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Extending the cleavage rules for the hammerhead ribozyme: mutating adenosine$^{15.1}$ to inosine$^{15.1}$ changes the cleavage site specificity from N$^{16.2}$U$^{16.1}$H^{17} to N$^{16.2}$C$^{16.1}$H^{17}

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ABSTRACT

In this paper, we show that an adenosine to inosine mutation at position 15.1 changes the substrate specificity of the hammerhead ribozyme from N$^{16.2}$U$^{16.1}$H^{17} to N$^{16.2}$C$^{16.1}$H^{17} (H represents A, C or U). This result extends the hammerhead cleavage triplet definition from N$^{16.2}$U$^{16.1}$H^{17} to the more general N$^{16.2}$Y$^{16.1}$H^{17}. Comparison of cleavage rates using I$^{15.1}$ ribozymes for NCH triplets and standard A$^{15.1}$ ribozymes for NUH triplets under single turnover conditions shows similar or slightly enhanced levels of reactivity for the I$^{15.1}$-containing structures. The effect of I$^{15.1}$ substitution was also tested in nuclease-resistant 2′-*O*-alkyl substituted derivatives (oligozymes), showing a similar level of activity for the NUH and NCH cleaving structures. The availability of NCH triplets that can be targeted without loss of efficiency increases the flexibility of ribozyme targeting strategies. This was demonstrated by an efficient cleavage of an HCV transcript at a previously inaccessible GCA site in codon 2.

INTRODUCTION

The hammerhead ribozyme self-cleaving motif was identified originally in plant pathogens such as the avocado sunblotch viroid (1). Dissection of the hammerhead motif into a catalytic and a substrate part allows the specific cleavage of RNA sequences (2,3). Since then, application of trans-cleaving hammerhead ribozymes for gene inactivation by specific hydrolysis of mRNAs has become a major topic of interest (4,5). A basic characteristic of the hammerhead ribozyme is that it cleaves its substrate after N$^{16.2}$U$^{16.1}$H^{17} triplets (6). Position U$^{16.1}$ of the substrate is strictly conserved in all naturally occurring *cis*-cleaving systems (5,7,8). A systematic analysis of possible substitutions at this position with naturally occurring nucleosides also confirmed that this A$^{15.1}$·U$^{16.1}$ base pair is essential for activity (9,10). A comparison of cleavage rates of individual NUH triplets revealed that the reaction rates (k_{cat}) decrease in the following order: AUC, GUC > GUA, AUA, CUC > AUU, UUC, UUA > GUU, CUA > UUU, CUU (9,11,12).

Several lines of evidence indicate that only a few regions on the RNA are accessible for fast hybridization with ribozymes, thus severely limiting the actual availability of many of the canonical NUH cleavage sites (13–18). This NUH specificity constrains the practical use of hammerhead ribozymes for gene inactivation, in as much as optimal triplets do not always occur within accessible regions of the mRNA, and the absence of such sites necessitates targeting of non-optimal triplets (19,20). Moreover, for certain biological systems, which by their nature necessitate the use of specific cleavage sites (BCR-ABL, oncogenes formed by mutations) (21,22), a greater flexibility in cleavage triplet selection would offer a significant advantage.

This problem has led to serious efforts involving the screening of randomized pools of RNA or DNA sequences for RNA cleaving capability at non-NUH sites. These experiments resulted in selection of the naturally occurring hammerhead sequence when GUC was the target triplet, or led to significantly altered sequences when the selection was performed for cleavage at a non-NUH site (23–25). The cleavage activities of these systems are lower than that of the standard hammerhead ribozyme which has a typical k_2 value of 3 per min at 37 °C in the presence of 10 mM Mg^{2+} at pH 7.5 (26).

In this paper, we describe an efficient solution to the hammerhead ribozyme target-site extension problem, which is based on the use of a nucleoside analogue at position 15.1. We found that although single functional group changes in either of the two conserved A$^{15.1}$ or U$^{16.1}$ residues are deleterious, the change of the complete A$^{15.1}$·U$^{16.1}$ base pair to I$^{15.1}$·C$^{16.1}$ (Fig. 1) results in retention of activity. Using inosine at position 15.1 therefore allows efficient cleavage at NCH sites.

MATERIALS AND METHODS

Materials

5′-*O*-Dimethoxytrityl-2′-*O*-allylribonucleoside-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidites) and 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilylribonucleoside-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidites) bearing *tert*-butylphenoxyacetyl

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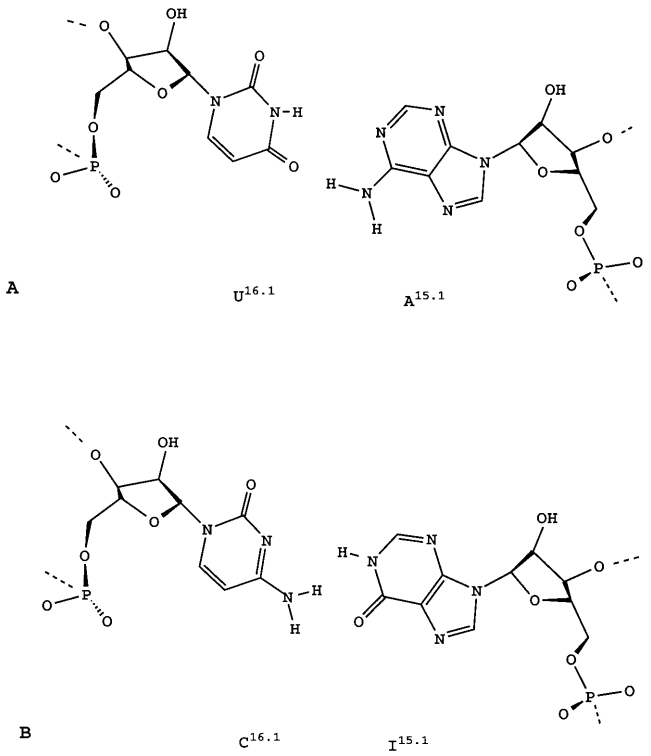


Figure 1. Relative orientation of the bases in the $U^{16.1}$·$A^{15.1}$ base pair in the 3D structure of the hammerhead ribozyme (**A**) and the isosteric $C^{16.1}$·$I^{15.1}$ base pair with an inverted arrangement of the H-bond donor and acceptor functional groups (**B**).

protection of the exocyclic amino functions of adenine, cytosine and guanine were obtained from PerSeptive Biosystems; 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilylinosine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) was obtained from CPG (Lincoln Park, NJ). 5′-Fluorescent-labelled oligoribonucleotides were obtained from OSWELL DNA service Southampton,UK. All other synthesis chemicals were from Perkin Elmer, Applied Biosystems or PerSeptive Biosystems, Hamburg. Molar extinction coefficients for oligonucleotides were calculated according to the nearest neighbour model (27).

Oligonucleotide synthesis

Ribozymes and 2′-*O*-allyl substituted analogues were synthesized on a 1 µmol scale by solid-phase β-cyanoethyl phosphoramidite chemistry (28) using the 2′-*O*-*tert*-butyldimethylsilyl (TBDMS) protection strategy for the ribonucleotides (29,30). Syntheses were performed on aminomethylpolystyrene, bearing an inverted thymidine linkage (31). Upon completion of the desired sequence, the oligomer was cleaved from the support and all base-labile protecting groups (2-cyanoethyl and 4-*tert*-butylphenoxyacetyl) were removed by treatment with aqueous ammonia–ethanol (3:1 v/v) for 2 h at 60°C in a sealed vial. After lyophilization of the filtered mixture, the residue was dissolved in 300 µl of anhydrous triethylamine–triethylamine tri(hydrofluoride)–*N*-methylpyrrolidinone (3:4:6 by volume) and kept at 60°C for 4 h to cleave the silyl protecting groups (32). The fully deprotected ribozyme was then precipitated by addition of sterile aqueous 3 M sodium acetate (30 µl, pH 5.2) followed by 1-butanol (1 ml). Purification was performed by anion-exchange chromatography on a Dionex NucleoPac column at elevated temperature and eluting with a sodium perchlorate salt gradient (32). All samples were treated with Chelex resin in order to remove traces of heavy metal ions before kinetic analysis. A different purification procedure was used for compounds containing the 5′-terminal hexanediol residue (Sproat *et al*., manuscript in preparation). Ribozymes with 5′-OH or 5′-hexanediol residues had identical kinetic properties in the single turnover assay.

The presence of inosine was verified by RNase T1 digestion [this enzyme has I and G specificity (33)] followed by mass spectroscopic analysis of the cleavage fragments. The fragments resulting from cleavage at G^{12} and $I^{15.1}$ gave the correct mass representing AAIp.

Mass spectroscopy

All modified and unmodified ribozymes used were characterized by mass spectroscopy on a Voyager DE Biospectrometry workstation (PerSeptive Biosystems). For MALDI TOF MS analysis, 0.04 A_{260} unit in 2 µl water was microdialysed using a 0.025 µm membrane. One microlitre from the resulting dialysed sample was mixed with 2,4,6-trihydroxyacetophenone:ammonium citrate matrix and samples were prepared according to the protocol provided by PerSeptive. Between 150 and 256 shots were averaged in the positive ion mode for each spectrum using an acceleration voltage of 256 kV. Spectra were calibrated with two external standards, $dT(pdT)_{25}$ and $dT(pdT)_{50}$. Mass spectra showed the correct molecular weights within ± 2 mass units.

Capillary electrophoresis

The homogeneity of all modified and unmodified oligoribonucleotides was analysed by capillary gel electrophoresis on a Biofocus 3000 Capillary Electrophoresis system (Bio-Rad). Probes were analysed under denaturating conditions using Dynamic Sieving Buffer (Bio-Rad 148–502) and 8 M urea and coated capillaries (44 cm × 15 µm). All compounds used for kinetic analysis were >98% pure under conditions where a base-line separation of 25 to 40mers was observed.

Conditions for kinetic experiments

To 75 µl H_2O, 2.5 µl of a 100 µM ribozyme solution and 2.5 µl of a 10 µM solution of 5′-fluorescein labelled substrate, 10 µl of 100 mM MES pH 6.0 or 100 mM Tris–HCl pH 7.4 were added. The solution was heated at 95°C for 2 min then allowed to cool to 37°C. The reaction was started by addition of 10 µl of 100 mM $MgCl_2$, giving a final concentration of 250 nM substrate, 2.5 µM ribozyme and 10 mM $MgCl_2$. Ten microlitre samples were withdrawn after 1, 2, 4, 6, 8, 10 and 20 min intervals and the reaction was quenched by the addition of 3 µl of 100 mM Na_2EDTA–95% formamide–0.05% bromophenol blue stop mix. Probes at 0 min were taken from reaction mixtures after the heating–cooling cycle, without addition of $MgCl_2$. Reactions with slower ribozymes were followed to completion (usually 80–85%) using appropriate reaction times. Cleavage products were separated from unreacted substrate by electrophoresis in 20% polyacrylamide–1.5% bisacrylamide–7 M urea gels (14 × 16 cm) using Tris–borate pH 8 buffer containing 2 mM Na_2EDTA, and the fluorescent bands were quantitated on a Molecular Dynamics FluoroImager using version 4.2 of the Molecular Dynamics

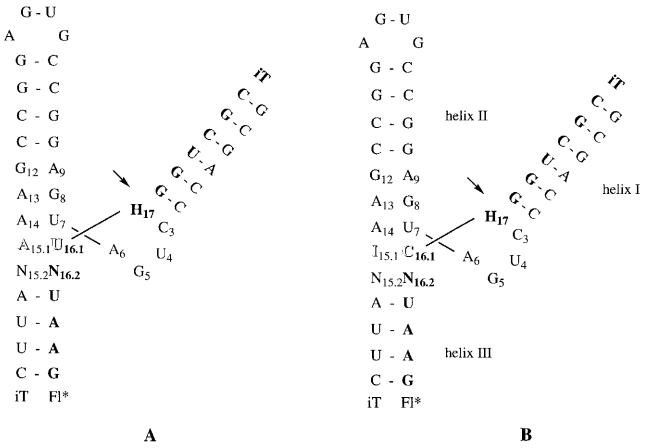


Figure 2. Sequence of the hammerhead ribozyme (**A**) and of the double mutated $I^{15.1}.C^{16.1}$ ribozyme (**B**). Substrate nucleotides are in bold, the 15.1–16.1 base pairs are outlined.

Image Quant software. Measurements were repeated at least twice. Fraction product curves were fitted to the equation $Frac[P] = H_0 \times (1 - e^{-k2t})/S_0$ (34) using KaleidaGraph (Synergy Software, Reading, PA) curve fittings routines with H_0 and k_2 as parameters. H_0 values, which can be interpreted as the ribozyme–substrate complex concentrations at t_0, were 80–85% of S_0; the error in k_2 was 10%. Alternatively, k_2 values were also determined from $\ln(S_{corr})$/time plots giving identical results. Substrate concentration changes (20–250 nM) and ribozyme excess (2–10-fold) had no effect on the cleavage kinetics, thus demonstrating that the reactions were performed under saturating conditions where the observed first-order rate constant is determined by k_2, i.e. the speed of the chemical step. Because at pH 7.4 the unmodified ribozymes have a k_2 value >2.5/min, which cannot be determined precisely from first-order decay curves, cleavage reactions with unmodified ribozymes were also performed at pH 6.

RESULTS

Chemical synthesis

The ribozyme and substrate sequences used in this study are shown in Figure 2. An inosine residue was incorporated into a sequence derived from the well characterized HH8 ribozyme (26,35). Sequence variants containing A, G, C or U at position 15.2 and A or I at position 15.1 were synthesized as nuclease-resistant 2′-*O*-allyl substituted analogues, which contain ribonucleotides at only five critical positions, viz. G^5, A^6, G^8, G^{12} and $I^{15.1}/A^{15.1}$ (36–38), and as unmodified oligoribonucleotides. The 12 NCH (Fl*GAAUNCHGGUCGC-iT) and NUH substrates (Fl*GAAUNUHGGUCGC-iT) were synthesized with a 5′-fluorescein label. The synthesis of the ribozymes was performed using standard oligonucleotide synthesis procedures using a general solid-phase support loaded with an inverted T, and oligoribonucleotides were purified by ion-exchange chromatography; an improved reverse-phase chromatography procedure was used for the purification of the modified structures (Sproat *et al.*, manuscript in preparation).

Cleavage assays

An initial screening using fluorescein-labelled substrates containing all possible NCH cleavage triplets with the 2′-*O*-allylated oligozymes demonstrated that each NCH triplet was cleavable by the complementary $I^{15.1}$-containing oligozyme, although the cleavage rates for most YCH containing substrates (Y = pyrimidine), except for CCA, were low (k_2 ~0.01 per min, ~30% conversion in 1 h). Further analysis using unmodified $A^{15.1}/I^{15.1}$ ribozymes was therefore concentrated on the faster cleaving $R^{16.2}C^{16.1}H^{17}$ and $R^{16.2}U^{16.1}H^{17}$ triplets having a purine at position 16.2 (R = A or G).

Figure 3 shows the cleavage products from a reaction between the GCA substrate and the $I^{15.1}.C^{15.2}$ ribozyme (Table 1, reaction 2). Comparison with the hydroxyl cleavage pattern and with the reaction product of a standard ribozyme reaction performed on a GUA substrate (reaction 8) shows that the $I^{15.1}$-containing ribozyme cleaves after the adenosine of the GCA triplet in the

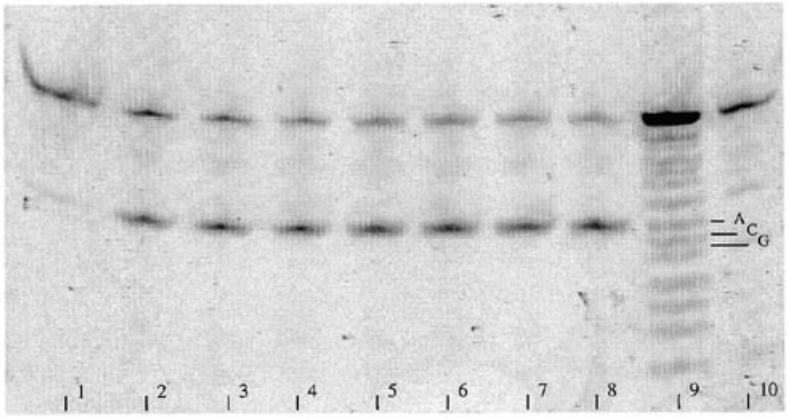
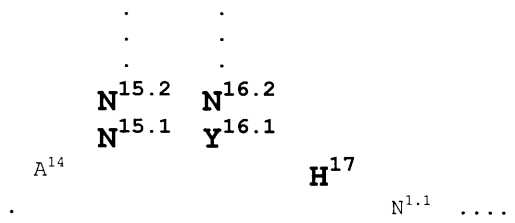


Figure 3. Gel scan of the time course of the $I^{15.1}$-ribozyme-mediated cleavage reaction of the 5′-fluorescent-labelled substrate, Fl*GAAUGCAGGUCGC-iT (Table 1, reaction 2). Bands indicate uncleaved 5′-fluorescein-labelled substrate and 5′-fluorescein-labelled product. Reaction was performed as indicated in the Materials and Methods (pH 6, 10 mM Mg^{2+}, 37°C, 250 nM substrate, 2.5 µM ribozyme). Lane 1, t = 0 min (sample was taken after the annealing and cooling step prior to the addition of Mg^{2+}); lanes 2–8, t = 1, 2, 4, 6, 8, 10 and 20 min reaction times; lane 9, sequence ladder generated from 5′-fluorescein-labelled substrate by treatment with 50 mM $NaHCO_3$ for 2 min at 90°C; lane 10, substrate incubated in the reaction buffer for 20 min with 10 mM Mg^{2+} without ribozyme.

Table 1. Single turnover rate constants for the cleavage of NCH and NUH substrates



	Ribozyme		Substrate			A ribozymes	B 2′-O-allyl substituted analogues
Reaction Nr	$N^{15.1}$	$N^{15.2}$	$N^{16.2}$	$Y^{16.1}$	H^{17}	k_2 (pH 6.0)	k_2 (pH 7.4)
1	**I**	C	G	**C**	C	0.19	0.11
2	**I**	C	G	**C**	A	0.39	0.40
3	**I**	C	G	**C**	U	0.03	0.02
4	**I**	U	A	**C**	C	0.17	0.07
5	**I**	U	A	**C**	A	0.41	0.30
6	**I**	U	A	**C**	U	0.03	0.014
7	**A**	C	G	**U**	C	0.15	0.10
8	**A**	C	G	**U**	A	0.12	0.31
9	**A**	C	G	**U**	U	0.04	0.06
10	**A**	U	A	**U**	C	0.14	0.05
11	**A**	U	A	**U**	A	0.11	0.17
12	**A**	U	A	**U**	U	0.05	0.04
Control reactions						k_2 (pH 7.4)	
13	**I**	C	G	**C**	A	> 2.5	
14	**I**	C	G	**U**	C	< 0.008	
15	**I**	C	G	**U**	A	< 0.003	
16	**I**	C	G	**U**	U	< 0.001	
17	**A**	C	G	**C**	C	< 0.008	
18	**A**	C	G	**C**	A	< 0.002	
19	**A**	C	G	**C**	U	< 0.001	
20	**I**	C	G	**A**	A	< 0.001	
21	**I**	C	G	**G**	A	< 0.001	
22	**I**	C	G	**C**	G	< 0.001	
23	**G**	C	G	**C**	A	< 0.001	

Upper part, partial sequence of the Hammerhead ribozyme indicating the five positions of substitutions. Position of cleavage in the substrate is between H^{17} and $N^{1.1}$.

A, with unmodified ribozymes at pH 6.0 in MES buffer at 37°C in the presence of 10 mM Mg^{2+} or, in the case of control reactions, pH 7.4 Tris buffer at 37°C in the presence of 10 mM Mg^{2+} (upper limits for cleavage rates estimated from reactions followed until 120 min).

B, with nuclease resistant 2′-*O*-allyl modified oligozymes containing five residual ribonucleotides at pH 7.4 in Tris buffer at 37°C in the presence of 10 mM Mg^{2+}.

Fl*GAAUGCAGGUCGC-iT substrate. All other sequences shown in Figure 2, which were investigated in this work, also gave identical 7mer products.

A comparison of the three $G^{16.2}C^{16.1}H^{17}$ and three $G^{16.2}U^{16.1}H^{17}$ cleavage triplets using $I^{15.1}$- and $A^{15.1}$-containing unmodified ribozymes at pH 6.0 in MES buffer is shown in Figure 4 (Table 1, reactions 1–3 and 7–9). Single turnover rate constants for all $R^{16.2}C^{16.1}H^{17}$ and $R^{16.2}U^{16.1}H^{17}$ triplets investigated using the nuclease-resistant and unmodified ribozymes are summarized in Table 1. This comparison revealed an $N^{16.2}C^{16.1}A^{17} > N^{16.2}C^{16.1}C^{17} > N^{16.2}C^{16.1}U^{17}$ order of reactivity at pH 6, with similar values for the $G^{16.2}$ and $A^{16.2}$ cases. The measured values for the GUC substrate and our k_2 value at pH 6 are very similar to the value reported by Taira under identical conditions (39), and similar to a recently published single turnover value of k_2 for the HH8 sequence when corrected to the

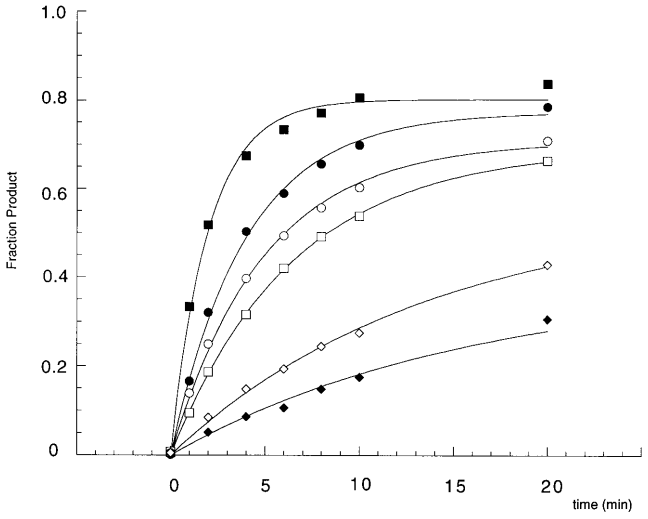


Figure 4. Comparison of the GCH- and GUH-cleaving ribozymes. Cleavage reactions were carried out at 37°C in 10 mM MES buffer at pH 6 under single turnover conditions. Reaction mixtures (100 µl) contained 250 nM 5′-fluorescein-labelled substrate and 2.5 µM ribozyme. Ribozyme and substrate solutions were mixed and heated to 95°C for 2 min. After cooling to 37°C over 10 min, the reaction was started by the addition of $MgCl_2$ to a final concentration of 10 mM. Ten microlitre samples were withdrawn after 1, 2, 4, 6, 8, 10 and 20 min. The t = 0 probe was taken after the heating–cooling cycle before addition of $MgCl_2$. Fraction product curves were fitted to the equation $Frac[P] = H_0 \times (1 - e^{-k2t})/S_0$ as described in the Materials and Methods, giving k_2 values listed in Table 1. Symbols: closed square, Fl*GAAUGCAGGUCGC-iT; closed circle, Fl*GAAUGCCGGUCGC-iT; closed diamond, Fl*GAAUGCU-GGUCGC-iT; with the $I^{15.1}$ substituted ribozyme; open square, Fl*GAAU-GUAGGUCGC-iT; open circle, Fl*GAAUGUCGGUCGC-iT; open diamond, Fl*GAAUGUUGGUCGC-iT with the standard $A^{15.1}$-containing ribozyme.

same conditions, viz. 3.2 per min at 37°C and 10 mM Mg^{2+} (40,41).

The influence of the $I^{15.1}{\cdot}C^{16.1}$ base pair on cleavage rates is context dependent. The data in Table 1 show that when H^{17} = A, the $I^{15.1}{\cdot}C^{16.1}$ base pair destabilizes the transition state less than the standard $A^{15.1}{\cdot}U^{16.1}$ base pair; when H^{17} = C, then the $I^{15.1}{\cdot}C^{16.1}$ and $A^{15.1}{\cdot}U^{16.1}$ cases are similar; however when H^{17} = U, $A^{15.1}{\cdot}U^{16.1}$ is preferred. A similar order of relative activities is visible in the series of 2′-*O*-modified compounds (Fig. 1B).

A comparison between the RCH/RUH triplets was also performed under biologically more relevant conditions (1 mM Mg^{2+}, pH 7.4). The order of reactivity remains unchanged, giving k_2 = 2.3/min for GCA, k_2 = 2.0/min for GCC, k_2 = 1.0/min for GUC and k_2 = 0.77/min for GUA.

Specificity of cleavage

In order to check the specificity of cleavage the activity of the $I^{15.1}$, all ribozyme was tested with the three corresponding GUH-containing substrates (Table 1, reactions 14–16). This experiment shows that the structure containing the $I^{15.1}{\cdot}U^{16.1}$ base pair catalyses cleavage of GUH substrates much less efficiently. The GUH substrates were cleaved in the order GUC > GUA > GUU, with a cleavage rate more than two orders of magnitude lower than observed in the GCH series. The cleavage rates for the slow ribozymes were estimated from the fraction product/time curves followed until 10–15% conversion; they have to be interpreted, therefore, as upper limits. We determined in control experiments that under our conditions the standard hammerhead also has a limited acceptance for the GCH-type substrates, with a preference for GCC (Table 1, reactions 17–19). Substrates containing a $G^{16.2}A^{16.1}A^{17}$ or $G^{16.2}G^{16.1}A^{17}$ triplet were completely unreactive with the $I^{15.1}$ constructs (Table 1, reactions 20 and 21); the observed formation of cleavage products after 120 min incubation at 37°C, pH 7.4, was 6 and 3%, respectively.

In order to check the validity of the hammerhead cleavage rules, which require H^{17} not to be guanosine (6), a reaction between the $I^{15.1}$-containing ribozyme and the corresponding $G^{16.2}C^{16.1}G^{17}$-containing substrate was also investigated (Table 1, reaction 22). Similar to the standard hammerhead, no cleavage (<3% conversion after 2 h) could be observed.

In another control reaction, the activity of a $G^{15.1}{\cdot}C^{16.1}$ hammerhead was examined, i.e. a case where a regular base pair can form (Table 1, reaction 23), and compared with the $I^{15.1}{\cdot}C^{16.1}$-containing construction. Less than 3% product formation was observed with the $G^{15.1}$-containing ribozyme, which allows an upper limit value for the rate constant of <0.001/min to be calculated. When compared with the >2.5/min value observed with the $I^{15.1}$ substituted case, this experiment strongly reinforces the importance of the single point contact between the nucleobases at positions 15.1 and 16.1 of the hammerhead ribozyme.

Influence of the N^7 position

According to the 3D structural model, cleavage at an NCH triplet instead of an NUH may also lead to a change in the $N^{16.1}/N^7$ stacking interaction (42). In order to examine the role of the N^7 nucleoside in the $C^{16.1}/N^7$ interaction in our structures, the influence of the other three natural bases was investigated. The nuclease stable 2′-*O*-allyl modified analogues were used for this experiment. The relative activities of these compounds were as follows: U^7 = 1, A^7 = 1, C^7 = 0.7 and G^7 = 0.5, similar to the order observed with the standard hammerhead.

Demonstration of cleavage with a long substrate at a GCA site

The cleavage activity at a GCA site was tested using a 1358mer transcript corresponding to the first 1358 bases of the human hepatitis C virus (DDBJ/EMBL/GenBank accession no. S62220). The sequence around the polyprotein AUG site (accgtgcacc atga**gca**cga atcc taaacc, region 330–350), which is known to be accessible from systematic binding studies (43), contains two GCA sites. An $I^{15.1}$-containing nuclease-resistant oligozyme targeting the gca site within codon 2 was synthesized (the region complementary to the recognition sequence is underlined). The k_2 observed with the corresponding short substrate was 0.4/min for this compound, similar to the model sequence in Table 1. Using 300 nM oligozyme and 30 nM substrate (pH 7.4, 10 mM Mg^{2+}), the long substrate gave ~60% product formation in 60 min. The two cleavage products were in the range of 350 and 1000 nucleotides, as determined by the use of length standards. The size of the shorter cleavage product was more accurately estimated by comparison with the cleavage products of a set of standard NUH-cleaving oligozymes cleaving the same transcript at the 322 guc, 351 auc, 354 cua, 360 cuc and 378 gua sites. In this experiment, the shorter product of the 345 gca-cleaving ribozyme was located between the shorter products created by the

322 guc and 351 auc ribozymes, thus showing that the cleavage products were approximately of the expected size.

DISCUSSION

According to the X-ray crystal structure (44–46) of the hammerhead ribozyme, the strictly conserved $A^{15.1}$·$U^{16.1}$ base pair (Fig. 1A), which determines the $N^{16.2}$ $U^{16.1}$ H^{17}-type cleavage specificity is part of the $A^{15.1}$·$U^{16.1}$, A^{14}·U^{7} branching element, where the $A^{15.1}$·$U^{16.1}$ base pair broadens, and the $A^{14}U^{7}$ base pair narrows the minor groove at the branching position of the stacked helices II and III and of helix I. The $A^{15.1}$·$U^{16.1}$ base pair is characterized by a single hydrogen-bond interaction between the 4-keto group of $U^{16.1}$ and the 6-amino group of $A^{15.1}$ (Fig. 1A); additionally, the 2-keto group of $U^{16.1}$ is involved in a set of interactions with the ribose of nucleotide A^{6} of the U-turn (45).

In the structure of this A·U base pair, the distance between the N^{1} atom of $A^{15.1}$ and the N^{3} atom of $U^{16.1}$ is 4 Å, which is significantly greater than the typical value of 2.8 Å for a regular Watson–Crick A·U base pair (Fig. 1A). Recent NOE distance measurements performed on a hammerhead ribozyme also confirm that the distance between the 16.1 pyrimidine N-H3 and 15.1 purine C-H2 atoms (3.95 Å) is greater than the 2.7 Å value observed in regular helices (47).

An overview of functional group modification studies involving the $A^{15.1}$·$U^{16.1}$ base pair shows that modifications which disrupt this structure have a large negative effect on catalytic activity. Substitution of $U^{16.1}$ by 2-pyrimidinone-1-β-D-riboside, i.e. deleting the 4-keto group and replacing NH by N, leads to complete inhibition of cleavage, and substitution of $U^{16.1}$ by 4-thiouridine leads to a 3-fold reduction of k_{cat} (48). Replacing $A^{15.1}$ by purine riboside, i.e. deletion of the exocyclic amino group leads to a 50-fold reduction (49,50). Changing A·U to isoG·U introduces an NH–NH repulsive interaction but retains the original 6-amino group at $N^{15.1}$ with the result that only a 2-fold reduction in activity is observed (51).

Our starting hypothesis for this work was that for any structural change at the $U^{16.1}$ position, appropriate compensating changes would have to be made in spatially neighbouring regions of the hammerhead ribozyme. When considering a $U^{16.1}$→$C^{16.1}$ mutation, which would have the important practical consequence that an $N^{16.2}C^{16.1}H^{17}$ triplet will be cleaved *in trans*, such a compensating change may be an $A^{15.1}$→$I^{15.1}$ substitution as the comparison of Figure 1A and B suggests. This change conserves the geometry of the $A^{15.1}$·$U^{16.1}$ single point contact and only reverses the polarity of the hydrogen bonds between the functional groups of nucleoside 15.1 and nucleoside 16.1. The interactions of the 2-keto group of the pyrimidine at position 16.1 with A^{6} may remain essentially unchanged and no new functional groups are introduced. Whereas the I·C base pair satisfies the most basic criteria for an A·U replacement, this substitution also changes the stacking interaction of the original $A^{15.1}$·$U^{16.1}$ base pair with A^{14}·U^{7} to $I^{15.1}$·$C^{16.1}$/A^{14}·U^{7}.

In order to test the acceptance of an $I^{15.1}$·$C^{16.1}$ pair in the hammerhead core, we incorporated inosine into a well characterized hammerhead sequence and tested its cleavage activity with complementary substrate sequences containing various NCH-type triplets. The results presented in Figure 4 and Table 1 confirm that the $A^{15.1}$·$U^{16.1}$ base pair of the hammerhead ribozyme can be replaced with an $I^{15.1}$·$C^{16.1}$ base pair without loss of catalytic activity. In some triplet contexts, even a slight increase in catalytic activity of the novel $I^{15.1}$·$C^{16.1}$ ribozyme relative to the $A^{15.1}$·$U^{16.1}$ molecule could be observed. The order of activity was changed from a preference for C^{17}, as observed with the standard hammerhead (5), to a preference for A^{17}. Comparison of the $k_{2\ (GCH)} : k_{2\ (GUH)}$ ratios gives 1.2 for the GCC:GUC, 3.2 for the GCA:GUA and 0.7 for the GCU:GUU triplet pairs. Thus, the increment of the I·C base pair on the cleavage rate depends on the triplet sequence. This is in agreement with a recent thermodynamic analysis of the hammerhead cleavage pathway, which concluded that the cleavage mechanism may be slightly different depending on the H^{17} nucleotide (41).

The acceptance of an $I^{15.1}$·$C^{16.1}$ base pair means that a new series of triplets will be cleavable in hammerhead ribozyme applications, since the cleavage specificity is determined by the 16.1 position. This increases the chance for finding an efficiently cleavable triplet in an accessible region. The chemical modification of the hammerhead structure requires the use of inosine, which is a non-toxic, readily available nucleoside analogue. With this potential application in mind, we wanted to prove the specificity of cleavage and investigated the cleavage rates of the $I^{15.1}$-containing ribozymes with other triplets. Similar experiments were previously performed with the $A^{15.1}$ ribozymes with the conclusion that only the $A^{15.1}$·$U^{16.1}$ combination gives efficient cleavage.

Our data in Table 1 demonstrate that substrate recognition is also specific with the $I^{15.1}$-containing compounds, the discrimination between other triplets is similar to that observed with standard hammerhead ribozymes. The cleavage rates with GUH triplets are at least 2 orders of magnitude lower than those with GCH triplets (Table 1, reactions 13 and 14–16). The level of acceptance of an $I^{15.1}$·$U^{16.1}$ pair is similar to an $A^{15.1}$·$C^{16.1}$ pair as shown in reactions 17–19 or to an $A^{15.1}$·$A^{16.1}$ pair (9,10). The cleavage rates of stuctures requiring an I·A or I·G base pair are extremely low.

An interesting structural aspect of these studies is the great loss of activity of a $G^{15.1}$-containing ribozyme when compared with the $I^{15.1}$-containing structure (Table 1, reactions 13 and 23). The single point contact in the catalytic core between the positions 15.1 and 16.1 thus appears to be an essential feature of the hammerhead structure. The reason for the inactivity of the $G^{15.1}$ structure may be at least 2-fold, (i) the 2-keto group at the 16.1 pyrimidine is no longer available for the required interaction with A^{6} and (ii) replacement of the I·C pair with a G·C pair significantly distorts the position of the sugar residue at the 16.1 position. Superimposition of the purine from the A·U (or I·C) base pair from the X-ray crystal structure (Fig. 1A) with that from a normal Watson–Crick G·C base pair indicates that the position of the ribose 2′-OH and the 2-keto group of the 16.1 pyrimidine, which are essential for transition state formation (46), are different by at least 5 Å in these two structures.

Using $I^{15.1}$ ribozymes for the cleavage of NCH substrates places the I·C pair base in the context of the $I^{15.1}$·$C^{16.1}$, A^{14}·N^{7} branching element. In order to evaluate the influence of the nucleotide identity at position 7, which is generally U, the other three natural bases (A, C and G) were tested. The nuclease stable 2′-*O*-allyl modified compounds were used for this comparison, where other structural factors may also influence the relative activity. This comparison shows an U ≈ A > C > G order of reactivity similar to the standard hammerhead (6,42).

The feasibility of cleaving longer target sequences at NCH-type positions with nuclease stable analogues was also investigated. We selected the HCV transcript because detailed accessibility analysis is available for this target (43). The cleavage rate of the

fluorescein-labelled short 15mer substrate corresponding to the target sequence was similar to that of the model compound. The cleavage of the radioactively labelled long substrate was performed without pre-annealing the reaction partners, using the 2′-*O*-allyl substituted oligozyme. The efficient cleavage of this target suggests that the NCH-cleaving ribozymes will be generally useful for the selective cleavage of long RNA sequences.

CONCLUSION

In summary, we have found that the cleavage rules characteristic of hammerhead ribozymes can be expanded from NUH to the more general $N^{16.2}Y^{16.1}H^{17}$, provided that a suitable nucleoside analogue, e.g. inosine, is used at position 15.1 to maintain the essential single point contact with the pyrimidine residue $Y^{16.1}$. The availability of NCH triplets, which can be targeted without loss of activity, makes a new set of previously inaccessible NCH target sites available for hammerhead ribozyme targeting.

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