rRNA loops shows that all the
mutations of the G of a G•A sheared base pair can be accommodated with a similar
geometry. Our results are in good agreement with Leontis and Westhof modelling, except for
the G21:U43 and G21:G43 mutants that retain some photoreactivity and for which they could
not model an isosteric pair (60). Interestingly, the U43 mutant is as photoreactive as the G43
mutant, but in contrast is completely inactive and not rescued by the a-3g•U12C substitution,
this suggests another role for A43.

In conclusion, it is likely that most of the G21•A43 mutants, are not so much altered in the
overall structure of loop B, but in another function which could be the interdomain interaction.

(c) Possible significance of the interaction between the loop B domain and the isolated
substrate
Results of the gel mobility-shift experiments demonstrate the formation of a complex between the substrate and domain B, with a dissociation constant of 0.6 µM and sufficient stability to be visualized by electrophoretic methods. No complexes between loop B and the substrate binding strand could be observed, either in the presence or absence of substrate. Particularly striking is the finding that formation of the complex is inhibited by the G21U substitution and rescued by the a-3g change, in correlation with the loss of cleavage activity that results from G21U and its restoration by the a-3g change. These results suggest that the mechanism through which G21U inhibits cleavage activity could involve destabilization of an interaction between domain B and substrate.

The nature of the complex between substrate and domain B is unknown. Examination of the sequences for potential Watson-Crick base pairing revealed the potential to form several short duplexes. The longest of them consists of five contiguous base pairs between the 3’ end of the substrate (u+5 through u+9) and loop B positions A22 through A26. However, four observations indicate that these potential short duplexes may not be responsible for the slow-migrating complex. First, no complex was observed when a DNA analog of the substrate was used. Second, modification of the sequence of helices 3 and 4 did not eliminate complex formation. Third, none of the identified potential interactions would be directly affected by the G21U or the c-3g substitutions. Fourth, Pinard et al. recently demonstrated that the g+1 position of the substrate interacts with C25 of loop B (27) consistent with this finding, we found that g+1a mutation destabilizes the observed complex.

The complex between substrate and domain B has no detectable cleavage activity, and is therefore unlikely to represent an intermediate on the normal cleavage pathway. However, there are several observations suggesting that the physical interaction that we observe may have some relevance to the catalytic structure. First, formation of the complex is disrupted and restored by the same mutations in the two domains that have been identified as inactivating and compensating mutations on the basis of activity-based in vitro selection experiments. Second, covalent crosslinking experiments indicate that hairpin ribozymes
derivatized with an azidophenacyl group at the A20-G21 linkage form specific adducts with the nucleotides at positions 11 and 12, the latter being the base pairing partner of nucleotide -3 (61). Third, a preliminary structure model of the hairpin ribozyme-substrate complex aligns the two domains such that the -3•12 base pair is closely opposed to the G21•A43 pair (16). Finally, hydroxyl radical footprinting shows that the ribozyme portion of helix 2 and A43 are internalized upon docking of the two domains (19).

(d) Nature of the functional compensation and role of the G21•A43 sheared base pair

The results summarized above indicate that G21 is important, but not essential, in maintaining the active configuration of loop B. The g-3•C12 substitution renders the activity relatively independent of the nucleotide present at the position 21. Despite the lack of an obvious specificity, this substitution can not be considered as a trivial general up mutation since it does not have a significant effect on most A43 mutants, and can not restore the phenotype of the G8 (Sargueil and Burke in preparation), g+1 and G11 mutants (data not shown). The increase of the wild type molecule upon the g-3•C12 substitution could be due to an improvement in loop A-Loop B interaction, or simply to the stabilization of a weak helix 2. We do not favor the latter hypothesis since and the rate limiting step of the hairpin ribozyme seem to be a structural rearrangement upon cleavage or the chemical reaction itself (discussed in (62)). Furthermore, the reverse base pair (c-3•G12) does not have such a drastic effect.

When run on a non-denaturing gel, the G21U mutant runs mainly as undocked molecules. Furthermore, our results suggest that both the G21•A43 and the -3•12 base pair are involved in interdomain interactions. Finally, we report here the existence of a loopB-substrate complex which is disrupted upon G21 mutation and restores by the a-3g substitution. Although we can not conclude on the nature and the functional role of this complex (see above), it clearly reflects that some interactions mediated by G21 and c-3 can be made between the substrate and the loop B domain. This led us to the conclusion that G21 variants
are “docking” mutants which can be restored by high Mg$^{2+}$ concentration. The lack of interpretable specificity of the mutation-suppression pattern do not argue in favor of a direct contact between the G21 and the -3•12 base pairs. In contrast, the identity of A43 seems constrained beyond the requirement for a sheared base pair. Furthermore, two lines of evidence suggest that A43 and U12 are implicated in interdomain interactions. First, using NAIM, Ryder and Strobel (1999) identified the Rp phosphate oxygen at A43 and the 2’ hydroxyl at U12 to be required for optimal activity (45). Second, these two positions are internalized upon docking of the two domains as monitored by hydroxy-radical footprinting. We propose that the A43 shallow groove face interacts with a-3•U12 minor groove. In the most straightforward scenario, A43 interacts with a-3•U12 through a Mg$^{2+}$ ion, which would be superfluous in presence of g-3•C12. Nevertheless, this hypothesis does not take into account the newly identified metal binding site in between A20 and G21. We are therefore proposing that the interdomain interaction is sensitive to the positioning of A43 by the G21•A43 base pairing, which in turn is likely to be influenced by the binding of a Mg$^{2+}$ ion (see below). G21 mutations may alter the configuration of A43, such as it that can no longer interact efficiently with a-3•U12. The substitution a-3g•U12C restores the interaction by providing a new dispatch of interacting functional group in the shallow groove, and the additional g-3 exocyclic amine. In any construct, this interaction may need a structural rearrangement to make A43 more accessible (reviewed in (2)). Although to date we have been unable to develop a compelling structural model for any of these contacts, our hypothesis seem to be a reasonable explanation for our results and the data accumulated by other authors (19,45,47,63). Such a contact could be incorporated in Earnshaw et al.’s model with minor rearrangements (E. Westhof, personal communication). Our data can not rule out that a-3•U12 and G21•A43 base pairs are independently involved in two functionally redundant interdomain contacts, although in this case, we would have expect the G21•A43 partner to appear in the selection experiment. The existence of an ion binding-site at G21 is already supported by two independent sets of experiments. First, in previous results of chemical modification
experiments, G21 shows protection from kethoxal (N1,N2) and NiCr (N7) modification upon folding Mg$^{2+}$ addition (49). Second, Butcher et al. (2000) have recently identified a Mg$^{2+}$ binding site between A20 and G21 phosphates. In their model, the ion bridges the two phosphate and is therefore likely to constrain the geometry of the backbone (see for example (64)). Interestingly enough, we isolated A20C as a suppressor of G21 mutations. In this case it seems likely that a local conformation effect partially restores the Mg$^{2+}$ binding site altered by G21 mutations.

The presence of one or two Mg$^{2+}$ binding sites in the hairpin ribozyme is still a matter of discussion. Walter et al. (2000) hypothesize that two ion binding sites were necessary for a ribozyme constituted of a four way junction, while only one was required for the two-way junction ribozyme (65). A careful examination of the sequences used in the different studies showed that the Mg$^{2+}$ requirement is correlated with the identity of the –3•12 base pair, independently of the nature of the junction (17,18,37,38,47,62,66). Interpreted in the light of the works cited above, our data show that the WT ribozyme has a requirement for two Mg$^{2+}$ ions, with the g-3•C12 mutation exempting the ribozyme from binding the second ion. Our results strongly suggest that the second Mg$^{2+}$ is bound by A20 – G21 and that the g-3•C12 mutation allows the ribozyme to make an interdomain contact without having to bind the second Mg$^{2+}$.

It is significant to note that the a-3g•U12C substitutions appear to increase the activity of the wild type ribozyme in low Mg2+. In addition, it is present in the majority of the molecules derived through in vitro selection (43), as well as in the two other known natural occurrences of the ribozyme (67,68). The reason for the presence of a functional but suboptimal base pair in the sTRSV version of the hairpin ribozyme is not known, and could be imposed by other viral functions.
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Table 1. G21 mutations inhibit trans-cleavage activity.

Cleavage properties of ribozymes with the wild type base (G) and all variants were examined. Initial cleavage rates were measured under conditions of substrate excess (20 nM ribozyme and 200 nM substrate see Material and Methods) in a buffer containing 40 mM Tris-HCl (pH 7.5) with the indicated MgCl₂ concentration, and were normalized to the wild type rate (See Material and Methods). nd, not determined.
| Clone | G21 mutation | Second-site mutation(s) | Activity |
|-------|--------------|-------------------------|----------|
| Wild type | none | none | +++ |
| 43 | U | none | + |
| 24 | U | a-3c•U12G | ++ |
| 31 | U | a-3u•U12G | +++ |
| 32, 42 | U | a-3g•U12C | +++ |
| 25 | U | A20C | +++ |
| 26 | C | none | + |
| 30 | C | a-3c•U12G | +++ |
| 29, 45 | C | a-3g•U12C | +++ |
| 23 | A | a-3g•U12C | +++ |

Table 2. Sequences and self-cleavage activities of pseudorevertants of G21 mutants.

Self-cleavage activity was scored on the basis of the fraction of molecules cleaved following a 3 h in vitro transcription reaction. + up to 10% cleavage, ++ 40 to 60% cleavage, 90-100% cleavage.
Table 3: a-3g •U12C supresses G21 mutations.

Trans cleavage time courses were conducted in a buffer containing 5 mM MgCl2, cleavage rates were determined and normalized to the wild type rate as described in Materials and Methods.

|        | a-3•U12 | g-3•C12 | u-3•A12 | c-3•G12 |
|--------|---------|---------|---------|---------|
| G21 (wt) | 1.0     | 3       | 1.0     | 1.0     |
| G21A    | 0.06    | 1       | 0.2     | 0.1     |
| G21U    | 0.01    | 1.1     | 0.2     | 0.07    |
| G21C    | 0.1     | 1       | 0.45    | 0.2     |
Table 4. A20C is a suppressor of G21U.

A. Effect of A20 and G21 substitutions in the context of a wild type base pair (a-3•U12) in helix 2. Cleavage rates were determined and normalized to the wild type rate, as described in Materials and Methods, and the legend to Table 1. B. Effect of A20 substitutions and -3•12 base pair substitutions in the context of a G21U mutant background.
Table 5: Effect of A43 mutations in different contexts

The values reported in the table (krel) are the observed cleavage rate constant relative to the wild type molecule in the following conditions 20 nM enzyme, 200 nM substrate, 5 mM MgCl2, 40 mM Tris•HCl pH 7.5. The reactions and the calculations were carried as explained in material and methods. *The same value was obtained in the presence of the mutation G21C instead of G21U.

| A43 | a-3•U12 | G21 | G21U |
|-----|---------|-----|------|
| G   | wt      | 0.4 | <0.001 |
|     | u•A     | 0.4 | 0.009 |
|     | g•C     | 0.3 | 0.04* |
|     | c•G     | 0.3 | 0.006 |
| U   | wt      | <0.001 | <0.001 |
|     | g•C     | <0.001 | <0.001 |
| C   | wt      | <0.001 | <0.001 |
|     | g•C     | <0.001 | 0.001 |
Figure legends

Figure 1. In vitro selection of pseudorevertants of G21 mutants.

Secondary structure of the ribozyme-substrate complex is shown, the indication of non canonical pairings are from (50) for loop A and (22) for loop B. Boldface type indicates sites at which sequence variation was introduced into the initial population of RNA variants, as described in the text. Nucleotides in the ribozyme are numbered 1-50. Substrate nucleotides to the 5’ side of the cleavage-ligation site (arrow) are indicated with negative numbers; those to the 3’ side have positive numbers.

Figure 2. G21•A43 sheared base pairs within internal loop B.

This structural model has been developed from NMR spectroscopy data (22,26). The color code is as follow: G21 (Black), A43 (Green), A20 (Gold) and C44 grey. A. Stereo view of the G21•A43 and A20•C44 base pairs. The hexahydrated Mg²⁺ ion is shown bound as modeled in (26) B. Stereo view of loop B global architecture. A20•C44, G21•A43 pairings and the related metal ion binding site are shown. This figure has been elaborated using the Ribbons software and the coordinates from (26).

Figure 3. Base-pair substitutions in helix 2 suppress G21 mutations.

A. Autoradiogram of trans-cleavage time courses, conducted in a buffer containing 5 mM MgCl₂, as described in Methods and Materials and the legend to Table 1. B. Cleavage rates were determined and normalized to the wild type rate (see material and methods).

Figure 4. Influence of G21 and a-3•U12 mutations on the Magnesium dependence of cleavage rate of G21 and a-3•U12 mutant ribozymes.
Single turn over reactions were carried out as described in material and methods in presence of increasing amount of MgCl$_2$. The values reported are the cleavage rate constant relative to the wild type construct.

*Figure 5. UV crosslinking analysis of mutants.*

A - Products of crosslinking reactions analyzed on a denaturing polyacrylamide gel. Reactions were carried out as described in Methods and Materials. Note that the G21 mutants have a 2 bp elongated helix 4, and therefore have a slightly reduced mobility. B - Crosslinking kinetics of variants. $F$ represents the fraction of uncrosslinked RNA.

*Figure 6. Binding of substrate to domain B of the hairpin ribozyme.*

A. Non-denaturing gel analysis of labeled substrate binding strand (SBS), in combination with substrate (S) and domain B. Respective concentrations were 1 nM SBS, 100nM substrate and 5mM loopB. No complex between SBS and loop B was observed with concentration in loop B up to 30 mM. Note that in the lane containing the 3 pieces of RNA, no SBS- S complex is detected because the substrate is titrated by the loop B (see fig. 6B). For detailed experimental procedure see material and methods. B. Non-denaturing gel analysis of complex formation between labeled substrate and unlabeled domain B. Experiments are described in Materials and Methods. C. Non-denaturing gel pattern of a binding assay using G21U Domain B, in combination with wild-type substrate and with a-3g variant substrate. D. formation of the complex between loop B and the substrate, as a function of loopB concentration. □ WT substrate and WT loopB. ● WT substrate and G21U loopB. ○ a-3g substrate and G21U loop B.
Mutagenized DNA oligonucleotide template pool

\[ \text{transcription} \]

Population of variant ribozyme-substrate complexes
(A, C, U replace G21)

\[ \text{Selection 1} \]

CLEAVAGE

Self-cleavage products

\[ \text{Selection 2} \]

LIGATION

Ligation products

\[ \text{RT-PCR} \]

cDNA of active ribozyme-substrate complexes

\[ \text{Cloning} \]

Individual clones

1. Self-cleavage assays
2. Sequencing
3. Synthesize trans-ribozymes
4. Activity assays
Figure 3
B

\[ \text{In} \ F \]

\[ \text{time (min)} \]

- WT
- G21A
- G21U
- G21C
- G21U U12C

\[ \text{In} \ F \]

\[ \text{time (min)} \]

- WT
- A43U
- A43G
- A43C
Figure 6A
Figure 6B

B

[loop B] (μM)

Substrate - loop B

Free substrate
Figure 6C
