15.0-h half-life, a spectral signature of a neutron burst would be present even several days after the burst. None was observed, leading to the conclusion that neither the d(d, p)t nor the d(d, n)^{3}He reaction was responsible for this anomalous heat burst.

In addition, we later learned that a low-level, d.c. heat excess was observed during our monitoring period (S. Pons, EPRI Conference, University of Utah, 16 August 1989); if this is the case, this excess did not originate from known nuclear processes. □

Received 26 September 1989; accepted 30 January 1990.

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ACKNOWLEDGEMENTS. We thank S. Pons, M. Anderson and M. Hawkins for their hospitality during our work in their laboratory. We thank K. Wolf for alerting us to the t(d, n)^{4}He reaction that follows d(d, p)t, and K. Drexler for suggesting neutron detection via activation of ^{23}Na within our detector. We also thank R. Lloyd, W. Schier, D. Leavitt, F. Steinhausler and P. Bergstrom for valuable assistance, R. Petrasso for a careful review of an earlier manuscript, P. B. Price, M. Solarz, S. Barwick, R. Huber, R. Price and C. DeTar for helpful conversations, and R. Cooper for technical assistance. This work was supported by the State of Utah. S.M.S. was supported by the Office of Health and Environmental Research, US Department of Energy.

DNA cleavage catalysed by the ribozyme from *Tetrahymena*

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An RNA enzyme derived from the self-splicing intervening sequence of *Tetrahymena thermophila* catalyses sequence-specific cleavage of an oligodeoxyribonucleotide substrate. Compared with RNA, the DNA substrate is bound very weakly and is cleaved very slowly, revealing the importance of the RNA 2′-hydroxyl group in both the binding and chemical steps. The finding that catalysis by RNA can extend to DNA substrates indicates new possibilities for the transposition of intervening sequences and for the design of DNA cleavage agents with novel sequence specificities.

ALTHOUGH catalytic RNAs, or ribozymes, have received much attention[1–4], they have been generally maligned as having poor catalytic efficiency and limited substrate repertoire. But the *Tetrahymena* ribozyme increases the rate of hydrolysis of RNA 10^{10} times over the estimated uncatalysed rate, well within the range of rate increases achieved by protein enzymes[5]. Also, this ribozyme cleaves essentially every substrate molecule it binds (D.H. and T.R.C., manuscript submitted) and has therefore attained catalytic perfection as defined by Albery and Knowles[6].

With respect to the versatility of substrate choice, it seemed that ribozymes were restricted to RNA substrates. But DNA substitutes for RNA in a stoichiometric addition reaction that results in reopening of the circular form of the *Tetrahymena* intervening sequence (IVS) RNA[7]. We now report that the *Tetrahymena* ribozyme catalyses the sequence-specific cleavage of single-stranded DNA, with multiple turnover.

Characterization of the DNA cleavage reaction provides new insight about the nature of substrate–ribozyme interactions. Equilibrium binding of the DNA substrate is about 10^4-fold weaker than binding of the analogous RNA, and the rate of chemistry with bound substrate is decreased by about another 10^4-fold. Therefore, one or more of the 2′-hydroxyl groups of the RNA substrate is involved in binding and catalysis. Despite these large differences, the maximum rate during steady-state turnover is only about 10-fold lower for the DNA substrate, because there is a change in the rate-limiting step when DNA is substituted for RNA.

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The ribozyme cleaves DNA

The ribozyme used in this study is the L-21 ScaI RNA, a shortened form of the *Tetrahymena* IVS[8]. This ribozyme is an endonuclease that cleaves RNA substrates directly after sequences resembling the 5′ exon, by means of attack by guanosine; the analogous reaction for a DNA substrate is shown in Fig. 1. The endonuclease reaction is an intermolecular version of the first step of self-splicing (Fig. 2).

The DNA substrate d(p*CCCUCUA$_5$) (p* denotes [^{32}P]/phosphate) was synthesized with dU residues to provide a strict DNA version of the RNA substrate studied previously. This DNA substrate is cleaved specifically at the same position as the analogous RNA substrate (Fig. 1). The product (Fig. 3*a*, lane 4) co-migrates with d(p*CCCUCU) (lane 3) and with the appropriate product from P1-nuclease digestion of the DNA substrate (lane 1). Mg^{2+} is required (lane 7), ATP cannot substitute for G (lane 6), and GTP can substitute for G (see below), each of which is also a property of the reaction with RNA substrates. A small amount of product is formed in the absence of added G, but only in the presence of ribozyme and Mg^{2+} (product seen in lanes 5 and 6, but not in lanes 7 and 8 in longer exposures; data not shown). This is analogous to the slow hydrolysis of RNA substrates that occurs in the absence of G (D.H. and T.R.C., manuscript submitted). Similar experiments with d(p*CCCUCUA) also gave specific cleavage between U and A dependent on the presence of the ribozyme (data not shown).

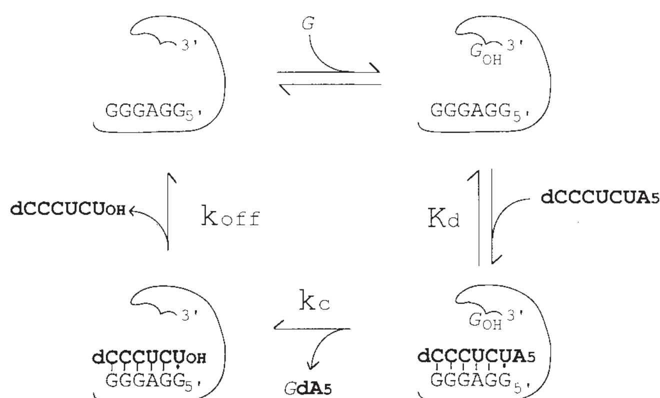

FIG. 1 The endonuclease reaction of the L-21 ScaI ribozyme with a DNA substrate (bold). Details of the reaction mechanism are analogous to those of the corresponding RNA reaction[32]. Because the equilibrium for the overall reaction in solution is expected to be near unity for this phosphotransesterification reaction, all steps are presumably reversible. The binding of G before the oligonucleotide substrate is shown for simplicity; but experiments with RNA substrates show that the two binding sites are essentially independent, so that either order of substrate addition can occur (D.H. and T.R.C., manuscript submitted). The 5′ sequence of the ribozyme (shaded), called the 5′ exon-binding site, is responsible for recognition of the substrate by base pairing[8,32-37].

Covalent addition of the guanosine cofactor to the 3′ product fragment as outlined in Fig. 1 was investigated using a DNA substrate that was 3′ end-labelled (dCCCUCUA$_5$p*3′dA; Fig. 3*b*). The product from the reaction with GTP (lane 11) migrates faster than that from the reaction with G (lanes 3 and 5), as expected from covalent attachment of GTP and G and the greater negative charge of the GTP product: pppGA$_5$p*3′dA versus GA$_5$p*3′dA. After treatment with ribonuclease T1, which cleaves after guanosine residues[9], the products from the reactions with G and GTP (lanes 4, 6 and 12) both co-migrated with A$_5$p*3′dA (lanes 2 and 9). This shows that G and GTP attack the substrate at the U–A bond, as depicted in Fig. 1.

The DNA endonuclease reaction is catalytic. For example, a reaction mixture with 25 μM DNA substrate (d(CCCUCUA$_5$)) and a trace of 5′-end-labelled substrate was incubated with 0.55 μM ribozyme and 800 μM G for 20 h (see Fig. 4 for conditions). Five per cent of the labelled DNA was converted to the product, corresponding to slightly more than two turnovers.

Steady-state kinetic parameters

The second-order rate constant for reaction of free ribozyme and substrate, $k_{cat}/K_m = 400\ M^{-1}\ min^{-1}$, ($k_{cat}$ is the catalytic constant, and K_m is the Michaelis constant) was determined from experiments with ribozyme in excess of labelled DNA substrate (Fig. 4). The value for the inhibition constant of $K_i = 30$ μM for d(CCCUCUA$_5$) was determined from inhibition by 1–320 μM DNA substrate of the reaction of ^{32}P-labelled RNA substrate (r(p*G$_2$CCCGCUA$_5$)) with the ribozyme in excess, but at a concentration ≪ K_m (20 nM ribozyme and ~1 nM RNA substrate; K_m for r(p*G$_2$CCCGCUA$_5$) = 300 nM; D.H. and T.R.C., manuscript submitted). These conditions give an observed K_i that is equivalent to K_m for the added deoxy inhibitor. This is explained in brief as follows. The reaction of the labelled RNA substrate is first-order with respect to free ribozyme. Therefore, the extent of slowing of the reaction on the addition of inhibitor reflects directly the amount of ribozyme that has bound DNA. This amount is dictated by the K_m of the DNA substrate[10]. The rate of reaction of the labelled RNA substrate decreases to zero with increasing concentrations of the DNA substrate, showing that the inhibition is competitive as expected for binding of the RNA and DNA substrates at the same site on the ribozyme.

The values of $k_{cat}/K_m = 400\ M^{-1}\ min^{-1}$, and $K_m = 30$ μM, give a k_{cat} of 0.01 min^{-1}. The k_{cat}/K_m is about 10^5-fold lower and k_{cat} about 10-fold lower than the values for the corresponding RNA substrate, r(G$_2$CCCUCUA$_5$) (Table 1). Note that the RNA substrate has two G residues at its 5′ terminus to facilitate its transcription by T7 RNA polymerase[11]. Removal of these G residues from the product oligonucleotide by ribonuclease T1 has only a minor effect on the binding constant, lowering it by about fourfold (D.H. and T.R.C., manuscript submitted). Similarly, the DNA product with two dG residues added to its 5′ terminus has the same binding constant, within about twofold, as the product oligonucleotide shown above (data not shown).

DNA binds weakly and is cleaved slowly

The following analysis shows that the large change in k_{cat}/K_m and relatively small change in k_{cat} with the DNA substrate

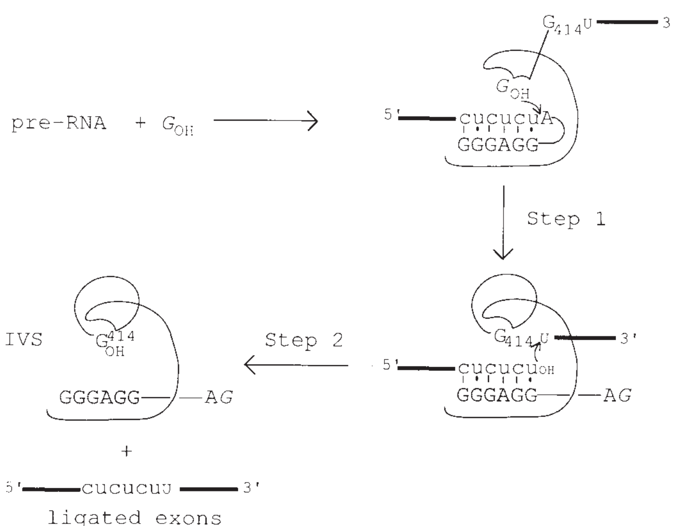

FIG. 2 Pathway for self-splicing of *T. thermophila* pre-ribosomal RNA[5,38,39]. The 413-nucleotide IVS is excised in two transesterification steps. In the first step, guanosine (or GTP) attacks the 5′ splice site so that the bond at this junction is cleaved and G is covalently attached to the 5′-end of the IVS. The nucleotides preceding the 5′ splice site are recognized by base pairing to the 5′ exon-binding site, a portion of the internal guide sequence[34-37]. This same binding motif is used in the endonuclease reaction described in Fig. 1. In the second step of splicing, the 3′-hydroxyl group at the end of the 5′ exon attacks the 3′ splice site after G_{414} to give exon ligation and excision of the IVS.

TABLE 1 Comparison of the kinetic parameters for cleavage of DNA and RNA substrates

	k_{cat}/K_m (M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	K_d (μM)	k_c* (min^{-1})
DNA ($CCCUCUA_5$)	400	0.01	30	30	0.01
RNA ($G_2CCCUCUA_5$)	10^8	0.1	0.001	0.002	~350
RNA/DNA ratio	3×10^5	10	$1/(3\times10^4)$	$1/(2\times10^4)$	4×10^4

Reactions were carried out at 50 °C in 50 mM MES buffer, pH 7.0 (at 25 °C), and 10 mM $MgCl_2$. The data for the DNA reaction are described in Fig. 4 and the text, and the data for the RNA reaction are described by us elsewhere (D.H. and T.R.C., manuscript submitted). As described in the text, the 5'-terminal G residues have little effect on binding for the RNA or DNA.

* The rate constant k_c is that for the chemical conversion of the E~G~S ternary complex (Fig. 1).

replacing the RNA substrate result from large decreases in the binding affinity and the rate of the chemical conversion for the DNA substrate. These large decreases are not fully expressed in k_{cat}/K_m and k_{cat} because there is a change from rate-limiting binding (k_{cat}/K_m) and product release (k_{cat}) with the RNA substrate, to rate-limiting chemistry with the DNA substrate.

Inhibition of the ribozyme by the DNA product, d(CCCUCU), performed as outlined above for determination of K_i for d($CCCUCUA_5$), gave a K_i of 20 μM. (Reactions were performed with 1–230 μM d(CCCUCU).) At the high concentrations of d(CCCUCU) the observed rate of reaction approached zero, showing that the inhibition is competitive. The value of K_i is equivalent to K_d because the inhibition represents removal of free ribozyme, which follows the equilibrium: $E+P=E\sim P$ ($K_d=[E][P]/[E\sim P]$), where E, P and E~P denote the ribozyme, the oligonucleotide product, and the complex that they form, respectively, and the brackets denote concentrations.

The binding of the DNA product is about 10^4-fold weaker than that of the analogous RNA product (K_d for r($pppG_2CCCUCU$) = 1 nM; D.H. and T.R.C., manuscript submitted). Therefore the DNA product should dissociate faster than the RNA product. Dissociation of the RNA product, r($G_2CCCUCU$), is rate-limiting for k_{cat} so that k_{cat} for the DNA reaction would be greatly increased if product dissociation were rate-limiting. But k_{cat} is decreased by about 10-fold with the DNA substrate. This presumably indicates that there is a change to a rate-limiting step involving the conversion of the ternary complex to products. This is presumably the chemical step (with rate constant k_c; Fig. 1). The value of $k_c(=k_{cat}) = 0.01$ min^{-1} for the DNA substrate is about 10^4-fold smaller than k_c for the RNA substrate (Table 1).

With subsaturating oligonucleotide substrate and saturating G, there is a second-order reaction of the E–G complex and S. For the RNA substrate, binding is rate-limiting; the value of $k_{cat}/K_m = 10^8$ M^{-1} min^{-1} for this reaction (Table 1) is essentially equal to the rate constant for formation of helices between oligonucleotides[12,13]. By contrast, the value of k_{cat}/K_m = 400 M^{-1} min^{-1} for the DNA substrate is much smaller than rate constants for helix formation. The strong implication is that binding of the DNA substrate is not the rate-limiting step, so that the subsequent chemical conversion is rate-limiting. This can also be shown by another quantitative argument. The value of $k_c = 0.01$ min^{-1} for the chemical conversion with the DNA substrate is much smaller than k_{off} (the rate constant for the

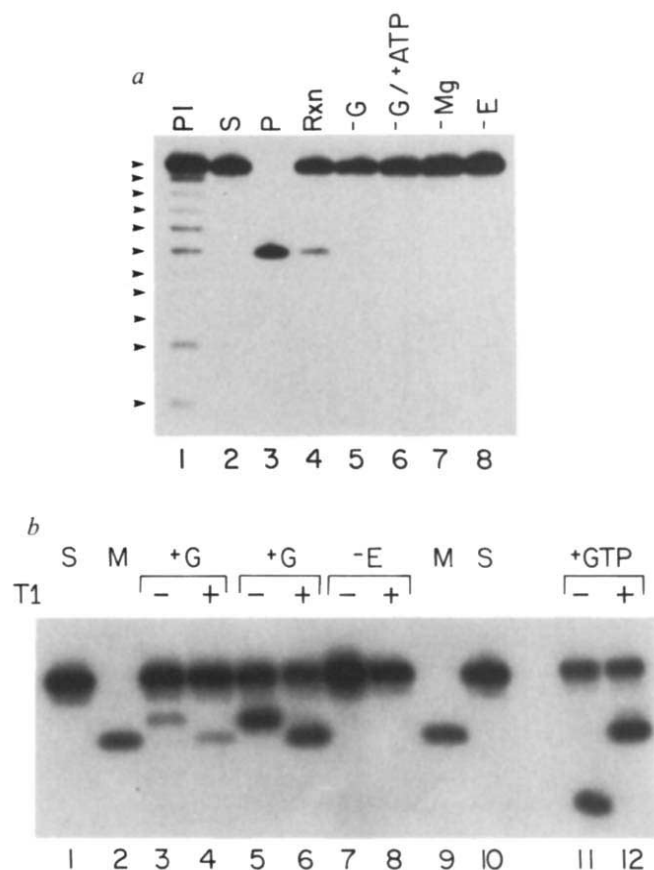

FIG. 3 Endonuclease reaction of a DNA oligonucleotide substrate catalysed by the ribozyme. *a*, Identification of the 5' product fragment: d(p*$CCCUCUA_5$) (~2 nM, 5' end labelled with ^{32}P), was incubated with 1 μM L-21 ScaI ribozyme for 15 h at 50 °C in the presence of 800 μM G, 50 mM MES, pH 7.0, and 10 mM $MgCl_2$ (lane 4, Rxn denotes reaction); without G (lane 5); with 800 μM ATP replacing the G (lane 6); without $MgCl_2$ (lane 7); and without ribozyme (lane 8). Lane 2 shows the substrate, d(p*$CCCUCUA_5$); lane 3 shows the putative product, d(p*CCCUCU); lane 1 shows the products from digestion of the substrate by 0.2 U μl^{-1} P1 nuclease in 1 mM Tris buffer, pH 7.5, 2 mM $ZnCl_2$, 0.1 mM EDTA, and 0.1 μg μl^{-1} transfer RNA for 60 min at 37 °C. Ribozyme reactions were initiated by addition of the DNA substrate after 10-min preincubation of the ribozyme in buffer and $MgCl_2$ at 50 °C. Reactions were quenched with EDTA and urea at 0 °C and electrophoresed on a 20% denaturing polyacrylamide gel[8]. The ribozyme was prepared as described previously[8]. DNA oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer; dU on a derivatized CPG support (American Bionetics) was used for oligonucleotides with a 3'-terminal dU residue. *b*, Identification of the 3' product fragment: d($CCCUCUA_5$p*3'dA) (~2nM) was incubated for 4 h with 4 μM L-21 ScaI ribozyme, 50 mM MES, pH 7.0, and 10 or 100 mM $MgCl_2$; one aliquot was quenched with EDTA and urea and another aliquot was treated with 0.05 U μl^{-1} ribonuclease T1 for 1 h at 37 °C. Denaturing PAGE was performed as in *a*. Lanes 3 and 4, reaction with 1 mM G and 10 mM $MgCl_2$; lanes 5 and 6 reaction with 1 mM G and 100 mM $MgCl_2$; lanes 11 and 12 reaction with 1 mM GTP and 100 mM $MgCl_2$. Lanes 7 and 8, from incubation with 1 mM G and 100 mM $MgCl_2$ in the absence of ribozyme. Lanes 1 and 10, substrate, d($CCCUCUA_5$p*3'dA); lanes 2 and 9, expected product after ribonuclease-T1 treatment, d(A_5p*3'dA) (M). Both d($CCCUCUA_5$) and dA_5 were labelled at their 3' end with p*3'dA by reaction with [α-^{32}P]3'dATP catalysed by terminal transferase. No product was observed when G or $MgCl_2$ was omitted from reaction mixtures containing the ribozyme (data not shown).

dissociation of substrate) = 0.2 min^{-1} for the RNA substrate (D.H. and T.R.C., manuscript submitted), and k_{off} for the DNA substrate should be even larger because of the lower affinity of the DNA oligonucleotides. The large value of k_{off} relative to k_c for the DNA substrate means that this substrate binds and dissociates many times before it reacts. Therefore, K_m is expected to equal K_d for the DNA substrate, and the rate-limiting step is presumably the chemical conversion, as with saturating substrate. The similar values of K_d = 30 and 20 μM for the DNA substrate and product, respectively, and for the corresponding RNA oligonucleotides (K_d = 2 and 1 nM for r(G_2CCCUCUA_5) and r(G_2CCCUCU), respectively; D.H. and T.R.C., manuscript submitted) are consistent with the assignment of $K_m = K_d$ for the DNA substrate.

The 2′-hydroxyl in binding

Comparison of the DNA and RNA reactions reveals the importance of the 2′-hydroxyl group in binding and in catalysis. RNA oligonucleotides such as r(G_2CCCUCU) bind to the ribozyme about 10^4-fold (6 kcal mol^{-1}) stronger than expected for simple helix formation (D.H. and T.R.C., manuscript submitted). By contrast, the DNA oligonucleotide binds much less strongly (about 10^4-fold) than the RNA oligonucleotide with the same sequence. This difference contrasts with the small difference in the stability of several RNA–RNA and RNA–DNA helices[14-16]. Therefore the DNA oligonucleotide binds to the ribozyme with roughly the affinity expected for a simple helix, indicating that one or more 2′-hydroxyl groups could be involved in tertiary interactions that provide additional stabilization with the RNA oligonucleotide.

The role of the 2′-hydroxyl group in binding could be direct (for example, hydrogen-bonding or Mg^{2+} coordination) or indirect (for example, favouring the correct helix geometry). The simplest model invokes a direct interaction of the ribozyme with a 2′ hydroxyl of the RNA oligonucleotide. It is tempting to propose that tertiary interactions involve the 2′ hydroxyl of the conserved U preceding the reaction site; substitution of other bases at this position greatly slows *G* addition in the first step of self-splicing and in a related reaction[17,18]. Alternatively, there could be an unfavourable helix geometry with the DNA substrate. Although RNA–RNA and RNA–DNA helices both tend to be of the A-form, there are some differences in conformation[19-22]. In addition, it remains possible that a different helical form occurs on the ribozyme that is much more easily adopted by an RNA–RNA duplex.

Similar conclusions about the importance of the 2′ hydroxyl were obtained from the study of a related reaction, the linearization of a circular form of the *Tetrahymena* IVS by d(CT), r(CU), and the mixed ribo-deoxy dinucleotides[7]. Based on comparisons of these substrates and the assumption that $K_m = K_d$, it was suggested that the 2′-hydroxyl moieties are involved in binding the IVS.

The 2′ hydroxyl in catalysis

The chemical step of the DNA reaction is about 10^4-fold slower than the chemical step of the analogous RNA reaction (Table 1). This could indicate that the 2′ hydroxyl has an increased role in stabilizing the transition state beyond its role in ground-state binding. But the possibility cannot be excluded that there is destabilization of the DNA-ribozyme duplex (Fig. 1) in the transition state from interactions with the active site that are not present in the ground state; in the ground state this helix

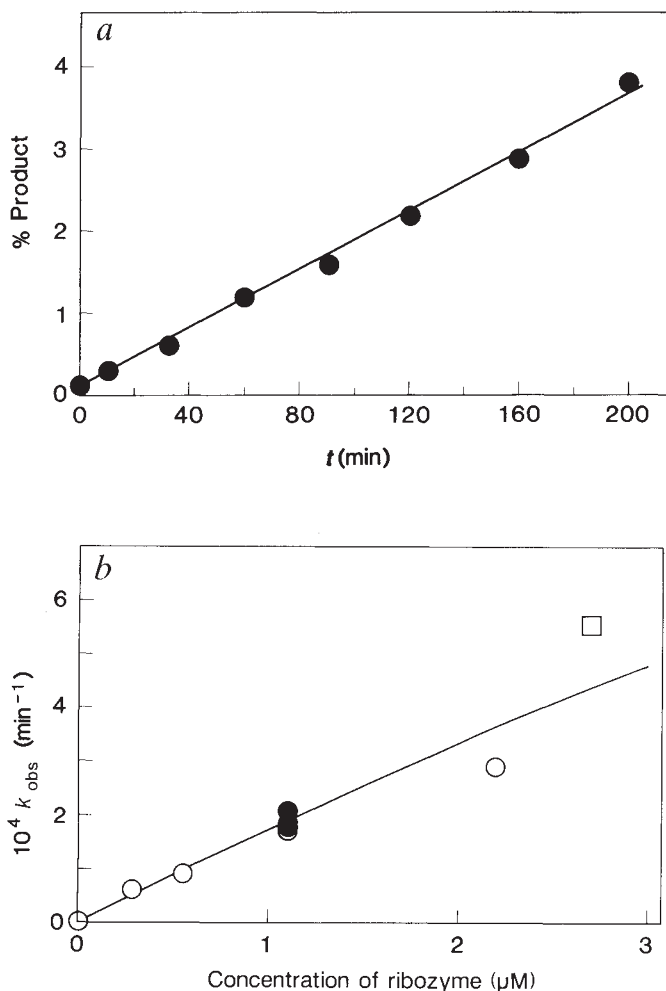

FIG. 4 Determination of k_{cat}/K_m for the DNA endonuclease reaction with d(p*CCCUCUA_5). *a*, Time course for formation of product (d(p*CCCUCU)) with 1.1 μM ribozyme, 800 μM G, 50 mM MES, pH 7.0, and 10 mM $MgCl_2$ at 50 °C. Reactions were quenched, products separated by denaturing gel electrophoresis, and quantitated with an AMBIS radioanalytical scanner. *b*, Observed rate constant (k_{obs}) determined from slopes of plots analogous to that in *a*, plotted against the concentration of ribozyme. Open and closed symbols are from independent experiments and the square is from a reaction with a different preparation of ribozyme. The initial slope of this line gives (k_{cat}/K_m) (apparent) = 180 M^{-1} min^{-1}; the line is theoretical for this value of (k_{cat}/K_m) (apparent) and K_m = 30 μM (see text). The value of K_m(G) = 1 mM (data not shown) and the concentration of G of 0.8 mM were used to extrapolate (k_{cat}/K_m) (apparent) to the value with saturating G of k_{cat}/K_m = 400 M^{-1} min^{-1} for the DNA substrate. This correction does not affect any of the conclusions derived from these data.

could be outside of the active site as the E-S complex has roughly the stability expected for a simple DNA-RNA hybrid (see above). The electron-withdrawing effect of the 2′-hydroxyl group and its ability to form an intramolecular hydrogen bond with the 3′ oxygen atom, which make the oxyanion of ribose a better leaving group than that of deoxyribose, could contribute to the faster reaction of the RNA substrate. But it seems likely that metal ion coordination or general acid catalysis would be required to achieve the large amount of catalysis observed in this reaction, and that such catalysis would reduce the charge development on the 3′ oxygen atom, thereby lowering the sensitivity of the reaction to the leaving-group ability of the ribose and deoxyribose.

As described above, the DNA substrate has both a lower affinity and slower rate of reaction after binding than the analogous RNA substrate. By contrast, an RNA substrate that forms a mismatch with the 5′ exon-binding site at position −3 from the reaction site, binds less strongly than the matched substrate (Fig. 1), but reacts at essentially the same rate as the matched RNA substrate once bound (D.H. and T.R.C., manuscript submitted). Therefore, not all changes in substrate binding cause a change in the rate of the chemical step.

Even though the reaction of the DNA substrate is much slower than that of the RNA substrate, the DNA reaction still represents enormous catalysis relative to the solution reaction. The ribozyme is estimated to catalyse the DNA reaction by about 10^9-fold. (This rate-enhancement is based on a k_c of 0.01 min^{-1} (Table 1) and an estimated rate constant for hydrolysis of dimethyl phosphate by water, k_w of $3\times10^{-12}\ \text{min}^{-1}$. This value has been calculated from $k_{OH}=8\times10^{-9}\ \text{M}^{-1}\ \text{min}^{-1}$ for reaction of dimethyl phosphate and hydroxide ion (attack at phosphorus; 50 °C)[23,24], the linear free energy relationship $\beta_{nuc}=\delta(\log k)/\delta pK_{nuc}=\sim0.3$, for reaction of oxygen nucleophiles with a phosphate diester[25], and the concentration of water of 55 M: $k_w=55\ \text{M}\times k_{OH}\times\exp\{\beta_{nuc}\times(\text{p}K_a^{HOH}-\text{p}K_a^{HO^-})\}$.) Therefore, although the 2′-hydroxyl group seems to be involved in one catalytic strategy by the ribozyme, there are additional strategies operative with both DNA and RNA substrates. Similarly, individual protein enzymes seem to use several different catalytic strategies to achieve rate increases comparable to that obtained with the ribozyme[26,27].

No cleavage of dC_5 was observed in earlier work with a different ribozyme derived from the *Tetrahymena* IVS[3]. The weaker binding of dC_5 than d(CCCUCUA$_5$), and the preference for U over C at the position preceding the reaction site[17,18] presumably contributed to the reason why no reaction was observed.

Implications and applications

Several group I IVSs are mobile genetic elements[28]. Each of these has an open reading frame (ORF) encoding a protein that acts as a sequence-specific endonuclease. This endonuclease cleaves double-stranded DNA and initiates conversion of the gene from IVS^- to IVS^+. The new finding of inherent DNA-cleavage activity in a group I IVS RNA indicates an extension of this idea: before the acquisition of an ORF, the RNA could have served as its own DNA endonuclease. Therefore the IVS could have initiated its mobility to an uninterrupted version of the same gene or its transposition to a new site, and could still do so in the case of group I IVSs, such as the *Tetrahymena* IVS, that do not contain an ORF. Considering that the IVS can use its own 3′-terminal G as an attacking group as an alternative to exogenous G (Fig. 2; ref. 3, and D. L. Robertson and G. F. Joyce, manuscript submitted), another version of this reaction would involve the insertion of the IVS RNA directly into a DNA genome by a two-step reaction analogous to the reverse of self-splicing (Fig. 2 and ref. 29). In either case, the DNA cleavage reaction that initiated insertion would necessarily involve the same sequence recognition as is required for RNA splicing; therefore, if the IVS inserted into the noncoding DNA strand, transcripts of the newly interrupted gene would undergo self-splicing at the RNA level, preventing gene inactivation. The slow reaction with DNA and the presumed requirement for single-stranded DNA would be expected to render such insertion events rare.

The finding of catalytic activity of RNA molecules rekindled speculation that an 'RNA world', in which RNA both provided catalysis and stored information, pre-dated life with protein catalysts[30,31]. Our finding that an RNA catalyst can act on a DNA substrate indicates that an RNA world could have expanded to include DNA before the involvement of proteins[7]. We note that the *Tetrahymena* ribozyme has been selected to catalyse reactions of RNA substrates, and it is therefore possible that there are different RNA catalysts considerably better at catalysing reactions of DNA substrates (D. L. Robertson and G. F. Joyce, manuscript submitted).

It is conceivable that the DNA endonuclease reaction will have some practical application. The reaction is sequence-specific (Fig. 3), and site-directed mutagenesis of the 5′ exon-binding site (Fig. 1) should allow cutting of a large variety of DNA substrates, as has been accomplished with the RNA endonuclease reaction[32,33]. Lengthening the internal guide sequence to improve binding should be one way to enhance the rate of the DNA endonuclease reaction. □

Received 28 December 1989; accepted 27 February 1990.

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ACKNOWLEDGEMENTS. We thank Pascale Legault for purification of ribozyme, and Dan Celander for helpful discussions. This work was supported by the NIH (T.R.C.) and the Helen Hay Whitney Foundation (D.H.).