Efficient control of group I intron ribozyme catalysis by DNA constraints†

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Double-stranded DNA constraints enable efficient control of catalysis by a large multi-domain group I intron ribozyme.

Rational attachment of double-stranded DNA as a structural constraint allows control over macromolecular conformation and function.[1] Others have modulated the structure or enzymatic activity of a protein by changing the hybridization state of a DNA strand that is attached at both ends to the protein.[2] We used DNA constraints to control the tertiary structure of the 160-nucleotide P4–P6 RNA domain of the *Tetrahymena* group I intron RNA.[3] This control was achieved by attaching the 5′-termini of two complementary DNA strands to different sites on the RNA. When the DNA strands form a duplex that is incompatible with the natively folded RNA structure, the RNA misfolds (Fig. 1). We subsequently reported that DNA constraints can modulate catalysis by the 63 nt hammerhead ribozyme.[4] As part of the latter efforts, we improved upon our previous chemically based strategy for DNA attachment[5] by using *in vitro* selection[6] to identify the 9FQ4 deoxyribozyme,[4,7] which attaches 5′-adenylated DNA to a specific internal 2′-OH group of an RNA target. In the present work, we address the question of whether DNA constraints attached by 9FQ4 can be applied successfully to control catalysis by a large multi-domain ribozyme, which might behave differently than a smaller single-domain ribozyme such as the hammerhead.

To implement the DNA constraint strategy of Fig. 1, a DNA strand must be attached to the RNA at each of two specific positions.[1,3] Here we focused on RNA adenosine nucleotides as attachment sites because of the functional requirements of the 9FQ4 deoxyribozyme.[4] For the large

RNA target, we chose the 388 nt L-21 ScaI ribozyme form of the *Tetrahymena* group I intron,[8–10] of which P4–P6 is one domain. Others have obtained X-ray crystal structures of group I introns,[11,12] including a 3.8 Å structure of a truncated form of the *Tetrahymena* ribozyme (containing the P4–P6 and P3–P9 domains)[13] that enabled us to choose appropriate sites for attaching DNA constraints. Candidate DNA attachment sites on the group I ribozyme were initially identified by inspection of the 3.8 Å X-ray crystal structure. We examined nine adenosine nucleotides scattered throughout the P4–P6 and P3–P9 domains, specifically choosing adenosines for which the 2′-OH group is not occluded within the RNA structure (Fig. 2). We then assayed these sites experimentally for the viability of DNA attachment by 9FQ4.

When the 5′-^{32}P-radiolabeled 388 nt ribozyme strand was used as the RNA substrate with a 16 nt 5′-adenylated DNA substrate and the appropriate 9FQ4 variant,[4] poor ligation yield was observed ($<10\%$ in eight cases and $\sim20\%$ in the ninth case). Complementary disruptor oligonucleotides were designed to prevent formation of key RNA secondary structure elements near the attachment-site adenosine within the ribozyme, thereby freeing the RNA nucleotides near the adenosine for binding to 9FQ4. At all nine sites indicated in Fig. 2 except for A324, inclusion of the appropriate disruptor oligonucleotide substantially improved the 9FQ4 ligation yield to 70–95%.

Because PAGE purification of the group I intron suffers from a low recovery efficiency (typically $\sim10\%$ in our hands),

Fig. 1 DNA constraint strategy to control RNA conformation.[1,3,4]

folded RNA
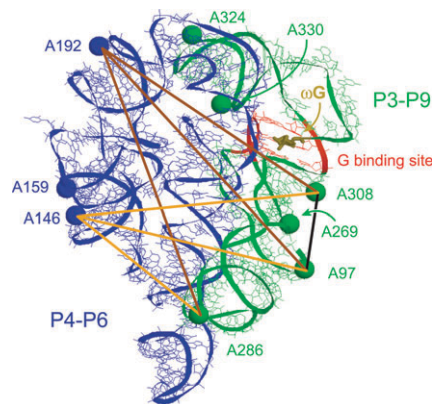*structurally incompatible*

misfolded RNA
structurally compatible

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Fig. 2 Structure of the *Tetrahymena* group I intron ribozyme with DNA attachment sites. Fig. S1 in the ESI† shows the secondary structure of the 388 nt L-21 ScaI ribozyme form of the intron RNA.[9,10] Depicted here is the 3.8 Å X-ray crystal structure obtained with a truncated version of the ribozyme that lacks the P1 and P2 regions.[11,13] The solid lines connecting 2′-OH groups (spheres) denote the seven pairwise combinations of attachment sites used to introduce DNA constraints.

we could not realistically perform two successive DNA attachments with PAGE purification after each reaction. We therefore needed to establish a procedure for simultaneously attaching two DNA strands to the RNA, which is complicated by the fact that the two strands being attached are inherently complementary to each other. To address this practical problem, each of the two DNA substrates was separately annealed to its corresponding 9FQ4 deoxyribozyme strand, while the ribozyme was annealed to the disruptor oligonucleotide in a third sample. This effectively sequesters the two DNA substrates from each other and also disrupts secondary structure in the RNA, leaving the appropriate portions of the two deoxyribozymes and the RNA free to interact as intended. The three separately annealed samples were immediately mixed, and Mg^{2+} was added to initiate the 9FQ4-catalyzed ligation reaction. This procedure led to $\sim 10\%$ isolated yield of doubly DNA-derivatized ribozyme after PAGE purification, which is about as high as possible for this large RNA using crush-and-soak elution from a polyacrylamide gel.

With the preparative route thus developed, specific pairs of DNA attachment sites were chosen for detailed study of the effects of DNA constraints on group I intron catalysis. Inspection of the intron's X-ray crystal structure suggests that a 15 bp DNA duplex (~ 51 Å in length) is too short to span the distance between A146 within P4–P6 and any of A97, A286, or A308 within P3–P9, because the 2′-OH groups of these three pairs of positions are 64, 70, and 60 Å apart (Fig. 2, orange lines). In each case, a 15 bp duplex formed between the attached DNA strands is predicted to clash structurally with the ribozyme. This incompatibility means that the DNA duplex would need to distort or separate for the RNA to fold properly. On the other hand, if the DNA duplex remains intact as designed, then the RNA should misfold and lose catalytic activity because the two remote 2′-positions are being pulled together by the DNA constraint. Similar predictions are made for a 15 bp DNA duplex attached at A192 and any of A97, A286, or A308, where the 2′-OH groups are 72, 74, and 64 Å apart (Fig. 2, brown lines). The seventh attachment-site combination of A97 and A308 has a short distance between 2′-OH groups of only 23 Å (Fig. 2, black line). In this case, a 15 bp DNA constraint should be structurally incompatible with the folded RNA state because the two remote 2′-positions are being pushed apart by the DNA constraint.

Single-turnover cleavage of the 11-mer RNA substrate 5′-^{32}P-CCCUCU^AAAAA-3′ was used to assess the catalytic abilities of the DNA-constrained ribozymes.[10] We initially evaluated the effects of DNA constraints using the three ribozyme variants with DNA attached at A146 and one of A97, A286, or A308 (Fig. 2, orange lines). In each case, the doubly DNA-derivatized ribozyme was annealed by heating to 95 °C and cooling to room temperature (23 °C). Then, 10 mM Mg^{2+} and 1 mM GTP (final concentrations) were added, the sample was incubated for 30 min at 50 °C, and finally the RNA substrate strand was added at 30 °C, all according to standard protocols.[14] However, contrary to the experimental design, little if any decrease in catalysis was observed relative to the unmodified (wild-type) ribozyme that lacks any attached DNA strands (data not shown).

One likely explanation for this initial lack of effect on intron catalysis is that under the experimental conditions,

the ribozyme folds rapidly to its catalytically active structure, circumventing successful formation of the DNA constraint duplex (Fig. 3, steps A1 and A2). If so, then inclusion of an 'inhibitor' DNA oligonucleotide directed against a key element of the RNA tertiary structure should prevent formation of the catalytically active ribozyme conformation, allowing the DNA constraint duplex to form (Fig. 3, step B). Subsequent addition of a 'removal' oligonucleotide that is fully complementary to the inhibitor oligonucleotide should then lead to formation of the DNA-constrained misfolded RNA state, which is catalytically inactive even after addition of Mg^{2+} and GTP (Fig. 3, steps C1 and C2).

We designed eight candidate inhibitor oligonucleotides complementary to different 30–40 nt regions of the ribozyme that are known to be important to the RNA structure. Each inhibitor oligonucleotide includes a 20 nt 3′-terminal extension to initiate binding of the removal oligonucleotide. Two of the eight tested inhibitor oligonucleotides substantially suppressed catalytic activity of the non-DNA-derivatized wild-type ribozyme, suggesting that these inhibitors would be useful in the approach of Fig. 3. These two successful inhibitors were directed against ribozyme nucleotides C102–G141 and A161–C197 (only 5% and 38% substrate cleavage in 30 min, respectively). In both cases, subsequent addition of the appropriate removal oligonucleotide restored full ribozyme catalytic activity (>95% cleavage in 30 min).

The same three sets of attachment sites as used in the initial experiment (A146 with A97, A286, or A308) were re-examined, now employing the inhibitor–removal oligonucleotide strategy of Fig. 3. In all three cases, the catalytic activity of the DNA-constrained ribozyme was substantially suppressed (Fig. 4), as designed. For the A146–A97 and A146–A308 ribozymes, the suppression of catalysis was nearly complete. In contrast, for the A146–A286 ribozyme a modest cleavage yield of $\sim 40\%$ was retained, suggesting that a fraction of the ribozyme folds to one or more active structures despite the attached DNA strands. These effects were independent of which inhibitor oligonucleotide was used. Furthermore, the wild-type activity of each ribozyme was restored after release of the DNA constraint by addition of a free oligonucleotide complementary to one of the strands (see Fig. 3, step D). In contrast to the results when two complementary DNA strands were attached to the ribozyme, no effect on ribozyme catalysis was observed when two non-complementary DNA strands were attached. Therefore, merely attaching DNA strands does not interfere with structure or catalysis.

The experiments in Fig. 4 were performed at pH 7.2, where k_{obs} is rather high (on the order of 1 min^{-1} in our hands).
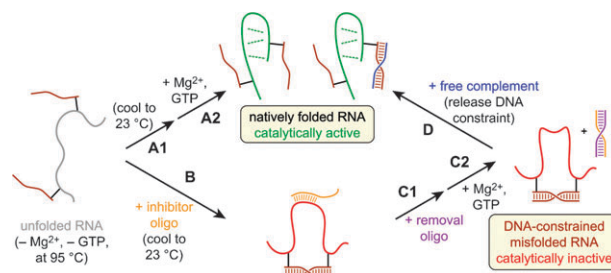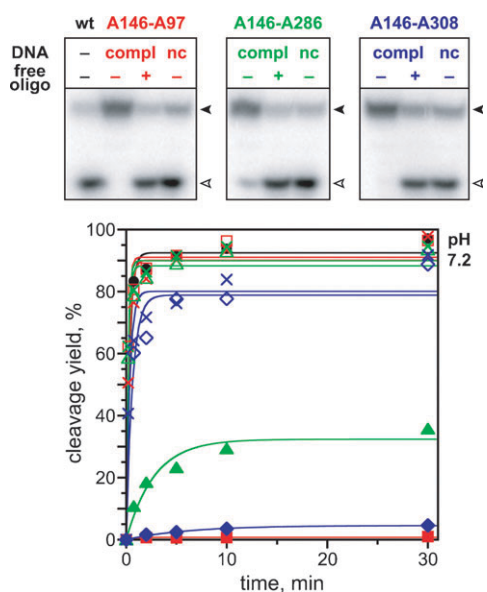

Fig. 3 Pathways for group I intron folding. See text for explanation.

Fig. 4 Suppression of group I intron ribozyme catalysis by DNA constraints at A146–A97, A146–A286, or A146–A308 (single-turnover substrate cleavage assays, 100 mM HEPES, pH 7.2, 10 mM Mg^{2+}, 100 mM NaCl, and 1 mM GTP at 30 °C). The 20% PAGE images show 10 min timepoints for cleavage of the $5'$-^{32}P-radiolabeled 11-mer RNA substrate by a DNA-constrained ribozyme (Fig. 2, orange lines). The RNA substrate (filled arrowhead) is cleaved after U6 to form the product (open arrowhead). The attached DNA strands were either absent (−), complementary (compl), or non-complementary (nc). The free oligonucleotide complementary to one of the two constraint strands was added for the lanes marked (+). The single-exponential kinetic fits are intended solely to guide the eye; the rates are too high under these incubation conditions to obtain precise k_{obs} values. Filled symbols indicate complementary DNA strands attached to the ribozyme; open symbols indicate complementary DNA strands after adding free oligonucleotide to release the constraint; crosses indicated non-complementary DNA strands.

To ensure that a quantitative effect of each DNA constraint on catalysis was not masked, similar experiments were performed at pH 6.0, where k_{obs} is lower and the chemical step of catalysis is rate-limiting.[15] The results (Fig. S2 in the ESI†) confirmed that catalysis can be restored with no decrease in k_{obs} relative to the unmodified ribozyme.

The other four combinations of DNA attachment sites from Fig. 2 were also examined, and nearly complete suppression of catalysis by each DNA constraint was observed (Fig. S3 in the ESI†). In all four cases, addition of the free complementary oligonucleotide to release the DNA constraint restored wild-type catalysis, and attachment of two non-complementary DNA strands did not affect catalysis. To assess the lifetimes of the DNA-constrained misfolded RNA states, the folding time for step C2 of Fig. 3 was increased from 30 min to 10 h for each of the A146–A97, A146–A308, A192–A97, A192–A308, and A97–A308 ribozymes. In all instances, the suppression of catalysis was unchanged. Therefore, the DNA-constrained misfolded RNA states have lifetimes ≫10 h.

The control by DNA constraints of group I intron catalysis appears to be more robust than the control we observed previously for the hammerhead ribozyme.[4] The residual level of catalytic activity was <10% for the DNA-constrained intron RNA (except for the A146–A286 variant), but 10–30% for the constrained hammerhead. This quantitative difference may relate to the multi-domain nature of the large group I intron, as compared with the single domain formed by the six-fold smaller hammerhead ribozyme.

In summary, we have shown that double-stranded DNA constraints (Fig. 1) can be used successfully to control catalysis of the 388 nt multi-domain *Tetrahymena* group I intron ribozyme. By strategically choosing seven different pairs of attachment sites on the ribozyme (Fig. 2), and by developing an appropriate strategy involving inhibitor and removal strands to allow the DNA constraints to form (Fig. 3), we showed that ribozyme catalysis can be almost completely suppressed (Fig. 4 and Fig. S3 in the ESI†). The importance of misfolded states in RNA folding has long been appreciated, especially in the context of off-pathway kinetic traps that can substantially slow the formation of natively folded RNA.[16] Early studies revealed that kinetic traps dominate the folding pathway of the *Tetrahymena* group I intron,[17] and subsequent work investigated the nature of several misfolded states of the ribozyme.[14,18] *Via* creation of indefinitely stable misfolded RNA states, we hope that DNA constraints will be a useful tool for investigating folding pathways of the group I intron ribozyme and other large RNAs.

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