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Allosteric Control of Ribozyme Catalysis by Using DNA Constraints

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Covalent, crosslinking tethers have previously been attached to nucleic acids for control of structure and catalysis.[1] Surprising results have been found, such as a study with the *Tetrahymena* group I intron ribozyme in which crosslinks that should substantially disrupt structure nevertheless did not suppress catalysis nearly as much as expected.[2] As an alternative approach to control macromolecular structure, we recently described the use of covalently attached DNA strands as constraints on RNA conformation.[3] When two complementary DNA strands are attached to a large and foldable RNA, DNA duplex formation can compete with native RNA structure. This can destabilize RNA folding (by >6 kcal mol^{-1} in one instance) because the DNA duplex must be disrupted in order for the RNA to fold properly. The integrity of the DNA constraint can be modulated by added enzymes, oligonucleotides, or small-molecule ligands that cleave or interact with the DNA strands.[4] These studies suggested the possibility that the catalytic activity of a ribozyme, and not merely the structure of a foldable RNA, could be controlled by strategic attachment of DNA strands. If this can be established, then we anticipate that the DNA constraint approach will be useful for studying RNA structure–function relationships that involve catalysis and not only folding. Here, we report the identification of a new deoxyribozyme[5] for attachment of DNA to RNA, which considerably aids the synthetic procedure. Using this deoxyribozyme, we describe the successful application of DNA constraints to control the catalytic activity of the hammerhead ribozyme. We provide initial data that allow us to understand the structural basis of catalytic control in terms of modulation of tertiary structure but not secondary structure.

To facilitate synthetic access to DNA-derivatized RNA, which previously required a laborious procedure that depended on assembly of numerous RNA fragments,[3] we used in vitro selection to identify new deoxyribozymes that ligate DNA to RNA. We previously reported the 7S11 deoxyribozyme,[6] which ligates an RNA 2′-hydroxyl group to a 5′-triphosphorylated or 5′-adenylated[7] RNA (Figure 1 A). Although 7S11 and an improved variant, 10DM24,[8] show modest activity when 5′-adenylated DNA is used in place of analogous RNA, the ligation yield was impractically low when examined with an RNA substrate that differed from the arbitrary sequence used during the original selection procedure (data not shown). Therefore, we performed a new selection experiment directly with 5′-adenylated DNA.[9]

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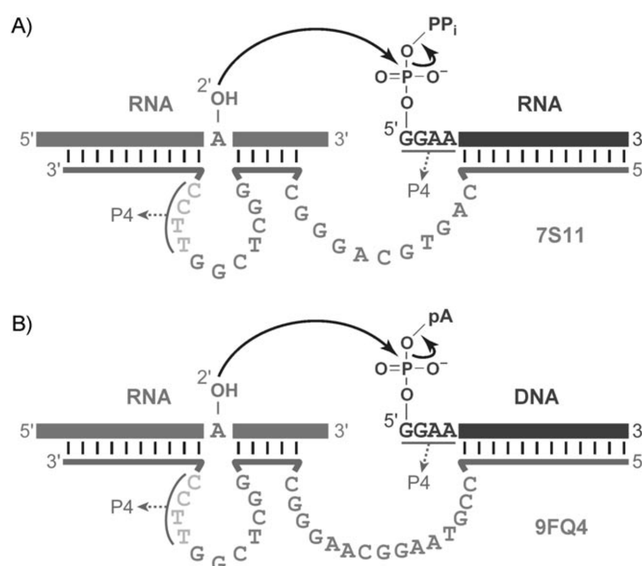

Figure 1. Branched nucleic acid formation by deoxyribozymes. A) The 7S11 deoxyribozyme, which forms branched RNA.[6] The indicated nucleotides form a fourth Watson–Crick paired region denoted P4. The leaving group is pyrophosphate (PPi). B) The new 9FQ4 deoxyribozyme, which attaches 5′-adenylated DNA to RNA. The leaving group is adenosine 5′-monophosphate (pA = AMP).

With a branch-site adenosine that provided the 2′-hydroxyl group in the RNA substrate, we iterated selection rounds (50 mM CHES, pH 9.0, 40 mM $MgCl_2$, 150 mM NaCl, and 2 mM KCl at 37 °C) using an incubation time as low as merely 1 min in the later rounds. The pool activity was 34 % at round 9, after which individual deoxyribozymes were cloned and characterized. One of these deoxyribozymes, 9FQ4 (Figure 1 B), was examined further. Deoxyribozyme 9FQ4 had relatively good RNA sequence tolerance, in that many changes to the RNA nucleotides other than the branch-site adenosine were accepted with good ligation rate and yield for model substrates.[10] However, the branch-site nucleotide itself could not be changed from adenosine; of the other three nucleotides, only C but neither G nor U gave a potentially useful yield. For the 5′-adenylated DNA substrate, changes to all nucleotides other than the 5′-terminal nucleotide were tolerated well. The 5′-nucleotide of the DNA could be G or A with high ligation rate and yield, or C with low activity, whereas 5′-T was not tolerated.

We surveyed the applicability of 9FQ4 for attachment of DNA to RNA using the biologically derived P4-P6 RNA domain, the sequence of which is unrelated to the substrates used during selection. P4-P6 is an independently folding domain of the *Tetrahymena* group I intron RNA,[11] and it was the basis for our first experiments with DNA constraints.[3,4] Ten adenosine nucleotides throughout P4-P6 were chosen for testing on the basis of the surface exposure of their 2′-hydroxyl groups; exposed hydroxyls could presumably have DNA attached without inherently perturbing the RNA structure. Using 9FQ4 deoxyribozyme with appropriate binding arms to target the desired adenosine 2′-hydroxyl groups, we found that four out of the

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ten sites were derivatized with DNA in high yield (>60%), and two other sites were modified in modest yield (~20%). The remaining four sites were derivatized poorly (<10%).[10] These results indicated that the 9FQ4 deoxyribozyme can be applied to the large and structured P4-P6 RNA target with a useful degree of success, although there is still room for improvement to the synthetic procedure.

To test the application of DNA constraints to control RNA catalysis we examined the hammerhead ribozyme, which is an often-studied natural self-cleaving ribozyme originally found in viroids and satellite RNAs of plant viruses.[12,13] Later investigations showed that the hammerhead ribozyme is the simplest RNA capable of self-cleavage at biologically relevant rates.[14] Because the hammerhead ribozyme has been extensively studied biochemically[15] and the structure of the catalytically active conformation was recently determined by X-ray crystallography,[16] this ribozyme is ideal for examining the allosteric control of RNA catalysis with DNA constraints. We chose to work with the hammerhead sequence that was used to obtain the recent X-ray crystal structure; this allowed us to use the structure for designing constraints. This hammerhead ribozyme comprises two RNA strands (Figure 2): a 43 nt (nucleotide) enzyme (e) strand, and a 20 nt substrate (s) strand that contains the scissile phosphodiester linkage.

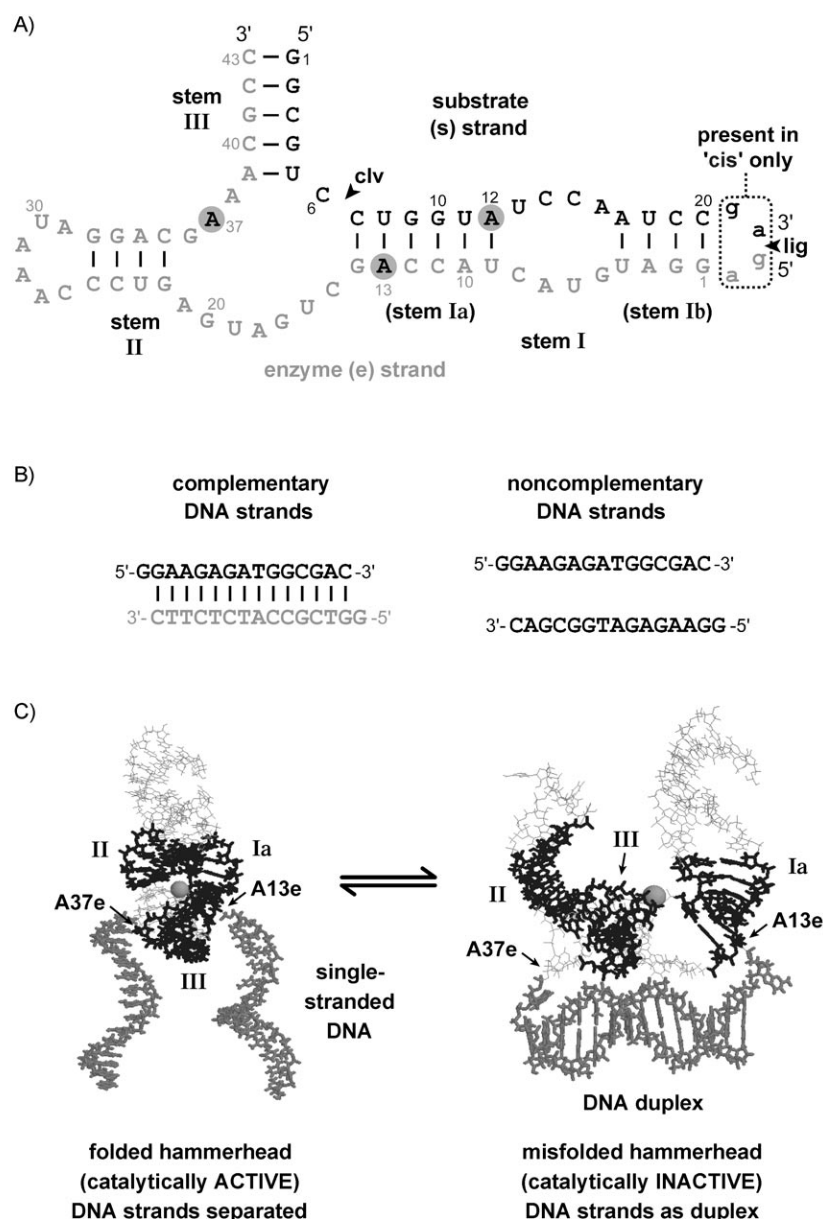

Figure 2. Allosteric control of hammerhead ribozyme catalysis by using a covalently attached DNA constraint. A) Secondary structure of the hammerhead ribozyme showing the enzyme (e) and substrate (s) strands and their numbering. The scissile phosphate is marked with an arrowhead (clv). The nucleotides shown in uppercase are present in the "trans" form of the ribozyme. The nucleotides shown in lowercase are additionally present as a tetraloop in the "cis" form of the ribozyme, with the enzyme and substrate strands joined at the indicated ligation site by using T4 RNA ligase and a DNA splint.[10] The two pairs of sites used for DNA attachment were A13e–A37e and A12s–A37e; all three nucleotides are circled. B) Complementary and noncomplementary 15 nt (14 bp) DNA strands used in this study. C) Strategy for allosteric control of ribozyme catalysis as depicted with 3D models (see the Supporting Information for color images and modeling procedure). Stems Ia, II, and III are labeled; the active site 2′-OH group is denoted with a sphere. On the left, the X-ray crystal structure of the hammerhead ribozyme is shown with the complementary DNA strands appended at A13e and A37e in arbitrary conformations. On the right, one of many possible models of the misfolded and therefore catalytically inactive hammerhead ribozyme is shown, which is formed when the complementary DNA strands come together to form a duplex. In this model, stems I and II of the hammerhead ribozyme clearly become separated upon formation of the DNA duplex.

We chose six adenosines in the enzyme strand to test DNA attachment using the 9FQ4 deoxyribozyme. All six adenosines have 2′-hydroxyl groups that are exposed to solvent; this suggests that attachment of a single DNA strand at these positions would not inherently disrupt the ribozyme structure. Similar to the overall observations with P4-P6, three of these sites were derivatized well with DNA (>60% yield); one site was modified in modest yield (~20%), and two sites were derivatized poorly (<10%).[10] The two best sites were A13e and A37e in the enzyme strand (see Figure 2A for locations), each of which was derivatized with DNA in >80% yield. We also targeted A12s in the substrate strand, which was modified with DNA in only 10% yield. However, we were able

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to improve this yield to ~30% by increasing the Mg^{2+} concentration from 40 to 120 mM, which provided a sufficient amount of RNA–DNA conjugate for subsequent experiments. By using 9FQ4, the A13e–A37e hammerhead ribozyme (i.e., with DNA strands attached at the two indicated positions) was prepared in both "cis" and "trans" forms, that is, with and without a tetraloop connecting the enzyme and substrate strands (Figure 2A, far right).[17] The DNA strands were each 15 nt in length and formed a 14 bp duplex (Figure 2B). Molecular modeling suggested that duplex formation by these attached DNA strands should induce misfolding of the ribozyme and therefore lead to a loss of catalytic activity (Figure 2C).

For both the "cis" and "trans" A13e–A37e ribozymes, when complementary DNA strands were attached the catalytic activity at 5 mM Mg^{2+} was substantially diminished relative to the activity of the unmodified, parent ribozyme (Figure 3). Subsequent addition of a free DNA oligonucleotide that was complementary to one of the constraint strands restored high activity. Addition of DNase I also substantially restored the catalytic activity.[10] As an important negative control, we examined a ribozyme to which two noncomplementary DNA strands (Figure 2B) were attached. As expected, these noninteracting DNA strands had only a modest effect on catalysis (Figure 3).

To explore the structural basis of the change in catalytic activity imposed by the duplex DNA constraint, nuclease probing experiments were performed for the A13e–A37e "trans" ribozyme. The use of a 5′-^{32}P-radiolabeled substrate strand allowed analysis of both stems I and III. Probing with RNase T2 (Figure 4), which cleaves after any nucleotide in a single-stranded region, indicated that both stems I and III remained entirely intact when either noncomplementary or complementary DNA strands were attached at A13e–A37e. Analogues probing with RNase T1, which cleaves only after single-stranded G nucleotides, led to a similar conclusion (data not shown). RNase T2 was also used to analyze stem II, which is formed intramolecularly within the enzyme strand. By using 5′-^{32}P-radiolabeled enzyme strand, the probing results confirmed that stem II is present in all cases.[10] Overall, the nuclease probing experiments demonstrated that all three of stems I, II, and III are intact within the A13e–A37e hammerhead ribozyme, even when the catalytic activity is substantially suppressed due to the attached DNA constraint. We conclude that as originally intended, the DNA constraint acts by disrupting the RNA tertiary structure while leaving the RNA secondary structure unchanged.

We also examined the catalytic activity of the hammerhead ribozyme with DNA strands attached at the alternative pair of sites, A12s and A37e. For the "cis" variant of the A12s–A37e hammerhead ribozyme, substantial reduction in catalytic activity due to the DNA constraint was observed as expected (Figure 5A). High activity was restored upon adding the complementary oligonucleotide, and only a modest effect on activity was observed when the DNA strands were noncomplementary. These results are very similar to those found for the A13e–A37e ribozyme; this indicates that the DNA constraint acts similarly when placed at the alternative set of attachment sites.

In contrast, for the "trans" variant of the A12s–A37e ribozyme, the catalytic activity was only modestly suppressed despite the attached complementary DNA strands (Figure 5B). This initially surprising result was readily explained upon closer consideration of the ham-

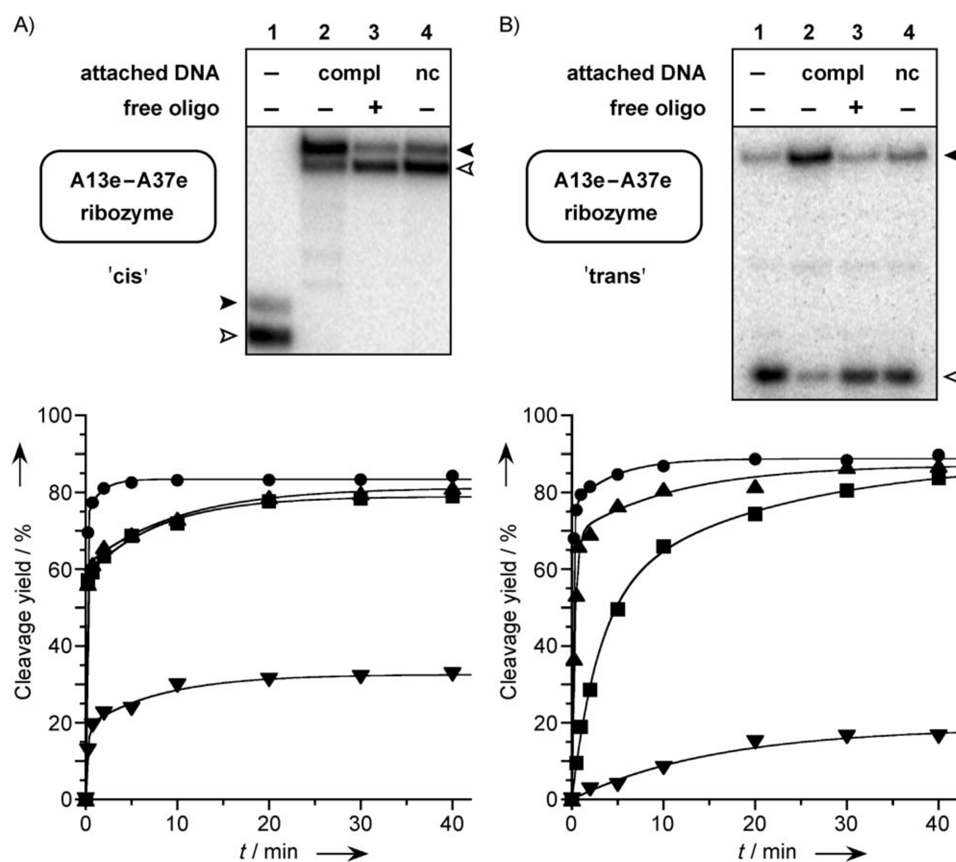

Figure 3. Allosteric control of hammerhead ribozyme catalysis by DNA strands attached at A13e and A37e. A) The "cis" ribozyme; B) the "trans" ribozyme. The substrate strand (top band; ▶) is cleaved after C6 to form the product (bottom band; ▷). The attached DNA strands were either absent (–), complementary (compl), or noncomplementary (nc). The free oligonucleotide that disrupts the DNA constraint was either absent (–) or present (+). The gel images show 20 min time points (12% PAGE for "cis"; 20% PAGE for "trans"). In the kinetic plots, data points are ● (lane 1), ▼ (lane 2), ▲ (lane 3), or ■ (lane 4). Assay conditions: Tris, pH 7.4 (50 mM), $MgCl_2$ (5 mM), 25 °C. For optimal curve fits the data required a biphasic kinetic equation, which is often observed with hammerhead ribozymes.[16,18]

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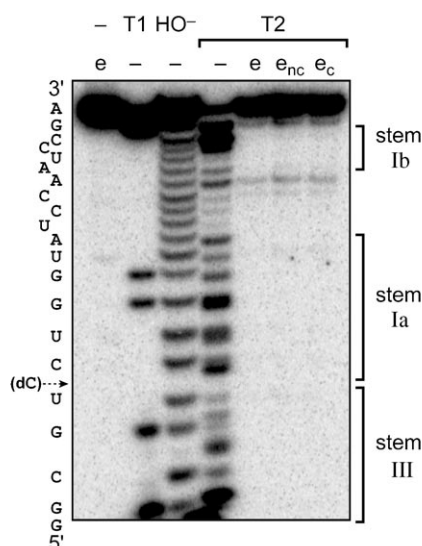

Figure 4. Nuclease probing experiments to characterize secondary structure elements within the "trans" A13e–A37e ribozyme. Nucleotide C6 of the substrate strand was 2′-deoxy (dC) to prevent ribozyme-catalyzed cleavage of the substrate strand, which was 5′-^{32}P-radiolabeled. The partial alkaline hydrolysis (HO^-) ladder was assigned by comparison with the RNase T1 cleavage ladder in the absence of enzyme strand (T1, −). The enzyme strand was either absent (−), unmodified (e), modified with noncomplementary DNA strands (e_{nc}), or modified with complementary DNA strands (e_c). Strong protection of the stem I and III nucleotides from cleavage by RNase T2 (which cleaves after any single-stranded nucleotide) is evident when the enzyme strand is present, regardless of DNA attachment. In contrast, the unpaired nucleotides that interrupt stem I (between stems Ia and Ib) are cleaved by RNase T2.

merhead structure and the choice of DNA attachment points. Nucleotide A12s is located at the end of stem Ia, which is remote from the active site (Figure 2A). As supported by computer modeling,[10] modest fraying of stem Ia would allow tertiary structure formation and therefore catalysis despite the presence of the attached DNA duplex (stem Ia can be reduced to only five base pairs and still permit catalytic activity[13,18]). Such fraying should be accompanied by disruption of short stem Ib, which is dispensable for catalytic activity although it does enhance the reaction rate by formation of an additional tertiary contact with the loop at the end of stem II.[19] However, the tetraloop capping stem Ib that is present only within the "cis" variant (Figure 2A) should disallow opening of stem Ib, and thus prevent fraying of stem Ia. The experimental data of Figure 5 are consistent with fraying of stem Ia (and thus significant maintenance of catalytic activity) in only the "trans" variant and not the "cis" variant of the A12s–A37e ribozyme. The modest decrease of catalytic activity in the "trans" variant can be attributed to loss of the tertiary contact between stem Ib and the loop at the end of stem II, although stem Ia remains intact and therefore catalysis by the core of the hammerhead ribozyme is still possible. One implication of these results is that care must be taken when attaching a DNA constraint near a dispensable structural element, such as the site of A12s near stem Ib, because such placement can result in a failure to control catalysis as desired. This lesson will be important as the DNA constraint strategy is expanded to larger macromolecular targets.

In summary, we have shown that strategically attached DNA strands can allosterically control hammerhead ribozyme cataly-

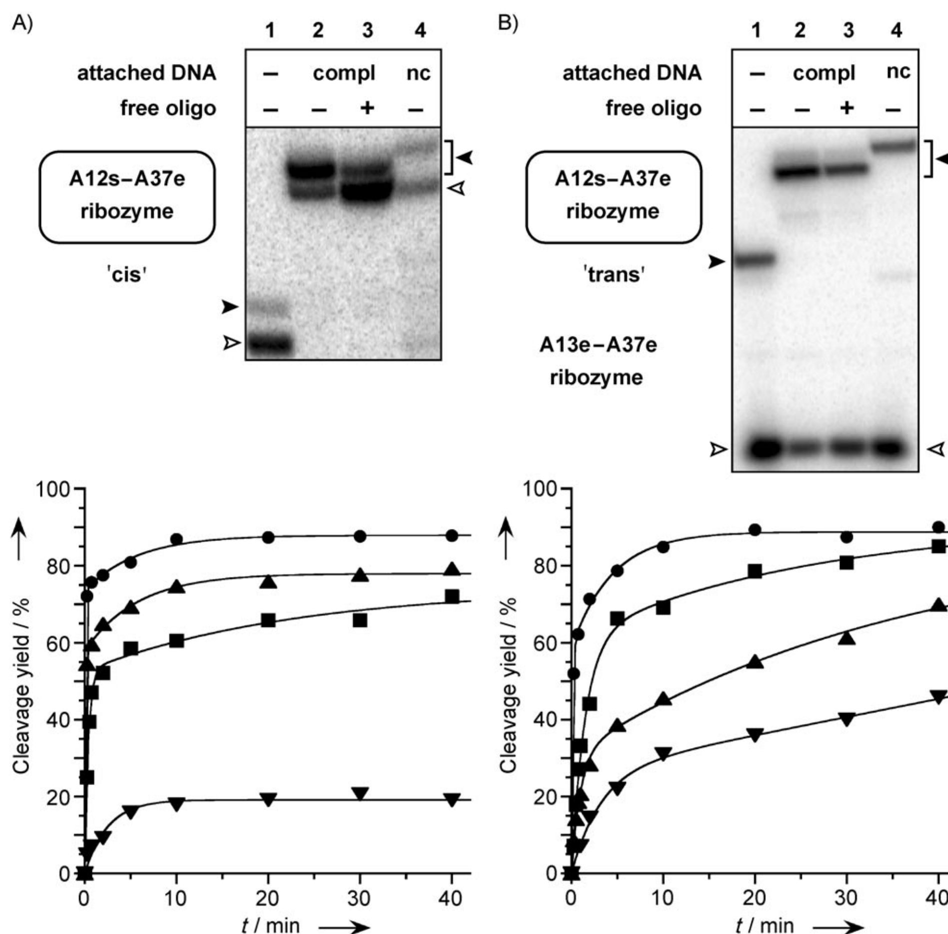

Figure 5. Allosteric control of hammerhead ribozyme catalysis by DNA strands attached at A12s and A37e. A) The "cis" ribozyme, for which suppression of catalysis by the DNA constraint is substantial; B) the "trans" ribozyme, for which the DNA constraint effect is modest. Assay conditions, data point symbols, and analysis are as in Figure 3. As expected, the uncleaved RNA (▶) migrated more slowly when DNA was attached at A12s. In contrast, all products (▷) migrated at the same rate because cleavage at C6 removed the portion of the RNA (including A12s) to which the DNA was attached.

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sis by forming double-stranded constraints that are incompatible with catalytic activity. As shown by catalytic activity assays in combination with nuclease probing, the DNA constraints act by disrupting the tertiary structure of the hammerhead ribozyme while leaving its secondary structure intact. We anticipate that the DNA constraint strategy should be applicable to other RNAs, including much larger ribozymes for which we currently lack a complete understanding of RNA structure–function relationships.[2] Ongoing efforts seek to improve the synthetic approach by identifying even more sequence-tolerant deoxyribozymes than 9FQ4. We note that the RNA–DNA conjugates synthesized by such deoxyribozymes have the connectivity of "multicopy single-stranded DNA" (msDNA), which is present in hundreds of copies within bacterial cells yet the function of which is unknown.[20] Therefore, deoxyribozymes that create RNA–DNA conjugates might have utility in elucidating the biochemical role of msDNA. Our overall strategy for using DNA to control macromolecular catalysis is related to that reported by Zocchi and co-workers in which DNA constraints were applied to several proteins,[21] in addition to our own previous work with controlling the structure of the P4-P6 RNA.[3,4,22] We anticipate that with the continued improvement of synthetic techniques, DNA constraints for controlling both structure and catalysis will be applicable to any large and foldable macromolecule.

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Keywords: deoxyribozymes · DNA constraints · macromolecular folding · ribozymes · RNA structures

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