Group II intron ribozymes that cleave DNA and RNA linkages with similar efficiency, and lack contacts with substrate 2'-hydroxyl groups

Edmund A Griffin Jr[1†], Zhifeng Qin[2†], William J Michels Jr[3] and Anna Marie Pyle*

Department of Biochemistry and Molecular Biophysics, The Columbia College[1], Department of Applied Physics[2] and the Integrated Program in Molecular, Cellular and Biophysical Studies[3], Columbia University, New York, NY 10032, USA.

Background: Group II introns are self-splicing RNAs that have mechanistic similarity to the spliceosome complex involved in messenger RNA splicing in eukaryotes. These autocatalytic molecules can be reconfigured into highly specific, multiple-turnover ribozymes that cleave oligonucleotides in *trans*. We set out to use a simplified system of this kind to study the mechanism of cleavage.

Results: Unlike other catalytic RNA molecules, the group II ribozymes cleave DNA linkages almost as readily as RNA linkages. One ribozyme variant cleaves DNA linkages with an efficiency comparable to that of restriction endonuclease *Eco*RI. Single deoxynucleotide substitutions in the substrate showed that the ribozymes bind substrate without engaging 2'-hydroxyl groups.

Conclusions: The ribose 2'-hydroxyl group at the cleavage site has little role in transition-state stabilization by group II ribozymes. Substrate 2'-hydroxyl groups are not involved in substrate binding, suggesting that only base-pairing is required for substrate recognition.

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Introduction:

The ribose 2'-hydroxyl group is widely regarded as important for molecular recognition and chemical catalysis by RNA [1–5]. It is a multifunctional recognition determinant and can act as either a hydrogen bond donor or acceptor [2–4,6–8]. For ribozymes such as the hammerhead, the 2'-hydroxyl group at the substrate cleavage site is the nucleophile in RNA cleavage reaction [9]. For other RNA-cleaving ribozymes, however, including RNase P, group I introns and group II introns, this is not the case (Fig. 1a) [10]. Nonetheless, the 2'-hydroxyl at the cleavage site is important in transition-state stabilization for both group I intron ribozymes and RNase P, [4,11,12], and neighboring 2'-hydroxyl groups also contribute to substrate recognition and binding [2,3,5,13,14]. The role of 2'-hydroxyl groups on substrates for group II intron ribozymes has not previously been investigated, although it has recently been shown that 2'-hydroxyls within a core region (domain 5) of group II introns are essential for binding and catalysis (D.L. Abramovitz and A.M.P., submitted).

Directed molecular evolution has been used to evolve a group I intron ribozyme that cleaves DNA linkages with relatively high efficiency [15,16]. But, to date, there have been no examples of naturally-occurring ribozymes (i.e., a ribozyme containing a wild-type active site) that cleave DNA with an efficiency comparable to their cleavage of RNA. Here we show that ribozymes derived from a group II intron can give efficient cleavage without engaging the 2'-hydroxyl groups on their target substrates; they cleave DNA linkages almost as well as RNA linkages.

Self-splicing group II introns have been found in the organellar genes of plants, lower eukaryotes and prokaryotes [17,18]. The excision of group II introns is essential for metabolism in many of these organisms. Group II introns can be arranged into six domains; domain 1 (D1) and domain 5 (D5) are essential for catalytic activity [19,20], whereas the other domains are dispensable [17]. Self-splicing can proceed via two pathways. In one pathway, initial 5'-splice site attack by the 2'-hydroxyl group from the bulged adenosine of domain 6 (D6), generates a 'lariat' intron that is released after the second step of splicing [21–23]. In the other, water acts as the nucleophile in the first step, resulting in ligated exons and release of linear intron [24–27]. A recent study has demonstrated that hydrolytic attack is a common pathway for *in vitro* self splicing of group II introns [27].

In spite of their biological importance, there have been relatively few investigations of the structural features and mechanistic enzymology of group II introns [28–32]. Recently, we established a minimal group II ribozyme system (derived from the group II intron ai5g) in which D1, acting as an enzyme with D5 as cofactor, cleaves short RNA oligonucleotides analogous in sequence to the 5'-splice junction ([20]; Fig. 1b). This system allowed a detailed kinetic study of the reaction. The nucleophile in this reaction is water or hydroxide, as it is for hydrolytic

*Corresponding author. †These authors contributed equally to this work.

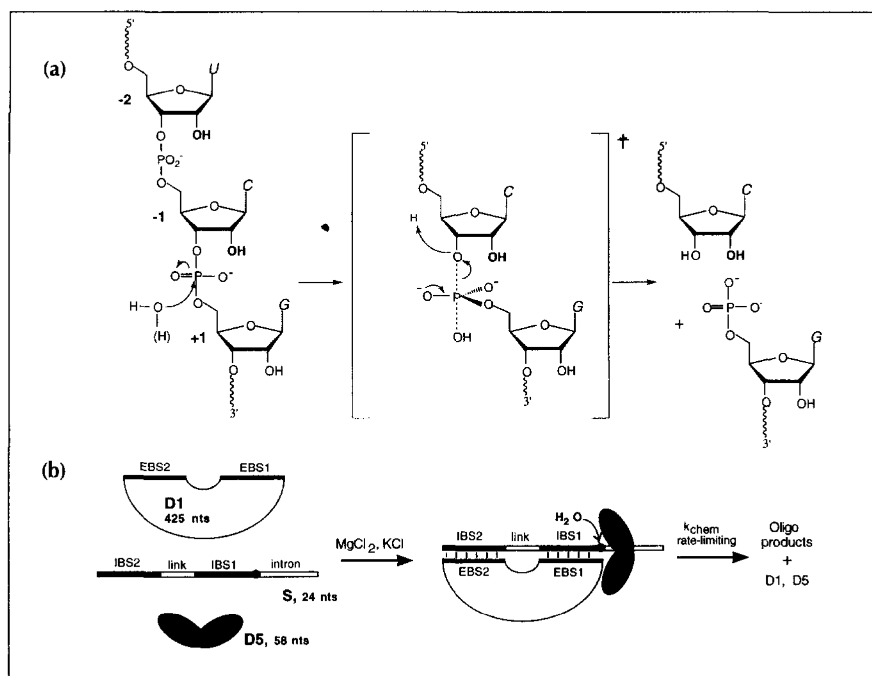

Fig. 1. Catalytic mechanism of RNA cleavage by the minimal group II ribozyme and schematic representation of substrate recognition and cleavage. **(a)** The catalytic mechanism of RNA cleavage is shown; water (or hydroxide) is the nucleophile. **(b)** The minimal group II ribozyme comprises domain 1 (D1, nts=nucleotides) of the six domains present in the full-length intron and contains two exon-binding sites (EBS1 and 2). The EBSs hybridize to intron binding sites (IBS1 and 2) on the oligonucleotide substrate, separated by a three nucleotide link. In the presence of a second RNA molecule containing domain 5 (D5) of the group II intron, the substrate is cleaved at the site indicated by the arrow.

splicing reactions (Fig. 1a). D1 binds to its substrate strongly with a K_d of 6.3 nM, due to base pairing between the intron-binding sites (IBS1 and 2) and exon-binding sites (EBS1 and 2). The cleavage reaction is rate limited by chemistry ($k_{cat} = k_{chem} = \sim 0.03\ min^{-1}$ at saturation). Using this kinetic framework, it is now possible to incorporate single-atom changes into the substrate to probe the reaction mechanism.

Here, we have examined the role of 2'-hydroxyl groups on the oligonucleotide substrate for a group II ribozyme by incorporating deoxynucleotides into specific positions along the strand. We show that, for two different ribozyme constructs that have different rates for RNA cleavage, 2'-hydroxyl groups along the substrate backbone are minimally involved in the tertiary contacts that stabilize binding to the ribozyme or stimulate catalysis during the chemical step of the reaction. In one case, deoxynucleotide linkages are cleaved with the highest efficiency (k_{cat}/K_M) observed for any ribozyme to date, having a k_{cat} comparable to that of certain protein restriction enzymes. Although incorporation of deoxynucleotides at the cleavage site and at sites immediately adjacent to it does give some reduction in cleavage efficiency, these effects are small compared to the ~10^3-fold effects on k_{chem} observed for other ribozymes in the same mechanistic class [3,4,11]. The k_{chem} for RNA cleavage of one of the group II ribozymes reported in this study is comparable to that of some group I ribozymes [33], but is noticeably lower than that for the best RNA-cleaving ribozymes known, those derived from bacterial RNase P and the *Tetrahymena* group I intron ribozyme [4,11].

Results

Deoxynucleotide rate effects — block substitution

We first synthesized chimeric substrates in which the cleavage site (–1C) was always a ribose and blocks of surrounding residues were substituted with deoxynucleotides. The blocks of deoxynucleotides were clustered into four functional regions: IBS2 (–16 to –11), link (–10 to –8), IBS1 (–7 to –2) and intron (+1 to +7) (Fig. 1b). IBS2 and IBS1 pair with complementary sequences on the D1 ribozyme [18,34]. The chimeric substrates were named according to the block substituted (e.g., in chimera d(IBS2) only the IBS2 sequence is composed of deoxynucleotides) and tested in single-turnover cleavage reactions under saturating ribozyme conditions. Cleavage rates were compared to those of an all-ribose substrate (rS) (Table 1).

Except for d(IBS1), the block-deoxynucleotide substrates showed small rate decreases relative to rS, indicating that the 2'-hydroxyl groups within IBS2, the linker and intronic nucleotide sections of the substrate are not essential for catalytic activity. Within experimental error, the d(link) substrate was as active as rS, so it was excluded

Table 1. Effects of block deoxyribose substitutions on ribozyme rate.

Modified substrate	1[a] Effect of dN (rel rate)$^{-1}$	2 250 nM D1[b] (rel rate)$^{-1}$	3 6 μM D5[b] (rel rate)$^{-1}$
d(IBS2)	3.2 ± 0.51	1.4 ± 0.22	2.9 ± 0.46
d(IBS1)	inactive	inactive	inactive
d(link)	1.4 ± 0.22	ND	ND
d(intron)	3.3 ± 0.53	3.3 ± 0.53	3.1 ± 0.50

[a]Conditions used: [D1] = 100 nM and [D5] = 3 μM, the lower limit for saturation of the unmodified rS substrate. See Table 2 for k_{obs} of rS. Relative rates are k_{obs} for the chimeric substrates divided by that of rS. Variance calculated to 95 % confidence, from multiple trials on a single derivative. ND, not determined.
[b]Rate relative to rS cleavage under equivalent conditions.

from further investigation. The rate of d(IBS2) cleavage was approximately three-fold slower than that for rS. This effect was due to decreases in hybrid stability [14,35,36] since d(IBS2) cleavage rates were restored at higher concentrations of enzyme components (Table 1, column 2). The three-fold difference in cleavage rate between rS and d(intron) substrates did not appear to be due to decreases in binding affinity, however, as it was unaffected by higher concentrations of D1 and D5 (Table 1, columns 2 and 3).

Under all experimental conditions tested, d(IBS1) was inactive. There are at least two explanations for its lack of activity: either important 2'-hydroxyl groups lie within the IBS1 region, or a DNA/RNA hybrid duplex adjacent to the cleavage site is destabilizing for other reasons. DNA/RNA duplexes are thermodynamically destabilized [36], their microstructural features can differ slightly from RNA/RNA duplexes [37] and they can deviate significantly from A-form geometry and pitch, an extreme case being the recent crystal structure of a B-form DNA/RNA helix [38]. We set out to distinguish between these models and to determine which, if any, of the 2'-hydroxyl groups of IBS1 were important in ground-state or transition-state stabilization.

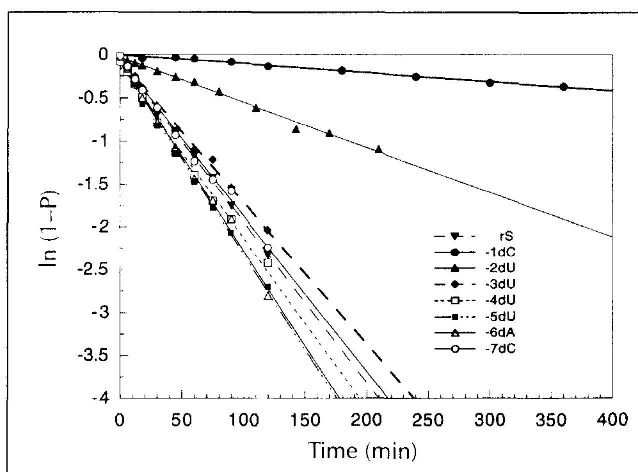

Fig. 2. Substitution with a deoxynucleotide at position –1 or –2 reduces the rate of the cleavage reaction. The pseudo-first order rate constants for the cleavage of chimeric substrates at ribozyme saturation were determined. Rates were measured simultaneously, under standard reaction conditions (see Materials and methods). Rates of reaction (k_{obs}) were determined by correcting each data set for the amount of total inactive substrate and plotting the data as shown, where P = fraction product at each timepoint. By solving the equation for each line, the half-time of reaction and subsequently the k_{obs} were determined as described previously [20]. For the rapidly reacting substrates shown, the rates were 0.021 min^{-1} for rS, 0.018 min^{-1} for –3dU, 0.026 min^{-1} for –4dU and –5dU, 0.025 min^{-1} for –6dA and 0.020 min^{-1} for –7dC. The variance in these rates was ± 16 % determined using eight separate trials. For –1dC the reaction rate was 0.0013 min^{-1}, with a variance of ± 23 % determined using eight different trials. For –2dU, the rate was 0.0042 min^{-1}, with a variance of ± 29 % based on three trials. All errors were calculated using a 95 % confidence limit and trials were performed on different days. For experiments performed side-by-side as shown here, relative error is expected to be much lower than the variances conservatively stated above.

Deoxynucleotide rate effects — single deoxynucleotide substitutions within IBS1

To investigate the importance of 2'-hydroxyl groups within IBS1 and at the cleavage site, rS was substituted individually with single deoxynucleotides (named according to the position of substitution relative to the cleavage site, at position –1). The mono-substituted chimeras were tested using single turnover reactions at saturating ribozyme conditions (Fig 2). The slowest cleavage (0.0013 min^{-1}) is observed for substrate –1dC, which has a deoxyribose at the cleavage site. Thus, the 2'-hydroxyl group at the cleavage site is the most important of all the substrate 2'-hydroxyl groups, although the decrease in rate is modest (only 16-fold lower than the rate of rS cleavage).

Cleavage of the –2dU substrate proceeded with a k_{obs} of 0.0042 min^{-1}, five-fold slower than rS. Single deoxy substitutions at all other positions within IBS1 (–3 to –7) had only minor effects on rate (Fig. 2). The most important 2'-hydroxyl groups along the substrate are therefore the one at the cleavage site (at position –1) and the one adjacent to it (position –2) in a region that is normally considered the 5'-exon sequence. Under the single-turnover, saturating ribozyme conditions used in these experiments, the changes in rate probably resulted from effects on the chemical step of the reaction. To show this conclusively, we needed to establish the rate-limiting step for k_{obs} in each case. Before doing this, we checked whether cleavage was taking place at the proper site on the chimeric substrates.

Characterization of reaction products

One reason that cleavage of a deoxynucleotide linkage might appear to be efficient is that the ribozyme might 'skip' the modified linkage and choose an adjacent ribose phosphodiester moiety for attack [39]. We therefore characterized the cleavage products from the D1/D5 ribozyme reactions using nucleases and alkaline hydrolysis, followed by high-resolution sequencing [40]. P1 nuclease cleaves both DNA and RNA linkages, resulting in fragments with the same 3'-hydroxyl and 5'-phosphate termini observed upon reaction with the ribozyme [40]. The products of ribozyme cleavage of both the rS and the –1dC substrates were found to migrate at exactly the same position as the 17-nucleotide RNA generated by partial P1 nuclease digestion of each substrate (data not shown), indicating that cleavage of the –1dC substrate occurs at the expected sequence.

High-resolution mapping of reaction products can be complicated by many factors, and we therefore also examined the terminus of the cleavage product directly. Correct cleavage of the –1dC substrate will generate a product with a 3'-deoxy terminus; this can be distinguished from a 3'-ribose terminus by $NaIO_4$ oxidation, converting the ribose sugar ring to a dialdehyde, which can be labeled using a hydrazine reagent [41]. The labeled oligonucleotide migrates more slowly than the unlabeled one on denaturing gels. We performed oxidation and

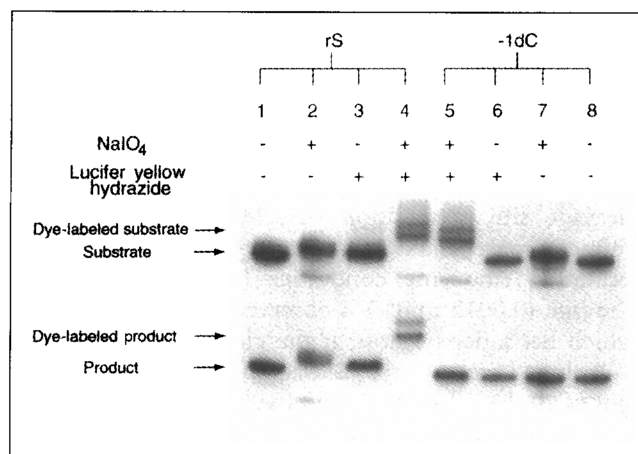

Fig. 3. The cleavage product of −1dC has a 3' deoxynucleotide terminus at the expected position. A 50/50 mixture of oligonucleotide substrate and product from a partial D1/D5 ribozyme reaction was subjected to oxidation (+ $NaIO_4$) and then 3'-end labeled with a hydrazine dye derivative (+ lucifer yellow). Lanes 1–4, rS substrate, lanes 5–8, −1dC substrate. After oxidation and covalent dye-labeling, both the precursor and product band of the rS substrate undergo a large mobility shift (lane 4). For −1dC, only the substrate band shifts to slower mobility (lane 5), indicating that the cleavage product of −1dC has a 3'-deoxy terminus, which cannot be oxidized with $NaIO_4$. Doublets in lanes 4 and 5 are most likely due to the products of lucifer yellow hydrazide attack on one or both of the aldehydes produced by oxidative attack of the ribose sugar [37]. Following oxidation with $NaIO_4$, there is a small mobility shift for species with a 3'-ribose because the sugar is oxidized to a larger dialdehyde moiety (compare lanes 1 and 2). Additional bands in the +$NaIO_4$ lanes are attributed to periodate attack on residues other than the ribose sugar [64].

labeling on a 50:50 mixture of substrate and product for both rS and −1dC substrates (Fig 3). The substrate served as an internal control, since it always has a 3'-ribose terminus and should always show a mobility shift. For the rS substrate, both substrate and product showed a shift (lanes 3 and 4), indicating that both have 3'-ribose termini. For the −1dC substrate, however, only the substrate showed a shift (lanes 6 and 5). Thus, the cleavage product of −1dC has a 3'-deoxynucleotide terminus, indicating that the −1dC substrate is cleaved at the correct site.

Mechanistic analysis of deoxynucleotide rate effects

The kinetic effects of deoxynucleotide substitution could result from a number of factors, including effects on ribozyme conformation, binding or the chemical step of catalysis. Furthermore, there have been several reports that deoxynucleotide substitution can change the rate-limiting step of a reaction [4,42]. We therefore investigated whether k_{obs} for cleavage of rS and −1dC under saturating D1/D5 conditions was governed by the same rate-limiting step. Cleavage of rS was previously established to be limited by chemistry under these conditions [30]; k_{chem} is expected to vary logarithmically with pH.

Values of k_{obs} for the rS, −1dC and −2dU substrates were obtained at several pH values using the same buffer (data not shown). In the pH range tested (pH 6.5 to pH 7.5), both rS and chimeric substrates have a linear pH/rate profile, with a slope close to 1 (0.87 for rS, 0.94 for −1dC, and 0.85 for −2dU). Such a profile is regarded as evidence that chemistry is rate-limiting [3,30,43,44] and that general base catalysis is occurring [45]. The fact that all three substrates give this type of behavior suggests that k_{chem} is limiting in each case and that their cleavage rates can be directly compared.

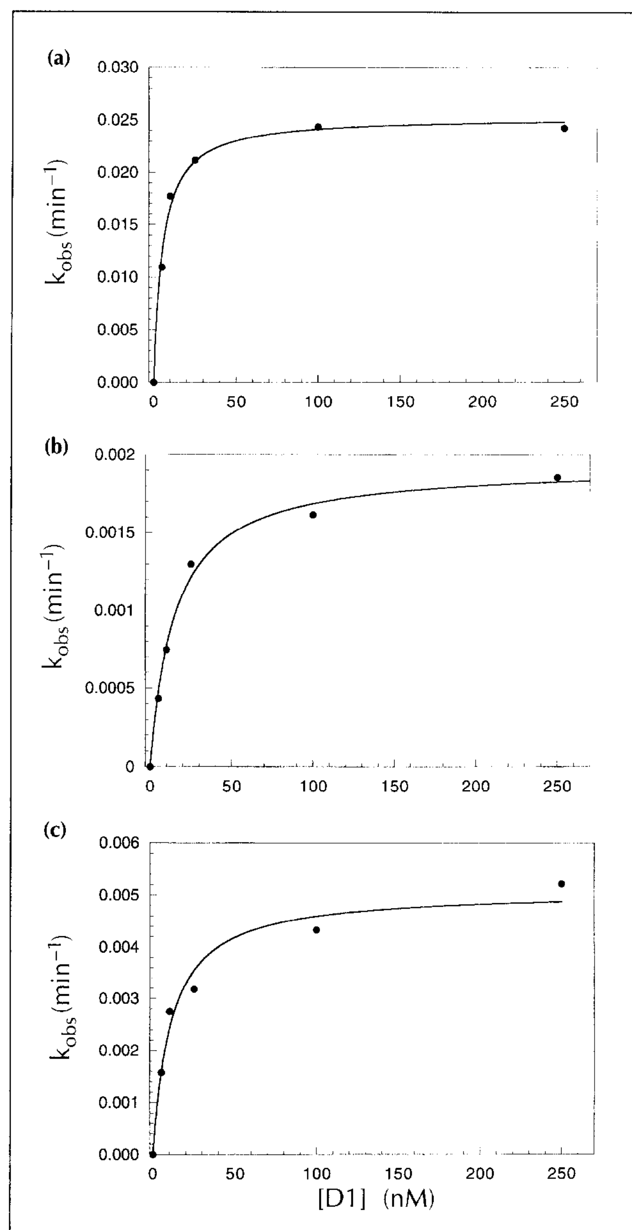

Fig. 4. Determination of kinetic parameters for rS, −1dC and −2dU substrates. By plotting k_{obs} as a function of D1 concentration at saturating D5 concentration, one can fit the data to a binding curve and obtain kinetic parameters for a reaction that follows Michaelis–Menten kinetics. Data were fit as previously described [20,30]. **(a)** For the rS substrate, K_M = 4.9 nM and k_{cat} = 0.025 min^{-1}. **(b)** For −1dC, K_M = 14 nM and k_{cat} = 0.0019 min^{-1}. **(c)** For −2dU, K_M = 10 nM and k_{cat} = 0.0051 min^{-1}. All k_{cat} values determined from these fits are in good agreement with the saturating k_{obs} values reported in Figure 2. Errors and k_{cat}/K_M values determined from this experiment are provided in Table 2.

Table 2. Kinetic parameters for D1/D5 ribozyme cleavage of the -1dC and -2dU chimeric oligonucleotide substrates.

Substrate[a]	k_{cat}[b] (10^{-2} min^{-1})	K_M[b] (nM)	k_{cat}/K_M[c] (10^6 M^{-1} min^{-1})
rS (all-RNA)	2.5 ± 0.070	4.9 ± 0.66	5.1 ± 0.83
-1dC	0.19 ± 0.0060	14 ± 1.7	0.14 ± 0.021
-2dU	0.51 ± 0.029	10 ± 2.4	0.51 ± 0.15

[a]Only substitutions that had significant effects on reaction rate are shown.
[b]Kinetic parameters and standard errors are derived from fits of the data shown in Figure 4. Kinetic data on the other chimeric substrates is presented in Figures 1,6 and Table 1.
[c]k_{cat}/K_M errors were calculated by propagation of the K_M and k_{cat} errors.

The pH/rate experiment indicates that 2'-hydroxyl groups at positions −1 and −2 directly affect the chemical rate of rS cleavage. To evaluate effects on binding, the rS, −1dC and −2dU substrates were cleaved at varying D1 concentrations. The k_{cat} and K_M values for cleavage of each substrate were calculated from the dependence of k_{obs} on D1 concentration, as described previously [20] (Fig. 4 and Table 2). The k_{cat} and K_M values for rS are in close agreement with published results (Fig. 4a) [20].

As well as the thirteen-fold decrease in the rate of the chemical step (k_{cat}), the −1dC substrate shows a three-fold decrease on the ground-state binding (K_M), resulting in a 36-fold effect on k_{cat}/K_M (Fig. 4b, Table 2). The −2dU substrate has a five-fold decrease in the chemical step and a two-fold decrease in the ground-state binding (Fig. 4c, Table 2), which may be attributed to duplex destabilization caused by deoxynucleotide substitution. The k_{cat}/K_M for the −2dU substrate is therefore 10-fold lower than that of rS. For both chimeric substrates, the small decreases in binding affinity (K_M) observed confirm that the ribozyme components were indeed saturating in the conditions used for our rate comparisons. Similarly, the decreases in k_{cat} for the chimeric substrates (thirteen-fold for −1dC, five-fold for −2dU) agree (within experimental error) with those seen in Figure 2 (sixteen-fold for −1dC, five-fold for −2dU).

Analogous rate effects for a fast group II ribozyme

One can modify the minimal D1 ribozyme construct by adding back intron domains 2 and 3 in *cis* (D123), to generate a ribozyme that cleaves RNA with high efficiency. Under standard reaction conditions in which D123 (100 nM) and D5 (3 μM) are in excess, this D123 ribozyme cleaves the rS substrate with an apparent k_{obs} of 0.88 min^{-1} (Fig. 5). This is 33-fold faster than the D1 ribozyme under the same conditions, and comparable to the rate of other ribozyme systems. D123 cleaves the −1dC substrate with a k_{obs} of 0.17 min^{-1} (Fig. 5), which is only five-fold slower than cleavage of the ribose linkage and ninety-fold faster than deoxynucleotide cleavage by D1. In both cases, cleavage occurs at the proper site (data not shown). Therefore, faster group II intron ribozymes cleave both DNA and RNA linkages with higher efficiency.

There is a smaller experimental window for detecting D1 reactivity, which is radically perturbed upon introduction of a hybrid EBS1/IBS1 duplex immediately adjacent to a ribose cleavage site (as in dIBS1, Table 1). Therefore, it was not possible to detect D1 ribozyme cleavage of an all-DNA oligonucleotide (dS). By contrast, the D123 ribozyme cleaves dS with a second-order rate constant of 2.6 ± 1.4 x 10^3 $M^{-1}min^{-1}$ under standard reaction conditions using a constant D5 concentration of 3 μM (data not shown). Together with effects from single deoxynucleotide substitutions, this result indicates that any barrier to cleavage of entirely-DNA molecules is likely to be due to global differences between RNA/RNA and DNA/RNA duplexes and not to a specific role for 2'-hydroxyl groups in the ribozyme mechanism.

The apparent five-fold preference of D123 for ribose over deoxynucleotide linkages compared to the sixteen-fold preference observed with D1 does not actually mean that D123 is less capable of differentiating between a ribose and a deoxyribose linkage; instead, the ratio between the two cleavage rates is smaller because they have different rate-limiting steps. The cleavage rate of −1dC by D123 (0.17 min^{-1}) is limited by the rate of chemistry, as demonstrated through pH/rate and other mechanistic analyses (data not shown). The rate of rS cleavage by D123

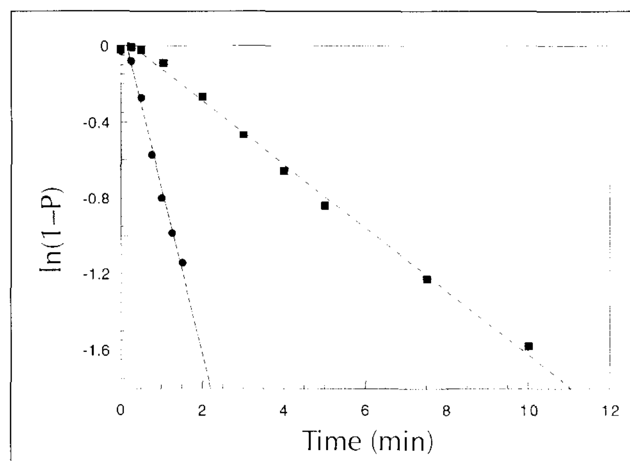

Fig. 5. D123 cleaves -1dC only five-fold slower than cleavage of rS. The saturating k_{obs} values for oligonucleotide cleavage by the fast D123 ribozyme were determined. Cleavage of rS (●) and -1dC (■) substrates by D123 (100 nM) and D5 (3 μM) yielded apparent rate constants (k_{obs}) of 0.88 ± 0.089 min^{-1} for rS and 0.17 ± 0.021 min^{-1} for -1dC. Errors were calculated with 95 % confidence from three trials, using average k_{obs} values of 0.84 min^{-1} and 0.16 min^{-1} for rS and -1dC, respectively. Because D123 cleaves oligonucleotides so efficiently, rate-limiting conformational processes become evident in the seconds timescale. A small initial lag exists at timepoints earlier than 15 s for both substrates. By conducting fluorescence binding experiments and altering the order of component addition, the lag was found to derive from a folding event related to the association of D5 (data not shown). After this initial lag, the data are almost perfectly linear; thus, at longer reaction times, the folding event is not rate-limiting. Rates were calculated from the fit to the linear portion of the line (after 15 s).

(0.88 min^{-1}) is not limited by the chemical rate of catalysis, however. Unlike all of the other processes reported in this paper, this rate is not pH-dependent; it has a similar magnitude to rates of folding processes observed in stopped-flow fluorescence experiments performed under identical reaction conditions (unpublished data). Preliminary estimates for the chemical rate of rS cleavage by D123 are ~3 min^{-1} (unpublished data), indicating that D123 has a similar, ~15-fold preference for ribose to that observed for the D1 ribozyme. Taken together, this suggests that significant transition-state stabilization can be achieved by fast group II intron ribozymes without engaging 2'-hydroxyl groups for high levels of transition-state stabilization. It is therefore clear that ribozymes in this mechanistic class do not necessarily exploit the stabilizing effects of the 2'-hydroxyl group at the cleavage site.

Discussion

The chemical role of substrate 2'-hydroxyl groups

We currently have only a modest understanding of how RNA acts as a catalyst. There are few natural ribozymes in existence, and only three in the mechanistic class to which the group II intron belongs [10,46]. It is therefore important to study each of these ribozymes, since each of them may offer different information on the mechanism of transition-state stabilization by RNA. Previous studies have examined the role of ribose sugars at the site of cleavage by the group I or RNAse P ribozymes [3,4,11,12,15,42,47,48]. In those cases, a 2'-hydroxyl group adjacent to the scissile bond is important for catalysis. Single deoxynucleotide substitution at the cleavage site of group I intron ribozymes resulted in large reductions in chemical rate: 590-fold for the *Tetrahymena* ribozyme [11] and a 1900-fold reduction for the *Anabaena* ribozyme [34]. Analogous studies on the RNA component of RNase P resulted in a relative chemical rate difference of 3400-fold [4]. It is thus surprising that, in a family of group II intron ribozymes, the absence of a 2'-hydroxyl group at the cleavage site causes only a 16-fold reduction in chemical rate (Fig. 6). Even this difference could simply be due to electron withdrawal by the 2'-hydroxyl group, stabilizing the 3'-oxyanion leaving group in the transition state.

Although the results presented here were unexpected, there is no direct reason that a 2'-hydroxyl group at the cleavage site should have a large effect on catalysis. As the 2'-hydroxyl group does not participate in the reaction either as nucleophile or leaving group (Fig. 1a), the chemical mechanism of ribozymes of this type does not implicitly require it [10]. A similar lack of dependence on the 2'-hydroxyl group at the 5'-splice site was observed in studies of pre-mRNA splicing [49], despite the fact that base mutations at that site do affect catalysis [50,51]. Group II introns are widely viewed as possible evolutionary predecessors of the eukaryotic spliceosome. There is also precedent for group II intron catalyzed attack on DNA linkages; the free bI1 intron (a group II intron from yeast mitochondria) can cleave a DNA substrate, albeit inefficiently [52].

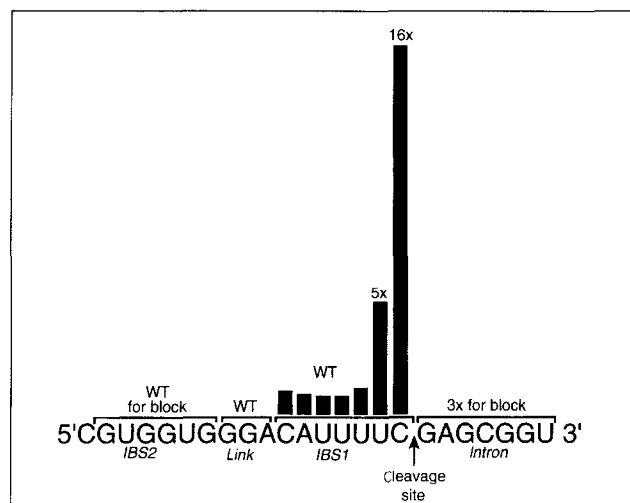

Fig. 6. Summary of deoxynucleotide effects on the chemical rate of D1/D5 ribozyme cleavage. The magnitude of k_{chem} diminution is plotted for the single and block deoxynucleotide substitutions along the oligonucleotide substrate strand. The k_{chem} effect for IBS2 is labeled as wild-type (WT) for block because the k_{cat} for cleavage of the d(IBS2) oligonucleotide was the same as the rS substrate, provided that additional D1 was added to overcome slight weakness in the chimeric EBS2–IBS2 helix.

Rapid deoxynucleotide cleavage by a group II intron ribozyme

D1/D5 cleavage of an all-ribose substrate is slower than that of many known ribozymes [47,53]. It is possible that the D1/D5 ribozyme does not contain all the functionalities necessary for efficient cleavage, including contacts to the 2'-hydroxyl group at the cleavage site. When domain 3 (~80 nucleotides in ai5g) is covalently attached to domain 1, however, one observes an apparent cleavage rate approaching 1 min^{-1} for rS (unpublished results and Fig. 5). For the fast variants, such as D123 described here, deoxynucleotide cleavage is also efficient, with a rate of 0.17 min^{-1} (Fig. 5). The D123 ribozyme can also cleave an all-DNA oligonucleotide, apparently overcoming difficulties associated with a totally hybrid IBS/EBS duplex. Thus it appears that this ribozyme, like its slower relative D1, does not use the 2'-hydroxyl at the cleavage site extensively for transition-state stabilization.

The k_{cat} value of 0.17 min^{-1} for −1dC cleavage by D123 is faster than that for deoxyribonucleotide cleavage by any known natural ribozyme [4,11]. It is similar to k_{cat} for cleavage of DNA by *Eco*RI and only an order of magnitude slower than the individual rate constant for the bond-breaking step, although this k_{cat} is slower than that of other, more efficient protein endonucleases [54,55]. The k_{cat} for −1dC cleavage by D123 is only two-fold slower than the rate observed for the best DNA-cleaving group I intron ribozyme evolved through 27 generations of mutation and selection [16]. But k_{cat}/K_M for the D123 ribozyme is $\geq 3.2 \times 10^7\ M^{-1}\ min^{-1}$ (estimating a K_M of 5 nM for substrate binding to D123), 10-fold faster than that for the evolved group I ribozyme [16]. While group II ribozymes are efficient for RNA cleavage, they are not

as fast as the best ribozymes that use a 2'-hydroxyl group to stabilize the transition state [4,11]. If a group II intron active site could be refashioned to exploit this stabilizing contact, it is possible that its chemical efficiency for RNA cleavage might improve.

DNA cleavage by group II intron ribozymes may have implications for the apparent mobility, or migration of group II introns into new genomic positions [56,57]. It is often suggested that free group II introns can bind to and then reverse splice into new RNA molecules, provided that these share sequence similarity to the parent site [58,59]. Group II introns might also attack and integrate into DNA sites, becoming directly incorporated into a new genome [52]. Although it is tempting to speculate on a biological role for DNA cleavage by these ribozymes, the catalytic behavior of group II intron ribozymes in cleavage reactions where the substrate is separate from the enzyme is often different from the behavior observed for *cis* splicing and for reactions in which the cleavage site is covalently attached to D1 [32]. *Cis*-cleaving constructs will need to be studied further before strong statements can be made about the function of 2'-hydroxyl groups in such reactions.

Ground-state effects of 2'-hydroxyls on the substrate

For the D1/D5 ribozyme, the effect of deoxynucleotide substitutions on ground state binding was measured by changes in the K_M value. This K_M is considered a reasonable measure of D1–substrate association energy because it was previously shown to equal K_d for binding of rS [20]. Because $k_{cat} = k_{chem}$ for each substrate investigated here, it is likely that $K_M = K_d$ for the chimeric substrates as well. K_M values for the −1dC and −2dU substrates are only three-fold and two-fold larger than for rS (Table 2). These effects are similar to the magnitude of the duplex-weakening effect observed previously upon single-deoxynucleotide incorporation into an RNA/RNA helix [14,36], suggesting that these 2'-hydroxyl groups do not form contacts with the ribozyme in the ground state. It is also noteworthy that in the group I *Tetrahymena* and RNase P ribozymes, small ground-state effects of approximately three-fold were also observed for substitution at the −1 position [3,4,11].

Single deoxynucleotide substitutions within the IBS1 region at positions −3 to −7 resulted in no observable effect on binding or chemistry (Figs 1 and 6). Being far away from the splice site, it is not surprising that these 2'-hydroxyl groups contribute little, if any, energetic stabilization in the transition state. The lack of any major effects on the rate at concentrations of D1 and D5 that represent the lower limit for saturation of the rS substrate also suggests that the ground state has not been substantially disturbed and that there are no contacts between any of these 2'-hydroxyl groups and functionalities on the ribozyme in the ground state. The idea that oligonucleotide binding by group II intron ribozymes does not involve backbone tertiary interactions is supported by the fact that ribozyme–substrate binding energies parallel the calculated amount of duplex stabilization for base-pairing of the two EBS–IBS interactions [20,52]. This contrasts with the behavior of the *Tetrahymena* group I ribozyme, in which 2'-hydroxyl groups make specific contacts that provide extra binding energy to stabilize both the ground and transition states [2,3,5,13,14]; exploiting the transition-state contact may help this ribozyme to cleave RNA with a higher k_{chem} than do the group II ribozymes used in this study.

Ribozymes that rely on extensive tertiary interactions with the backbone of their substrates can suffer from a loss of sequence specificity. Extensive backbone contacts may allow a mismatched substrate to be 'tolerated' long enough in the binding site to undergo cleavage [60]. Because 2'-hydroxyl groups appear to make minimal contributions to stabilizing D1–substrate association, group II introns may be able to obtain a higher level of specificity in substrate recognition.

Significance

Self-splicing group II introns are mechanistically related to the eukaryotic spliceosome, perhaps representing a form of its evolutionary precursor. These introns are essential for RNA processing in plants, yeast and other organisms. The structural complexity and size of group II introns make them interesting, but difficult subjects for chemical and enzymological analysis. We have therefore used a simplified system, in which minimal group II intron ribozymes cleave small oligonucleotide substrates, to study catalysis. Since the 2'-hydroxyl group of ribose is central to substrate association and/or cleavage by other ribozymes, we first examined the role of 2'-hydroxyl groups on the substrate in the catalytic mechanism. Surprisingly, there is little role for the 2'-hydroxyl group in the transition-state, suggesting that the group II introns may rely on a catalytic strategy that differs from other ribozymes. In addition, 2'-hydroxyl groups make only minor contributions to ground-state stabilization by these ribozymes. This lack of backbone interactions may cause the group II intron ribozymes to be particularly specific for targeting the correct RNA sequence, a potentially important feature for applications such as gene therapy.

Materials and methods

Preparation of RNA

RNA transcriptions of D1 (425 nucleotides (nts)) and D5 (58 nts), from plasmids pT7D1 and pJDI5'-75 respectively, were carried out as previously described [20]. Ribozyme D123 (710 nts) was transcribed from plasmid pT7D123, which was cut with Hind III. This plasmid was constructed by Kunkel mutagenesis [61] from plasmid pT7I3'-675 [28,30]. Mutagenesis removed the 5' exon from the parent plasmid, such that the first nucleotide of the resulting transcript corresponds to the first nucleotide of the ai5g group II intron from yeast mitochondria. Plasmids pJDI5'-75 and

pJDI3'-675 were kindly provided by Dr. Philip S. Perlman. Oligonucleotide substrates, all ribose (rS) or with deoxynucleotides (chimeric substrates) selectively placed at specific positions, were synthesized on an ABI 392 DNA/RNA synthesizer, and worked up according to standard procedures [62]. Oligonucleotides were composed of the same base sequence which, for the all ribose substrate (rS) was 5'-CGUGGUGGGACAUUUUC˅*GAGCGGU*. Underlined positions represent IBS2 and IBS1 in that order, italic letters represents the intronic sequences and ˅ indicates the cleavage site. The sequence of the d(IBS1) block substrate was 5'-CGUGGUGGGA**dCdAdUdUdUdU**C˅*GAGCGGU*, in which all IBS1 residues other than the nucleotide at the cleavage site were deoxynucleotides (bold). The d(IBS2) substrate contained the sequence 5'-C**dGdUdGdGdUdG**-GGACAUUUUC˅*GAGCGGU*. The d(link) substrate contained deoxynucleotide substitutions at the three positions between IBS1 and IBS2 (as above). The d(intron) substrate contained the nucleotides 5'-CGUGGUGGGA-CAUUUUC˅***dGdAdGdCdGdGdU***. Substrates containing single deoxynucleotide substituents are named according to the position of substitution relative to the cleavage site. For example, the sequence of −1dC is 5'-CGUGGUG-GGACAUUUU**dC**˅*GAGCGG*, and that of −2dU is 5'-CGUGGUGGGACAUUU**dU**C˅*GAGCGGU*, and so on.

Reaction conditions for kinetic analyses

Characterization of preliminary reaction rates for the d(IBS2), d(link), d(IBS1) and d(intron) substrates was carried out under single turnover conditions with saturating ribozyme. The reaction buffer contained 1 M KCl, 100 mM $MgCl_2$, and 80 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) pH 7.5. Reactions were usually carried out in 20 μl volumes at 42°C, with excess ribozyme (100–250 nM D1 or D123, 3–6 μM D5, as indicated) and trace 5'-^{32}P labeled substrates (0.25–1 nM). Initiation of the reaction, measurement of k_{obs} values and single turnover kinetic analysis were conducted as described previously [20] with one exception: preliminary results from fluorescence experiments showed that D1 can slowly fold and bind to the substrate without D5 (unpublished data). To ensure that both rS and chimeric substrates reached binding equilibrium with ribozyme before reaction, substrates containing single deoxynucleotides were preincubated for 20 min with D1 or D123 before initiation of the cleavage reaction by addition of D5. Appropriate amounts of substrate and D1 were mixed into 4 μl of 80 mM MOPS pH 7.5, and heated at 95°C for 1 minute to denature potentially misfolded structure. After cooling to 42°C, 8 μl concentrated salt in 80 mM MOPS pH 7.5 was added to this D1–substrate mix to give a final concentration of 1M KCl and 100 mM $MgCl_2$. The solution was incubated at 42°C for 20 min. The reaction was initiated by the addition of 8 μl D5 mixture, preincubated in parallel under the same salt concentration. This additional procedure was merely a precaution, however, since control experiments showed that for rS and mutant substrates, preincubation of D1 or D123 with substrate results in the same kinetic parameters as analyses without preincubation, performed as described previously [20].

Determination of individual kinetic parameters

To determine K_M and k_{cat} for reaction of substrates with the D1 ribozyme, k_{obs} was monitored at varying concentrations of D1, while holding the concentration of D5 at saturation. The data fit an equation describing bimolecular association, from which the kinetic parameters K_M and k_{cat} were extracted. The data were quantitated and analyzed as described previously [20,30]. To determine whether, in each case, k_{cat} represented k_{chem}, it was necessary to conduct parallel pH/rate profiles of the reaction. Values of k_{cat} for rS, −1dC and −2dU substrates were compared at pH 6.5, 7.0 and 7.5, in 80 mM MOPS, 100 mM $MgCl_2$ and 2 M KCl. MOPS was chosen because it maintains high buffering capacity throughout this pH range. In addition, MOPS maintains a stable pH over a wide temperature range. For these experiments, the KCl concentration was raised to 2 M to quantitate the slow rate of −1dC cleavage at pH 6.5 accurately. Doubling the KCl concentration speeds up the reaction by ~10 fold for all substrates, at all pH values. With the exception of −1dC cleavage at pH 6.5, which is too slow to measure, the same pH/rate behavior is seen for all three substrates (with lower precision) at 1 M KCl.

Mapping of cleavage products and chemical analysis of 3'-ends

Partial alkaline degradation and enzymatic digestions with nucleases T1, *B. Cereus* (Pharmacia), and P1 (Boehringer) were carried out as described [20]. To examine whether cleavage of the −1dC substrate was indeed occurring at the deoxyribose cleavage site, 5'-^{32}P labeled substrates, rS and −1dC, were cleaved to 50% completion, under conditions described above. Excess salt was removed with oligo purification cartridges (OPC, from Applied Biosystem, Inc.). The reaction products were then oxidized with 0.1 M $NaIO_4$ in 0.1 M sodium acetate pH 5.0 for 90 min at room temperature in the dark [63]. Excess $NaIO_4$ was removed using an OPC cartridge. The oxidized mixture was then reacted with 0.1 M lucifer yellow hydrazide (lucifer yellow CH, Aldrich) in 0.1 M sodium acetate pH 5.0 at room temperature in the dark for ~4 h. If the 3' terminus of an oligonucleotide is a ribose, reaction with $NaIO_4$ will oxidize the terminal sugar to a dialdehyde that reacts readily with hydrazine dyes [41,63] such as lucifer yellow. Any 3'-terminal deoxyriboses are resistant to attack by $NaIO_4$. After passing the dye reaction through an OPC cartridge to remove excess lucifer yellow, the mixture was combined with denaturing dye, and loaded onto a 20% denaturing polyacrylamide gel. Attachment of lucifer yellow to the 3'-terminus of an oligonucleotide substantially reduces its mobility in a denaturing gel; the identity of 3'-terminal sugars was then analyzed by autoradiography of the products after electrophoresis.

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