Minimum Ribonucleotide Requirement for Catalysis by the RNA Hammerhead Domain†

Jing-hua Yang,‡ Nassim Usman,*,§ Pascal Chartrand,‡ and Robert Cedergren*,‡

Département de Biochimie, Université de Montréal, Montréal, Québec H3C 3J7, Canada, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received December 11, 1991; Revised Manuscript Received March 13, 1992

ABSTRACT: Several mixed DNA/RNA and 2′-*O*-methylribonucleotide/RNA analogues derived from the "hammerhead" domain of RNA catalysis have been prepared to study the minimum ribonucleotide requirement for catalytic activity. Oligodeoxyribonucleotides containing from seven to as few as four ribonucleotides are active in cleaving a substrate RNA. Predominantly deoxyribonucleotide-containing analogues have k_{cat} values 20–300 and k_{cat}/K_M values approximately 100–2000 times lower than those of the all-RNA ribozyme. In the case of predominantly 2′-*O*-methyl analogues, at least five ribonucleotides are needed to assure catalytic activity. In addition, both predominantly deoxyribonucleotide and 2′-*O*-methyl oligomers are at least 3 orders of magnitude more stable than an all-RNA ribozyme in incubations with RNase A and a yeast extract. These results suggest that the ribophosphate backbone is not a strict requirement for ribozyme-type catalysis. The identification of the four required ribonucleotides in the hammerhead catalytic domain provides valuable information for the rational design of chemical species having ribonuclease activities.

The RNA World hypothesis, that primitive organisms made use of an RNA-based genetic system rather than the DNA-based system present in all current cell types (Gilbert, 1986), was revived by the discovery of RNA molecules, the ribozymes, capable of simple catalysis (Kruger et al., 1982; Guerrier-Takada et al., 1983). Since RNA could thus accumulate both genotypic and phenotypic functions in this scenario, neither DNA nor proteins would be required for self-replication in early cells (Darnell & Doolittle, 1986; Cedergren & Grosjean, 1987). The lack of examples of catalytic properties associated with other nucleic acids, i.e., DNA, is consistent with the primacy of primordial RNA. On the other hand, the conformational preferences of ribonucleotides and deoxyribonucleotides provide little information as to why a deoxyribonucleotide oligomer modeled after a known catalytic RNA domain would not be active (Saenger, 1984).

Over the past years, we have been interested in evaluating the importance of 2′-hydroxyls in RNA structure and functionality. This interest originally took the form of the structural comparison of tDNA vs tRNA (Perreault et al., 1989; Paquette et al., 1990); however, more recently, a chemical synthesis strategy for RNA (Usman et al., 1987; Scaringe et al., 1990) compatible with the well-known protocol for DNA synthesis has permitted the synthesis of mixed oligonucleotides in our laboratory having either ribonucleotides or deoxyribonucleotides at any given position (Wu et al., 1989). We have thus initiated a series of experiments to evaluate the importance of 2′-hydroxyls in the ability of the hammerhead domain of RNA to catalyze the cleavage of ribose phosphodiester bonds (Perreault et al., 1990, 1991; Yang et al., 1990).

For our purposes, the hammerhead domain of approximately 50 nucleotides found in naturally occurring RNAs has been partitioned between a 35-unit catalytic fragment, the ribozyme, and a 14-nucleotide unit substrate (Haseloff & Gerlach, 1988; illustrated in Figure 1). This structure consists of three base-paired regions which delimit a central core of consensus nucleotides (Ruffner et al., 1990). We have shown previously that the 2′-hydroxyl of the ribocytidine at position 17 in the substrate is essential for catalysis, a fact that corroborates the proposed cleavage mechanism involving a nucleophilic attack by this hydroxyl on the adjacent scissile phosphate bond [Perreault et al., 1990; Yang et al., 1990; see also Koizumi et al. (1989)]. In contrast, no 2′-OH in the ribozyme fragment is absolutely required, even though substitution of deoxyribonucleotides at positions G5 and A9 leads to reduced catalytic activity (Perreault et al., 1991; Olsen et al., 1991). Recently, evidence for the importance of the 2′-OH of G8 has also been obtained (Williams et al., 1992). In this report, we make use of this information to design several predominantly deoxyribonucleotide mixed oligomers and show that some have catalytic activity.

Materials and Methods

Synthesis of Mixed Polymers. Mixed DNA/RNA oligonucleotides[1] were synthesized, deprotected, and purified as described previously (Wu et al., 1989; Scaringe et al., 1990). 2′-*O*-Methyl analogues were synthesized using 200 μL per cycle of 0.05 M solutions of each 2′-*O*-methylribonucleoside phosphoramidite (Glen Research Corporation, Sterling, VA) in anhydrous acetonitrile and a 10-minute coupling time on a Pharmacia gene assembler. Deprotection was performed in saturated ammonia/ethanol for 16 h at 55 °C followed by incubation in a solution of 1 M tetrabutylammonium fluoride in tetrahydrofuran for 10 h at room temperature. Crude oligomers were purified by passing them through a 20-mL Sephadex G-50 column and by 15% PAGE containing 7 M

† This work was funded by the Medical Research Council of Canada.

* Corresponding authors.
‡ Université de Montréal.
§ Massachusetts Institute of Technology.

[1] Catalytic species are abbreviated as follows: The first two letters, DR and MR, designate analogues composed of predominantly deoxyribonucleotides and 2′-*O*-methylribonucleotides, respectively. The following number indicates the number of ribonucleotides in the molecule, and the final number refers to the particular combination of ribonucleotides. The OMe prefix denotes the presence of predominantly 2′-*O*-methylribonucleotides in a molecule. The positional numbering system follows that suggested by Hertel et al. (1992, personal communication). These catalytic analogues are not ribozymes, since they are derived from deoxyribonucleotides in one case and 2′-OMe ribonucleotides in the other. PAGE is polyacrylamide gel electrophoresis.

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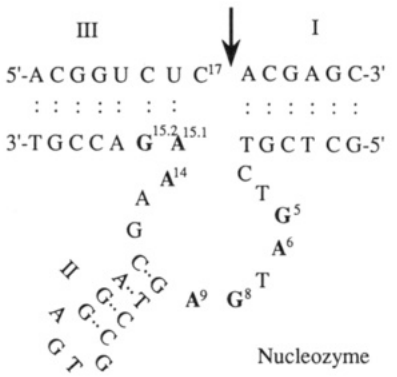


	Ribonucleotide positions						
	5	6	8	9	14	15.1	15.2
Nucleozyme							
DR2R	rG		rA		-	-	
DR3R	rG		rA		rA		
DR4R1	rG		rA		rArG		
DR4R2	rG		rGrA		rG		
DR4R3	rG		rGrA		rA		
DR5R2	rG		rGrA		rArG		
DR5R3	rGrA		rGrA		rG		
DR6R2	rGrA		rGrA		rArG		
DR7R	rGrA		rGrA		rArArG		
OMe-nucleozyme							
MR2R	rG		rA		-	-	
MR3R	rG		rA		rA		
MR5R	rG		rGrA		rArG		

FIGURE 1: Structure and sequence of ribozyme analogues.[1] The structure and sequence of ribozymes and analogues used in this study were derived from the consensus structure (Haseloff & Gerlach, 1988) as modified by Perreault et al. (1990). The top fragment is the all-RNA substrate. The lower fragment is represented in its deoxyribonucleotide form where thymidines have been taken the place of uridines found in the parent ribozyme. In the 2′-*O*-methyl analogue, 2′-*O*-methyluridine has been used for uridine. Roman numerals refer to the three presumed duplex regions of the domain. The arrow indicates the cleavage site. Positions in bold are the targeted sites for ribonucleotide substitution. The deoxyribonucleotide and 2′-*O*-methyl analogues used in this study are presented in the bottom of the figure.

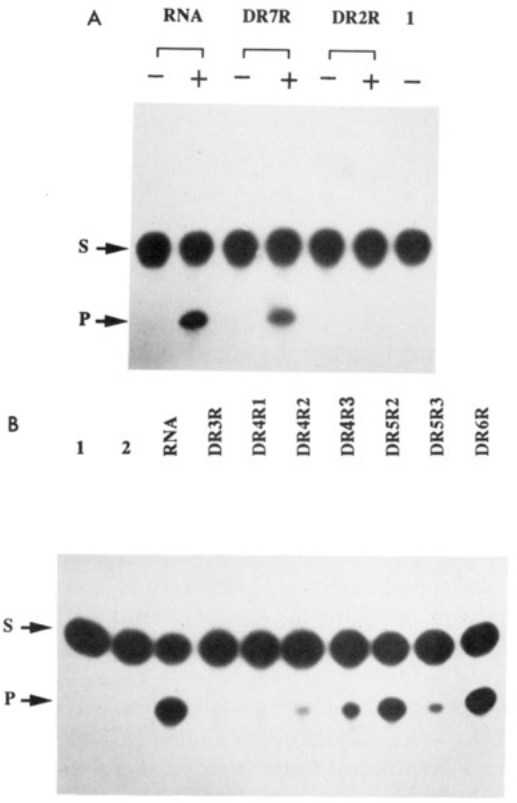


FIGURE 2: Cleavage of 5-^{32}P-labeled RNA substrate by deoxyribonucleotide analogues. The letters S and P indicate the gel position of the substrate and the product, respectively. RNA is the all-RNA ribozyme, and nucleozymes are abbreviated as in Figure 1. (A) Incubations were performed under the standard condition except that the presence or the absence of Mg^{2+} is shown by (+) or (−), respectively. Lane 1 is a control lacking both the catalytic fragment and Mg^{2+}. (B) Lane 1 shows the result of the incubation of the substrate with neither Mg^{2+} nor nucleozyme for 4.5 h, and lane 2 shows the incubation of substrate in the presence of Mg^{2+} but with no added catalytic fragment. All other lanes refer to the standard incubation conditions with the catalytic fragments from Figure 1.

urea. The positions and identities of ribonucleotides in oligonucleotides were identified by partial Mg^{2+}/formamide degradation and specific RNase digestion followed by gel sequencing (Usman et al., 1988).

Cleavage Conditions. Standard reactions were performed with 0.5 pmol of 5′-^{32}P-labeled RNA substrate and 0.5 pmol of the catalytic fragment (0.1 pmol in the case of the all-RNA ribozyme) in 9 μL of 50 mM Tris-HCl (pH 7.4). This solution was heated to 70 °C for 2 min and then cooled to 30 °C, and 1 μL of 100 mM $MgCl_2$ was added. Incubation was for 3 h at 30 °C. Reactions were stopped by the addition of 5 μL of 20 mM EDTA and analyzed by electrophoresis on a 12.5% polyacrylamide gel in 50 mM Tris–boric acid buffer (pH 8.3) containing 1 mM EDTA and 7 M urea. The product and unreacted substrate were located by autoradiography and excised from the gel. The percentage of cleavage was determined by scintillation counting of gel slices or by direct scanning of the autoradiogram.

Stability of Modified Hammerhead Domains. A sample of 0.001 pmol of 5′-labeled catalytic fragment was incubated with 1 μg of carrier tRNA, 1 μL of RNase A or yeast extract at different concentrations in a 50 mM Tris-HCl (pH 7.4), and 10 mM $MgCl_2$ buffer (total volume of 10 μL). Incubations with RNase A were for 40 min and with the yeast extract for 30 min at 37 °C. The reactions were stopped by the addition of 5 μL of 20 mM EDTA, loaded, and analyzed on 15% PAGE in 7 M urea. The yeast extract was prepared from a 1-mL culture of yeast strain BWG2-9A grown to late-log phase, harvested, and washed with 25 mM sodium phosphate buffer (pH 7.8). The pellet was suspended in 100 μL of the same buffer and sonicated for 20 s (60 W). Centrifugation for 5 min in an Eppendorf centrifuge gave a supernatant; 1-μL samples of this supernatant and dilutions thereof were incubated with the all-RNA ribozyme and various analogues.

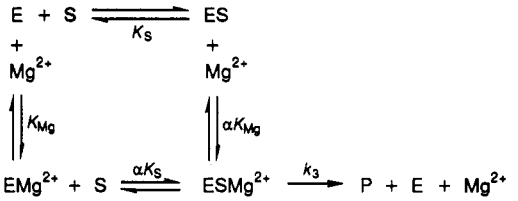
Results

Predominantly Deoxyribonucleotide-Containing Analogues. An all-DNA analogue of the hammerhead domain is inactive in catalysis; therefore, we synthesized and analyzed a predominantly deoxyribonucleotide, mixed oligomer substituted with ribonucleotides at positions G5 and A9 (Figure 1).[1] The 2′-OHs of these ribonucleotides were shown previously to have an effect on catalytic activity (Perreault et al., 1991). As this analogue was also inactive under standard conditions (see Figure 2), a mixed oligomer having ribonucleotides at positions 5, 6, 8, 9, 14, 15.1, and 15.2 of the ribozyme was tested and showed good catalytic activity (Figure 2A). The additional nucleotides were simply chosen to increase the ribonucleotide character of the central core region of the hammerhead. The activity of this analogue suggested that other mixed oligomers containing between two and seven ribonucleotides could possess catalytic activity, and a series of minimally ribonucleotide-substituted oligodeoxyribonucleotide analogues were prepared and tested (Figure 1). From Figure 2B, it can be seen that

Table I: Kinetic Parameters of Nucleozyme-Catalyzed Reactions[a]

substrate/nucleozyme	K_M (μM)	k_{cat} (min^{-1})	K_S (μM)	k_3 (min^{-1})	K_{Mg} (mM)	k_{cat}/K_M [(min·μM)$^{-1}$]
RS/DR4R3	4.2	0.004	3.8	0.013	26	0.001
RS/DR5R2	3.8	0.010	3.6	0.035	26	0.003
RS/DR7R	3.8	0.056	3.2	0.21	28	0.015
RS/RR	0.7	1.2	0.7	1.9	6.1	1.7
DSrU16.1rC17/RR[b]	4.5	0.17	ND	ND	ND	0.038

[a] The kinetic experiments were performed following the same protocol as the standard conditions with the following differences: the final concentrations were the nucleozyme at 0.05 μM, the substrate from 0.5 μM to 5 μM, and $MgCl_2$ from 5 to 50 mM; the reaction times were from 30 min to 6 h at 30 °C. Initial rates were determined from the first 10% of the reaction. The values for K_S, k_3, and K_{Mg} were determined using the random assembly model for ternary complex formation (Yang and Cedergren, unpublished data; see Segel, 1975):



when

$$\alpha K_S = K_S \quad \text{and} \quad \alpha K_{Mg} = K_{Mg}$$

then

$$v = k_3[E]\frac{[S]}{K_S + [S]}\frac{[Mg^{2+}]}{K_{Mg} + [Mg^{2+}]}$$

[b] Taken from Yang et al. (1991). ND is not determined.

analogues containing five and six ribonucleotides were active and that the active analogues DR4R3 and DR4R2 contain the fewest ribonucleotides: only 4 of 35 nucleotides composing the catalytic fragment and 3 of the 10 presumed nonhelical, consensus nucleotides and ribonucleotides. Predominantly deoxyribonucleotide substrates were not cleaved by any predominantly deoxyribonucleotide-containing analogue under standard conditions, although they are cleaved by the all-RNA ribozyme (Yang et al., 1990).

Thus, demonstrably active analogues (having at least $^1/_{5000}$ of the activity of the all-RNA catalyst) require the presence of the two influential 2′-OH's at G5 and A9 as well as two others. The apparent requirement of a 2′-OH at G8 was not completely unexpected, since deoxyribonucleotide substitution at this position in the ribozyme reduces the catalytic rate by a factor of approximately 2 (Perreault et al., 1991), and in a similar study elimination of this 2′-OH led to a dramatic reduction in activity (Williams et al., 1992). In addition, a ribonucleotide is preferred over a deoxyribonucleotide in position 15.1. This observation may signify the requirement of a stable base pair at this position: deoxyadenosine and ribouridine form only weak base pairs (Martin & Tinoco, 1980).

In spite of slower cleavage rates for these analogues than for the all-RNA ribozyme, the reactions are truly catalytic since substrate is turned over in each case. For example, a 3-h incubation of 5 μM RNA substrate with 0.05 μM of the nucleozyme DR7R and 10 mM Mg^{2+} yields eight substrate turnovers (8% cleavage).

To assess more precisely the activity of the predominantly deoxyribonucleotide-containing analogues, kinetic parameters including K_M and k_{cat} were determined for some of the reactions. The knowledge that ternary complex formation follows the random assembly model allowed the computation of the K_{Mg}, K_S, and k_3 of these reactions as well (J. Yang and R. Cedergren, unpublished data). A summary of these results is presented in Table I along with the generalized reaction scheme starting from the catalytic species, the substrate, and the Mg^{2+} cofactor, traversing a ternary complex and yielding a product. As shown, the apparent K_M's and K_S's of the three analogues are very similar and approximately 6 times that of the all-RNA ribozyme acting on the same RNA substrate. This factor is almost identical to that found for the ratio of K_M's from the predominantly deoxyribonucleotide substrate and the RNA substrate when cleaved by the all-RNA ribozyme (Yang et al., 1990) and is, therefore, likely due to the presence of a heteroduplex formed between the ribonucleotide substrate and the deoxyribonucleotide catalyst. The temperature optimum of 30 °C is the same as that of the reaction when the predominantly deoxyribonucleotide-substituted substrate is cleaved by the ribozyme via a similar heteroduplex complex.

The K_{Mg}'s of the deoxyribonucleotide analogues are virtually identical and approximately 3 times that of the all-RNA system indicating a higher optimal Mg^{2+} requirement. Since this ratio is similar to that of the ribozyme analogues having a deoxyribonucleotide substitution at G5 or A9 compared with the all-RNA ribozyme, the existence of an influential 2′-OH which was not detected in previous experiments could explain this result. Alternately, the K_{Mg} may reflect a generally more relaxed hammerhead domain when an analogue is extensively deoxyribonucleotide-substituted (Perreault et al., 1991; Paquette et al., 1990).

The k_{cat} and k_3 of the three predominantly deoxyribonucleotide-containing analogues vary over a factor of 16 between the DR7R and the DR4R3 derivatives containing seven and four ribonucleotides, respectively. The fact that the k_3 of DR5R2 containing five ribonucleotides is midway between those of DR7R and DR4R3 is suggestive of a general decrease in catalytic activity as the number of deoxyribonucleotides is increased rather than an effect due to the lack of a specific 2′-OH. The rate of the catalytic step k_3 for the all-RNA ribozyme is 9 times greater than that for DR7R. A reduced rate factor was also observed when a predominantly deoxyribonucleotide substrate is cleaved by the all-RNA ribozyme [see Yang et al., (1990)] which could suggest that the factor is due to the presence of a heteroduplex in the enzyme/substrate complex. A specific 2′-OH or a nonspecific effect of

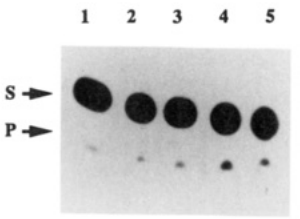


FIGURE 3: Cleavage of radioactive substrate by an OMe analogue. The letter S indicates the mobility of the intact substrate and P indicates that of the product. The incubations with MR5R were for 4 h under the standard conditions and the following modified Mg^{2+} concentrations: Lane 1, no Mg^{2+}; lane 2, 10 mM; lane 3, 20 mM; lane 4, 30 mM; lane 5, 50 mM.

increasing the deoxyribonucleotide content of the analogue cannot be entirely ruled out, however. The ratio of k_{cat}/K_M for the different catalytic fragments have been calculated; they vary from approximately $^1/_{100}$ to $^1/_{2000}$ of the ratio for the all-RNA ribozyme. For comparison, the kinetic parameters for the cleavage of the deoxyribonucleotide containing substrate analogue DSrU16.1rC17 (containing ribonucleotides only at U16.1 and C17, see Figure 1) with the all-RNA ribozyme are given.

2′-O-Methyl Analogues. At this stage, many reasons could be invoked to explain the reactivity or lack of it by the minimally ribonucleotide-substituted hammerhead analogues. For example, a structural alteration impinging on the activity could derive from the known differences in preferred nucleotide conformations: Ribonucleotides prefer the intranucleotide torsion angles of the 3′-endo conformation, whereas deoxyribonucleotides prefer the 2′-endo conformation (Saenger, 1984). Consideration of this aspect certainly highlights the unexpectedly high number of ribonucleotides which can be removed from the catalytic core without eliminating activity. However, the energy difference between the two conformations is not great (Levitt & Warshel, 1978), so that the final conformational choice could be dependent on other, perhaps tertiary, interactions.

Analogous to ribonucleotides, 2′-*O*-methylribonucleotides prefer the 3′-endo conformation, so that their use in the synthesis of minimally ribonucleotide-substituted analogues could provide some estimate of the conformational effect due to deoxyribonucleotide incorporation (Saenger, 1984). Since it had been shown that an all-OMe-analogue of the ribozyme does not support catalysis (N. Usman, unpublished), 2′-*O*-methyl analogues, containing 2, 3, and 5 ribonucleotides (Figure 1), were prepared from the corresponding phosphoramidite derivatives. Although the OMe analogue MR5R containing five ribonucleotides showed some activity, it was low and difficult to characterize fully (Figure 3). Significantly, the K_M, which can only be considered as approximate, is very close to that of the all-RNA ribozyme. Consistent with the expectation that the 3′-endo conformation is preferred, the OMe analogue forms a more stable enzyme/substrate complex than the predominantly deoxyribonucleotide analogues. With a k_{cat} of 0.001, it was impossible to determine a reliable K_{Mg}.

The surprisingly low activity of MR5R could result from either increased steric hindrance in the central core region due to the replacement of the 2′-OH group by the more bulky 2′-*O*-methyl group or the interference of the hydrophobic methyl groups with a normally hydrophilic catalytic center in the all-RNA ribozyme. In either case, deoxyribonucleotide substitution in the ribozyme is unexpectedly less detrimental to the catalytic core than the use of OMe nucleotides in spite of the potential conformational problem. Even so, the activity of a OMe derivative adds to the emerging notion that major

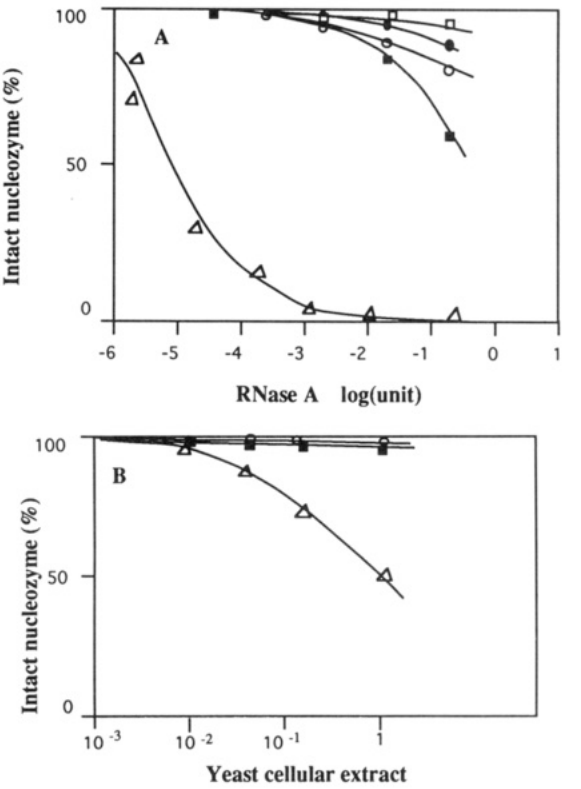


FIGURE 4: Stability of catalytic species. The 5′-labeled all-RNA (△), DR7R (□), DR5R2 (○), DR4R3 (●), and MR5R (■) were exposed to (A) RNase A and (B) a yeast cellular extract as described in Material and Methods. The degradations were analyzed by PAGE, and the percent of degradation was calculated by comparing the quantity of full-length material from the incubations to that of a control with no added RNase A or yeast extract.

parts of the catalytic core of the hammerhead ribozyme can be assembled from a variety of nucleotide building blocks [see also Pieken et al. (1991)].

Relative Stability of the Analogues. In addition to being biochemical curiosities, oligodeoxyribonucleotide analogues such as those described above may well have in vivo applications in targeting specific cellular or viral genes (Haseloff & Gerlach, 1988; Sarver et al., 1990; Pieken et al., 1991). RNAs are well-known to be ephemeral cellular entities due to their relatively high chemical and biochemical instability. DNA on the other hand is more stable; thus the in vivo half-life of these novel species could be considerably longer than that of the ribozyme. This possibility was tested (shown in Figure 4A,B) where the stability of various analogues was determined in the presence of RNase A and a cellular extract of yeast. In both cases the analogues reported here were from 10^3 (yeast extract) to 10^5 (RNase A) times more stable than the ribonucleotide catalyst. Interestingly, the rate of hydrolysis in RNase A does not seem to reflect the ribonucleotide content of the oligomers.

DISCUSSION

The experiments reported here show that mixed oligonucleotides containing as few as four ribonucleotides have catalytic activity. Furthermore, in the case of DR7R, the loss of activity when compared to that of the all-RNA ribozyme is so similar to the decrease when a predominantly deoxyribonucleotide-substituted substrate is used that it is possible that the activity loss can be attributed to the presence of the heteroduplex formed between the enzyme and the substrate in both cases. Viewed in this manner, the lower activity of DR7R could be explained solely on the basis of the hetero-

duplex effect, and deoxyribonucleotide substitution in the single-stranded core would not affect its activity. Increasing the deoxyribonucleotide substitution by three to obtain the DR4R3 analogue only decreases the k_{cat}/K_M by a factor of 15, an effect smaller than the heteroduplex effect (approximately 100). Even though the importance of the four influential ribonucleotides in these analogues must not be underestimated, their presence is not essential on an individual basis as demonstrated by the activity of the predominantly ribonucleotide analogues (Perreault et al., 1991).

This discussion underlines the surprising ability of deoxyribonucleotides to emulate the ribonucleotides in single-stranded regions, in spite of differences in preferred conformations for the two nucleotides. Observations that some tDNAs can be charged with amino acid by aminoacyl-tRNA synthetases (Khan & Roe, 1988; Perreault et al., 1989) and that the tertiary structure of tDNA greatly resembles that of tRNA (Paquette et al., 1990) were equally unexpected. On the basis of these admittedly limited studies, it would seem that both deoxy- and ribonucleotides can adopt a similar conformation (2'-endo, 3'-endo, or variants thereof) depending on the context. It may be that the energy of only one additional hydrogen bond in a structure could be sufficient to direct a nucleotide to one or the other conformation.

The implication of these results for the RNA World hypothesis is also of some interest. The question of how ribonucleotides and deoxyribonucleotides could be distinguished in a primordial world, that is, how could this primitive chemistry assemble pure RNA species, has not been adequately addressed. Our results suggest that the primordial RNA need not be pure and could in fact be "contaminated" by a large proportion of deoxyribonucleotides without the elimination of catalysis. In addition to the activity of the OMe analogues, we raise the issue whether ribozyme-type catalysis is strictly limited to RNA, that is, whether other sugar–phosphate backbones could provide an appropriate, "catalytic" conformation. Indeed, extensive deoxyribonucleotide substitution is tolerated in the substrates of both Group I and RNase P examples of RNA catalysis (Herschlag & Cech, 1990; Robertson & Joyce, 1990; Pyle & Cech, 1991; Forster & Altman, 1990). Even though the activities reported here are less than that of the all-RNA molecule, this loss could be more than offset by the accrued stability of these mixed polymers in the primordial world. Furthermore, we cannot rule out the possibility that nucleotides other than deoxy- or ribonucleotides could furnish the desired stability without having such a pronounced effect on the activity [for example, see Pieken et al. (1991)]. The ultimate question of whether DNA itself could have catalytic activity would depend on its ability to allocate other functional groups such as those on the nitrogen bases for the creation of the critical interactions which at least in the hammerhead domain are maintained by four ribonucleotides.

Finally, the stability of these deoxyribonucleotide analogues suggests possible applications in gene-targeting therapies, since even though they have reduced activities, their overall stability (more than 1000-fold) would make them attractive alternates for in vivo strategies. Furthermore, the identification of the positions where ribonucleotides are required could be very useful in the design of other effective and stable catalytic nucleic acids. In fact, the above heteroduplex hypothesis suggests that stable analogues could be made with higher activities.

Acknowledgments

We express our thanks to Professor A. Rich for his encouragement and support and to Professor W. F. Doolittle for his comments on the manuscript. This work was supported by a grant from the Medical Research Council of Canada and Fonds pour la Formation de Chercheurs et l'Aide à la Recherche of Québec. P.C. holds a predoctoral studentship from the MRC of Canada. N.U. was supported by funds from NASA and an NIH Fogarty International Research Postdoctoral Fellowship. R.C. is a fellow of the Canadian Institute for Advanced Research.

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