ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT 10:53–61 (2000)
Mary Ann Liebert, Inc.

Nucleotides −1 to −4 of Hepatitis Delta Ribozyme Substrate Increase the Specificity of Ribozyme Cleavage

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ABSTRACT

In the past, the use of delta ribozyme as a therapeutic tool was limited because substrate specificity was thought to be determined by only 8 nucleotides. Recently, we have accumulated evidence suggesting that the substrate sequence upstream of the cleavage site, which is not involved in the binding with the delta ribozyme, appears to be essential in the selection of an appropriate cleavage site. To understand the role of this region in efficient cleavage, we synthesized a collection of small substrates that possessed single and multiple mutations in positions −1 to −4 and determined the kinetic parameters of their cleavage using a model antigenomic delta ribozyme. Some substrates were found to be uncleavage, whereas others showed >60-fold difference in relative specificity between the least and most efficiently cleaved substrates. The base at each position from −1 to −4 contributes differently to the ability of a substrate to be cleaved. An optimal sequence for positions −1 to −4 was determined to be $_{-1}$HRHY$_{-4}$ (H = U, C, or A). These results shed light on new features that contribute to the substrate requirement of delta ribozyme cleavage and should increase interest in the use of this unique ribozyme.

INTRODUCTION

BOTH GENOMIC AND ANTIGENOMIC HEPATITIS DELTA VIRUS (HDV) RNA have self-cleavage activity in the presence of divalent metal ions that produces a 2′,3′-cyclic phosphate and 5′-hydroxyl-termini (for reviews see, respectively, Been and Wickham, 1997; Mercure et al., 1997). A pseudoknot model secondary structure has been proposed for the self-cleaving delta sequence (Perrotta and Been, 1991). This structure, which is well supported by experimental data, consists of two stems (P1 and P2), two stem-loops (P3 and P4), and three single-stranded regions (J1/2, J1/4, and J4/2) (Fig. 1) (Been and Wickham, 1997). An additional pseudoknot, named P1.1, that is formed by 2 GC base pairs (bp) between nucleotides from the J1/4 junction and the P3 loop, was recently demonstrated (Ferré-D'Amaré et al., 1998; Wadkins et al., 1999). *Trans*-acting ribozymes have been developed by removing the J1/2 junction, thereby producing one molecule possessing the substrate sequence and the other possessing the catalytic site (Been and Wickham, 1997).

Like other catalytic RNA, delta ribozymes have a potential application in inhibiting gene expression by cleaving specific mRNA molecules. In fact, delta ribozyme offers several unique properties. (1) It uniquely possesses the natural ability to function in the presence of human proteins. In hepatocytes, HDV naturally replicates via a mechanism involving self-cleavage activity of both the genomic and antigenomic strands (Been and Wickham, 1997; Mercure et al., 1997). In fact, it is the only catalytic RNA motif that has been discovered in human. (2) It functions at low magnesium concentrations (~1 mM). (3) It is unique in that it is fully active in the presence of calcium (Wu et al., 1989). Previous work aimed at using delta ribozyme as a therapeutic tool was limited, however, because its substrate specificity was believed to be determined by only 8 nucleotides (nt), specifically those of the P1 stem and a single-stranded pyrimidine 5′ to the cleavage site (Fig. 1). Several investigations have been performed to determined the substrate specificity of both *cis*-acting and *trans*-acting delta ribozymes (Perrotta and Been, 1991, 1992; Been et al., 1992; Wu and Huang, 1992; Wu et al., 1992, 1993; Kumar et al., 1993; Kawakami et al., 1996; Nishikawa et al., 1997, 1999). Cleavage activity is not destroyed by the interchanging of 1–4 bp base pairs of the P1 stem, even though substrate recognition of delta ribozyme is primarily based on the formation of the P1 stem, which includes 1 G-U wobble base pair and 6 Watson-Crick base pairs. Furthermore, it was shown that the positions located at both extremities of the helix formed by the substrate and the ribozyme were more likely to tolerate a mismatch than are the internal po-

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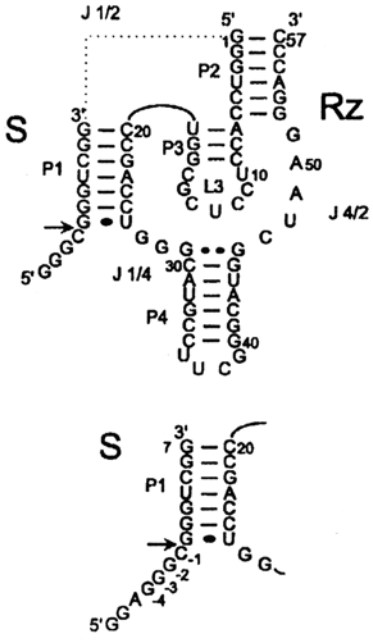

FIG. 1. Secondary structure and nucleotide sequence of an antigenomic *trans*-acting delta ribozyme. The base-paired regions are numbered according to the model pseudoknot structure (Been and Wickham, 1997). S and Rz represent substrate and ribozyme, respectively. The dotted line represents the J1/2 single-stranded region joining the substrate and ribozyme molecules present in the *cis*-form. The arrow indicates the cleavage site. The homopurine base pair at the top of the P4 stem is represented by two dots (G· ·G), and the wobble base pair is represented by a single dot (G· U). At the bottom, the sequence of the 14-nt substrate used in this work is shown. Only the P1 region and the two single-stranded guanosines (G) of the J1/4 junction of the ribozyme are illustrated. Note that the positions of the substrate discussed in this report are identified (i.e., −1 to −4).

sitions. In addition, the minimal sequence upstream of the substrate cleavage site has been determined to be 1 nt (Been and Wickham, 1997; Perrotta and Been, 1991).

We recently reported the first demonstration of the potential of delta ribozyme for inhibiting gene expression by using an antigenomic version to cleave specifically the HDV antigen mRNA in *trans* (Roy et al., 1999). In the course of this research, we noted that the proposed substrate specificity was not accurate. Therefore, a better understanding of the sequence specificity of delta ribozyme is critical for the engineering of ribozymes efficient for this purpose. Initially, we undertook a systematic analysis of how each base pair in the P1 stem influences the catalytic activity of the delta ribozyme (Ananvoranich and Perreault, 1998; Ananvoranich et al., 1999). We observed that the base pairing between substrate and ribozyme is important for cleavage activity and that the identity of the substrate's nucleotide sequence is also important. In fact, these results show the nucleotide located in the center of the P1 stem (Fig. 1, position +4 of the substrate) to be important not only for substrate recognition but also for subsequent steps of the cleavage pathway (Ananvoranich and Perreault, 1998; Ananvoranich et al., 1999).

During the development of delta ribozymes cleaving mRNA encoding the HDV antigen (Roy et al., 1999), we gathered evidence suggesting that the region 5′ of the cleavage site (i.e., that extending beyond position −1) also contributed to substrate specificity. Preliminary experiments allow us to propose that positions −4 to −1 exhibit nucleotide preferences and contribute significantly to efficient cleavage by delta ribozyme. Specifically, substrates with a higher guanosine content upstream of the cleavage site (i.e., positions −4 to −2) seem to cleave more efficiently than does wild-type substrate (Roy et al., 1999). As a consequence of these initial results, we undertook a systematic investigation of the effects of nucleotide changes at positions −4 to −1 on delta ribozyme cleavage. A collection of substrates, including single and multiple mutations, was synthesized, and the efficiency of cleavage was determined. The results presented here shed light on several new features that contribute to the substrate specificity of delta ribozyme cleavage. General rules of substrate specificity are suggested with the goal of facilitating the design of efficient delta ribozymes for use as therapeutic tools.

MATERIALS AND METHODS

Materials

[γ^{32}P]ATP was purchased from Amersham (Arlington Heights, IL), yeast pyrophosphatase from Boehringer Mannheim (Mannheim, Germany), DNase I (RNase free) from Promega (Madison, WI), and T4 polynucleotide kinase, calf intestine alkaline phosphatase, RNA Guard® and G-50 Sephadex gel matrix from Amersham Pharmacia Biotech (Paris, France). T7 RNA polymerase was purified as described (Zawadzki and Gross, 1991), using the expression system of Studier (Davanoo et al., 1984).

In vitro *synthesis of substrates and ribozymes*

Substrates and ribozymes were synthesized by *in vitro* transcription as described previously (Ananvoranich and Perreault, 1998; Ananvoranich et al., 1999). Substrate molecules were prepared by annealing two pairs of complementary and overlapping oligonucleotides in a 20-μl mixture containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 50 mM KCl by incubating at 85°C for 2 minutes and then allowing the mixture to slowly cool to 37°C. Using the resulting partial duplex as template (500 pmol), *in vitro* transcription reactions were carried out in a final volume of 50 μl containing 27 U of RNAGuard, 4 mM of each rNTP (i.e., ATP, GTP, CTP, and UTP), 80 mM HEPES-KOH, pH 7.5, 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 0.01 U yeast pyrophosphatase, and 25 μg purified T7 RNA polymerase at 37°C for 4 hours. The samples were then DNase treated, and the transcripts were purified on 20% polyacrylamide gels (PAGE) using 50 mM Tris-borate, pH 8.3/1 mM EDTA/7 M urea solution as running buffer. The reaction products were visualized by ultraviolet shadowing over a fluorescent thin-layer chromatography plate, and the bands corresponding to the correct sizes were cut out. Transcripts were eluted from these gel slices by incubating overnight at 4°C in 0.1% SDS/0.5 M ammonium acetate solution. They were then

precipitated by adding 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.2 vol of ethanol and finally washed with 70% ethanol and dried. Purified transcripts were quantified by spectrophotometry at 260 nM. The *trans*-acting ribozymes (RzP1.1/P4.1) (Fig. 1) were constructed as described previously (Ananvoranich and Perreault, 1998; Ananvoranich et al., 1999; Mercure et al., 1998) and were synthesized as described for substrates.

Chemical synthesis of RNA substrates

RNA substrates, including a hairpin upstream of the cleavage site and a small complementary RNA oligonucleotide (i.e., Comp-6), were synthesized on an automated oligonucleotide synthesizer (Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT) and deprotected according to previously described procedures (Perreault and Altman, 1992). The polymers were purified by 20% PAGE. Major bands were excised and eluted as described.

5′-End-labeling of RNA

Purified substrates (10 pmol) were dephosphorylated in a 20-μl reaction mixture containing 200 mM Tris-HCl, pH 8.0, 10 U of RNAGuard, and 0.2 U of calf intestinal alkaline phosphatase at 37°C for 30 minutes and were then purified by extracting twice with an equal volume of phenol/chloroform (1:1) and ethanol precipitating. Dephosphorylated transcripts (1 pmol) were end-labeled in a mixture containing 1.6 pmol [γ^{32}P]ATP, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, and 3 U of T4 polynucleotide kinase at 37°C for 30 minutes. Excess [γ^{32}P]ATP was removed by passage through a G-50 Sephadex spin column. The concentration of labeled transcripts was adjusted to 0.01 pmol/ml by the addition of water.

Cleavage reactions and kinetic analyses

Unless otherwise stated, cleavage reactions were carried out in 20-μl mixtures containing 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ at 37°C. Prior to the reaction, trace amounts of [^{32}P]5′-end-labeled substrate and nonradioactive ribozyme (200 nM) were mixed together and denatured at 95°C for 2 minutes, chilled on ice for 2 minutes, and finally equilibrated at 37°C for 5 minutes. The reactions were then initiated by addition of the buffer. Aliquots (2–3 μl) were removed at various times and quenched by the addition of 8 μl ice-cold formamide/dye mixture (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Substrate and products were separated on a 20% PAGE gels and analyzed with a Storm Imager (Molecular Dynamics, Sunnyvale, CA). The extent of cleavage was determined from measurements of radioactivity in the substrate and the 5′ product bands. At least two independent experiments were performed for each measurement.

Kinetic analyses were performed under presteady-state conditions as described previously (Ananvoranich and Perreault, 1998; Ananvoranich et al., 1999; Mercure et al., 1998). Various concentrations of ribozyme, ranging from 5 nM to 600 nM, were mixed with trace amounts of substrate (final concentration <0.1 nM) in a final volume of 18 μl, and the reactions were performed and analyzed as described. The fractions of substrate cleaved were determined, and the rate of cleavage (k_{obs}) was obtained from fitting the data to the equation $A_t = A_\alpha(1 - e^{-kt})$ where A_t is the percentage of cleavage at time t, A_α is the maximum cleavage (or the end point of cleavage), and k is the reaction rate (k_{obs}). Each rate constant was calculated from at least two measurements. The values of k_{obs} obtained were then plotted as a function of concentrations of ribozyme for determination of the kinetic parameters: apparent catalytic rate constant (k_2) and apparent Michaelis-Menten constant (K_m') values.

RESULTS

We have derived a 57-nt delta ribozyme from the antigenomic HDV genome (Fig. 1) in which the P2 stem has been elongated and the P4 stem-loop has been substituted by a shorter, ultrastable hairpin derived from the UNCG family (Varani, 1995). The thermodynamic behavior of this ribozyme has been extensively characterized under both presteady-state and steady-state conditions using an 11-nt model substrate that produces products of 4 and 7 nt (Ananvoranich and Perreault, 1998). We designed a novel substrate 14 nt long that produced two distinct 7-nt products (Fig. 2) so as to systematically study the effect of the sequence immediately upstream of the cleavage site on the ribozyme's cleavage activity. This 5′-extended substrate allowed substitution of the nucleotides at positions −4 to −1 without modifying the *in vitro* transcription efficiency, as the 5′-end sequence remained intact (i.e., 5′-GGA).

Cleavage assays with substrates containing single mutations

A collection of 13 substrates possessing single mutations in positions −4 to −1, as compared with the original $_{-4}$GGGC$_{-1}$, were synthesized, and cleavage reactions were performed under pre-steady-state conditions. For each mutant, trace amounts of [^{32}P]5′-end-labeled substrate (<1 nM) were incubated in the presence of an excess of ribozyme (200 nM), and the maximal

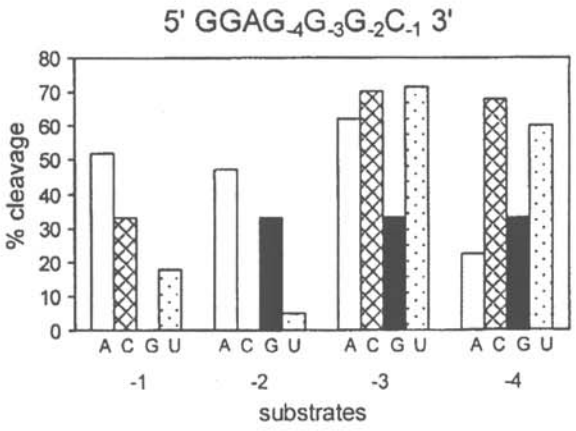

FIG. 2. Histogram of the maximum cleavage percentages (end point) of a collection of singly mutated substrates. For each substrate, trace amounts of [^{32}P]5′-end-labeled substrates (<1 nM) were incubated in the presence of an excess of ribozyme (200 nM), and the maximal cleavage percentages (end point) were determined as a comparative parameter. The sequence of the original substrate is illustrated above the histogram.

TABLE 1. KINETIC ANALYSIS OF SUBSTRATES POSSESSING SINGLE MUTATIONS[a]

Position	Identity	k_M' (nM)	k_2 (min^{-1})	k_2/k_M' ($nM^{-1}min^{-1}$)	S.I.
-1	C	31.5±1.7	0.22±0.03	$6.98x10^{-3}$	1.00
	U	33.2±3.1	0.11±0.02	$3.31x10^{-3}$	0.47
	A	14.3±1.2	0.27±0.04	$1.89x10^{-2}$	2.70
	G*				
-2	G	31.5±1.9	0.22±0.02	$6.98x10^{-3}$	1.00
	A	28.7±3.6	0.33±0.05	$1.15x10^{-2}$	1.65
	C*				
	U	94.3±5.2	0.08±0.02	$8.48x10^{-4}$	0.12
-3	G	31.5±4.4	0.22±0.03	$6.98x10^{-3}$	1.00
	A	9.9±1.0	0.20±0.02	$2.02x10^{-2}$	2.89
	C	11.3±2.4	0.24±0.03	$2.12x10^{-2}$	3.04
	U	8.8±1.5	0.20±0.01	$2.27x10^{-2}$	3.25
-4	G	31.5±3.7	0.22±0.04	$6.98x10^{-3}$	1.00
	A	27.1±2.8	0.12±0.03	$4.43x10^{-3}$	0.63
	C	11.8±0.4	0.27±0.01	$2.29x10^{-2}$	3.28
	U	16.4±1.3	0.23±0.03	$1.40x10^{-2}$	2.01

[a]Pseudo-first-order cleavage rate constants (k_2 and K_m') were measured using an excess of ribozyme (5–600 nM) and trace amounts of end-labeled substrate (>0.1 nM). Apparent second-order rate constants (k_2/K_m') were calculated, and their relative specificities as compared with the original substrate were determined. The values were calculated from at least two independent experiments. The sequences for positions −4 and −1 are indicated for each substrate.

*Substrate for which the maximum percentage of cleavage was too small, and, therefore, further determination of the kinetic parameters was performed.

cleavage percentage (i.e. end point) was determined (Fig. 2). We found that the base requirement varies for each position. At position −1, the base preference was A > C > U >> G, where the presence of a guanosine produced an uncleavable substrate. At position −2, the presence of an A improved the cleavage efficiency as compared with the original G, and substrate with a U was poorly cleaved and that with a C was uncleavable. At position −3, C, U, and A gave substrates that possessed a 2-fold improvement in cleavage activity as compared with the wild-type G. Finally, at position −4, the presence of a pyrimidine (i.e., C or U) improved the maximum cleavage at least 2-fold as compared with when a purine (i.e., G or A) was present.

To accurately assess the base requirement at each position, kinetic analyses were performed under presteady-state conditions. Pseudo-first-order cleavage rate constants (k_2 and K_m') were measured with an excess of ribozyme (5–600 nM) and trace amounts of end-labeled substrate (<0.1 nM) (Table 1). Apparent second-order rate constants (k_2/K_m') were calculated, and a relative specificity value was determined by arbitrarily fixing as 1.00 the value for the original substrate. At position −1, the presence of uridine gave a similar relative specificity (0.47), whereas the presence of adenine increased it to 2.70. This increase appears to be primarily a result of a 2-fold decrease in K_m'. At position −2, the presence of a purine (i.e., G or A) gave similar relative specificities (1.65 compared with 1.00, respectively). In contrast, the presence of a uridine produced a poorly cleaved substrate, and the presence of cytosine rendered it uncleavable. Specifically, the presence of uridine at position −2 reduced the specificity from 1.00 to 0.12 compared with the original substrate. This decrease in specificity appears to result from a 3-fold increase in the K_m' and a 3-fold decrease in the k_2 values. These results show a clear preference for the presence of a purine in position −2 and indicate that a pyrimidine should be avoided at all costs.

At position −3, replacement of the guanosine of the original substrate by any other base (i.e., A, C, or U) lowered the K_m' 3-fold, whereas k_2 remained unchanged. This resulted in an increase in specificity ranging from 2.89 to 3.25. Finally, at position −4, the presence of a purine (G and A) produces a substrate with approximately the same specificity as the original (i.e., 0.63 and 1.00). The presence of a pyrimidine in position −4, however, improved the specificity by at least 2-fold to 3.28 and 2.01 for C and U, respectively. Specifically, the presence of C or U lowered K_m', whereas k_2 remained essentially unchanged. Clearly, the base requirements for positions −4 to −1 of the substrate provide a significant but different contribution to the ability of the substrate to be cleaved.

Based on the observation that mutations at position −3 most significantly increased the relative specificity, we investigated whether or not the presence of a larger amount of Mg^{2+} in the cleavage reaction would affect the kinetic parameters of these substrates. Under single turnover conditions, in which the ribozyme and substrate concentrations were kept at 200 nM and 1 nM, respectively, we found that the ribozyme effectively cleaved these substrates at Mg^{2+} concentrations as low as 1 mM, which is the estimated physiologic magnesium concentration (Traut, 1994). A maximum k_{obs} for each substrate was ob-

served when the concentration of Mg^{2+} was 10 mM. The requirement for magnesium at half-maximal velocity (K_{Mg}) was virtually identical for these mutated substrates and the original substrate, varying between 1.5 and 2.2 mM. Similar experiments were also performed with several other substrates from the collection, and identical results were obtained, suggesting that the differences in the kinetic parameters for the various substrates were not related to different affinities for magnesium.

The effect of pyrimidines

The cleavage assays performed with the initial collection of substrates (i.e., those possessing single mutations) indicate that the presence of a pyrimidine at position −2 either reduces the cleavage activity or yields an uncleavable substrate (Table 1). Most likely, this is because when a C is present at position −1 followed by a pyrimidine (i.e., C or U) at position −2 of the substrate, both nucleotides may interact with nucleotides located on the ribozyme, resulting in the formation of an inactive substrate/ribozyme complex. It seems reasonable to suggest that base pairing with the ribozyme's guanosines at positions 27 and 28 of the J1/4 junction may occur, thereby creating new base pairs that will compete with formation of the P1.1 pseudoknot (Fig. 1). In this case, the presence of a cytosine in position −2 will form 2 consecutive GC base pairs. Similarly, the presence of a uridine in position −2 allows formation of $RzG_{27}SC_{-1}$ followed by $RzG_{28}SU_{-2}$, which is less stable than 2 GC base pairs and results only in reduced activity as compared with the absence of activity in the case of consecutive GC base pairs. To learn more about the nucleotide preference at position −2 and to consider the effect of the neighboring positions, we synthesized a second collection of substrates harboring more than one mutation (Fig. 3B,C,D,E,F).

First, to support the hypothesis that the presence of two consecutive pyrimidines at positions −1 and −2 has a detrimental effect on cleavage, substrates with the sequences $_{-1}UCGG_{-4}$ ($S\text{-}U_{-1}C_{-2}$) and $_{-1}UUGG_{-4}$ ($S\text{-}U_{-1}U_{-2}$) were synthesized and tested for cleavage (Fig. 3B,C). These substrates were found to be poorly cleaved, with cleavage end points of <3% and <6%, respectively. Because the extent of cleavage was so low, no kinetic analysis was performed. These results support the notion that the presence of consecutive pyrimidines at positions −1 and −2 has a detrimental effect on cleavage. Moreover, the presence of a cytosine at position −2 drastically reduced the percentage of cleavage compared with when a uridine was present.

Second, we verified whether or not the presence of a cytosine at position −2 after a noncytosine at position −1 had a detrimental effect on cleavage. Based on the previous results (Fig. 2 and Table 1), a substrate with an adenine in position −1 and a cytosine in position −2 (i.e., $S\text{-}A_{-1}C_{-2}$) (Fig. 3D) was synthesized and tested for cleavage efficiency. It yielded a moderate extent of 14% at 200 nM ribozyme, less than that observed for the substrates including the sequences $C_{-1}G_{-2}$ (i.e., original sequence) or $A_{-1}G_{-2}$ (mutant in position −1). The original substrate ($A_{-1}G_{-2}$) and the $S\text{-}A_{-1}C_{-2}$ substrate possessed apparent K_m' that were virtually identical, whereas the cleavage constant (k_2) for the $S\text{-}A_{-1}C_{-2}$ was reduced approximately 4-fold, resulting in a 4-fold reduction in the relative specificity (i.e., from 2.70 to 0.56). These results indicate that the presence of a cytosine at position −2 significantly reduced the amount of substrate cleavage.

Third, we verified whether or not the presence of a cytosine at position −2 with a cytosine at position −3 yielded a cleavable substrate, in essence investigating whether or not the pres-

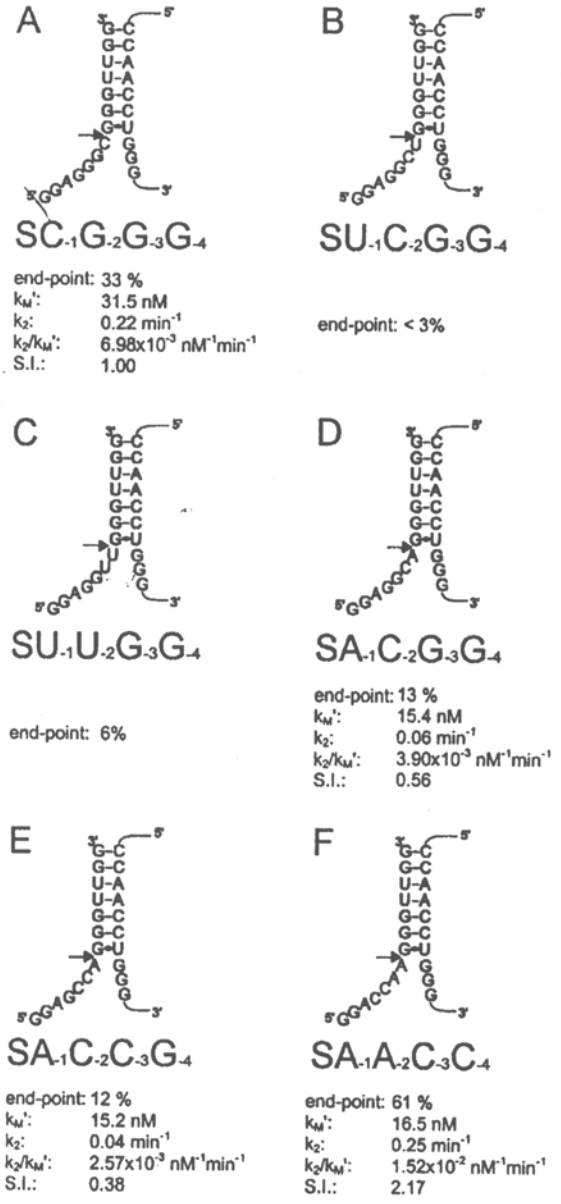

FIG. 3. Sequence and cleavage activity of a collection of multiply mutated substrates. The sequence of each substrate is shown base paired with the ribozymes (**A,B,C,D,E,F,** with **A** being the original substrate). For each substrate, trace amounts of [^{32}P]5′-end-labeled substrate (<1 nM) was incubated in the presence of an excess of ribozyme (200 nM), and the maximum cleavage percent (end point) was determined. Pseudo-first-order cleavage rate constants (k_2 and K_m') were calculated. Kinetic characterizations were not possible for substrates **B** and **C** because there was insufficient activity for an accurate determination.

ence of two consecutive cytosines, regardless of their positions, resulted in an uncleavable substrate. We found substrate S-$A_{-1}C_{-2}C_{-3}$ (Fig. 3E) to be cleaved with kinetic parameters almost identical to those of substrate S-$A_{-1}C_{-2}$, except that k_2 was slightly reduced (0.04 min^{-1} as compared with 0.06 min^{-1}), resulting in a small reduction in the relative specificity (i.e., from 0.56 to 0.38) (Fig. 3). These results show that the presence of a cytosine at position −3 following a cytosine at position −2 slightly reduced the cleavage activity but did not significantly affect the ability of a substrate to be cleaved. Clearly, the presence of a cytosine at position −3 does not have the same influence as it does at position −2.

Fourth, we investigated whether or not the presence of two consecutive cytosines at positions −4 and −3 give similar results. A substrate containing cytosines at both positions −3 and −4 and adenines at positions −1 and −2 was synthesized (S-$A_{-1}A_{-2}C_{-3}C_{-4}$) (Fig. 3F). Adenines were included in positions −1 and −2 because these residues appear to yield a readily cleaved substrate as compared with the single mutation collection. The S-$A_{-1}A_{-2}C_{-3}C_{-4}$ mutant was found to have a maximum cleavage of 61% (Fig. 3F), a K_m' of 16.5 nM, and an increased k_2 value of 0.25 min^{-1}, resulting in a substrate with a relative specificity of 2.17 as compared with the original. Thus, the presence of two consecutive cytosines at positions −3 and −4 has no detrimental effect on cleavage.

Better substrates

The substrates characterized to this point suggest that adenine is preferred at positions −1 and −2, and the presence of pyrimidines at positions −3 and −4 increases the extent of cleavage. To verify this hypothesis, we analyzed substrates with the sequences $_{-1}AAAU_{-4}$ (S-$A_{-1}A_{-2}A_{-3}U_{-4}$) and $_{-1}AAUC_{-4}$ ($SA_{-1}A_{-2}U_{-3}C_{-4}$) and found them to have the highest maximum cleavages observed in this study, 73% and 82%, respectively (Fig. 4). These substrates had almost identical K_m' (17.4 nM and 14.1 nM, respectively) and k_2 values (0.61 min^{-1} and 0.69 min^{-1}, respectively), with the K_m' values being comparable to those of the best previously characterized substrates (Table 1 and Fig. 3) and the k_2 values being 3-fold higher (compared with ~0.25 min^{-1}), resulting in relative specificities of 5.26 and 7.35, respectively, as compared with the original substrate (Fig. 4). More importantly, the results provided by the substrates possessing single mutations can be used to design an ideal substrate, indicating that they are not restricted to the context of the original sequence. Furthermore, the efficient cleavage of the substrates with an adenosine in position −2 (Fig. 4), as compared with those with a cytosine (Fig. 3D and E), supports the notion that the base at this position is of primary importance in determining the substrate specificity. All of these substrates (Fig. 3D, E and Fig. 4) gave virtually identical K_m' values while have k_2 values that varied up to 17-fold. One possible explanation for this variation is that the presence of a cytosine in position −2 leads to the formation of inactive ribozyme/substrate complexes.

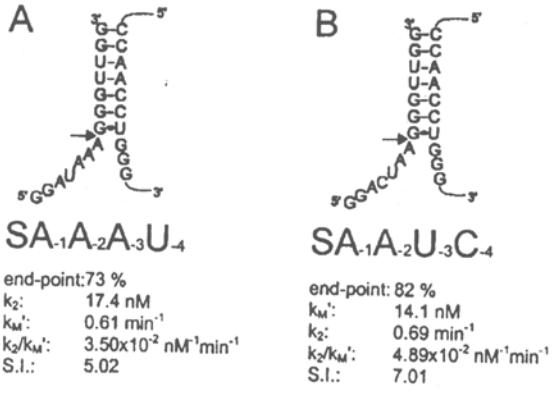

FIG. 4. Sequence and cleavage activity of the better substrates. Trace amounts of [^{32}P]5′-end-labeled substrates (<1 nM) were incubated in the presence of an excess of ribozyme (200 nM), and the maximal cleavage percentages (end point) were determined. Pseudo-first-order cleavage rate constants (k_2 and K_m') were calculated.

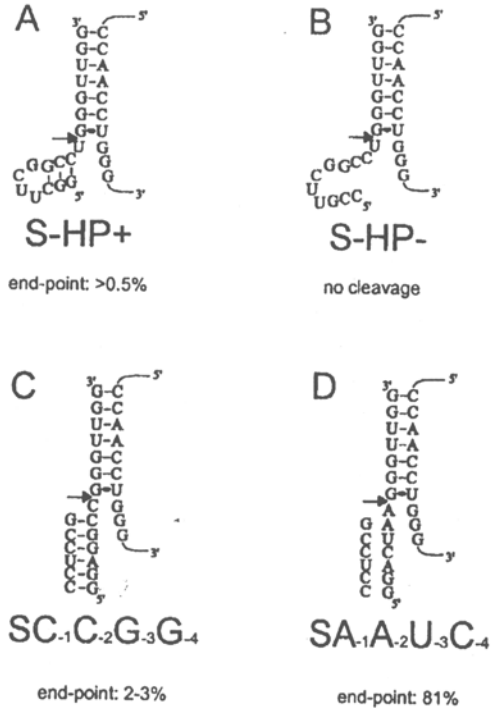

FIG. 5. Cleavage activity of complex substrates. (**A** and **B**) 18-Mer substrates that included or lacked a sequence forming a 5′-end hairpin, respectively. The maximum cleavage percentages (end point) were determined in the presence of 200 nM ribozyme. (**C** and **D**) The two substrates tested in the presence or absence, respectively, or the Comp-6 oligonucleotide facilitator (50 nM).

The effect of double-stranded cytosines

Previous results support the hypothesis that the presence of consecutive pyrimidines (and, more specifically, cytosines) at positions −1 and −2 has a detrimental effect of the ability of a substrate to be cleaved by the delta ribozyme. Therefore, we wondered whether it is possible to compensate for this effect of the presence of two consecutives cytosines at positions −1 and −2 by including the one located at position −2 in a double-stranded structure. Consequently, a longer RNA substrate (i.e., an 18-mer as compared with the 14-mer used previously),

which included a hairpin at the 5′-end that involved C_{-2} in the last base pair of the helix (S-HP+, Fig. 5A), was chemically synthesized and tested. This substrate yielded only trace amounts of cleavage products (maximum percentage cleavage of <0.5%), and no extensive characterization was possible. If the sequence was designed so as to avoid formation of the 5′-end hairpin (i.e., C_{-2} remains single stranded; S-HP−), no cleavage was observed (Fig. 5B). These two results show that the presence of a base-paired cytosine at position −2 gave minimal activity as compared with when this cytosine is single stranded. However, the improvement was very limited. This limitation in activity can be explained in several ways: (1) the presence of the 5′-end hairpin provides a steric inhibition in the ribozyme-substrate complex, and (2) the substrate with the 5′-end hairpin adopts another secondary structure that includes either intramolecular or intermolecular base pairings, resulting in only a small proportion of the substrates being available to form the ribozyme-substrate complex. Even the presence of a large excess of ribozyme did not produce any significant quantity of cleaved substrate.

To reduce the potential problem of steric inhibition of cleavage caused by the presence of a hairpin, we used a 14-mer substrate harboring the two cytosines at positions −1 and −2 and incubated in either the presence or the absence of a small RNA oligonucleotide (Comp-6) complementary to its 5′-end (Fig. 5C). Comp-6 should tightly bind to the substrate (involving 5 GC/CG and 1 UA base pairs) under the conditions used, thereby placing the cytosine in position −2 in a double-stranded region. Although the substrate alone was not cleaved, the hybrid substrate/Comp-6 showed increasing amounts of product with increasing Comp-6 concentration. The maximum percent cleavage increased from 0 to ~2.0%–3.0% when the concentration of Comp-6 increased from 0 to 25 nM. Comp-6 concentrations as high as 50 and 100 mM did not produce an increase in the maximum percent cleavage. Thus, Comp-6 acts as a facilitator, and the detrimental effect of the presence of a cytosine at position −2 can be partially alleviated if this base is in a double-stranded region. However, this effect is limited and probably results from a lesser steric effect than occurs in the presence of the hairpin. Similar results were obtained regardless of whether the Comp-6 was added before or after the substrate/ribozyme was denatured/renatured. To verify if the gain in activity was limited because the facilitator Comp-6 interacted with the ribozyme, producing an inactive conformation, similar experiments were performed using the most efficient substrate characterized (S-$A_{-1}A_{-2}U_{-3}C_{-4}$) (Fig. 5D). The presence of Comp-6 in concentrations ranging from 0 to 100 nM did not prevent cleavage of $SA_{-1}A_{-2}U_{-3}C_{-4}$, indicating that the facilitator did not interact with the ribozyme. In contrast, when a Comp-6 possessing the appropriate sequence for base pairing with the $SA_{-1}A_{-2}U_{-3}C_{-4}$ substrate was used, the end point was drastically lower than 10% (data not shown), indicating that it produced a steric effect.

DISCUSSION

14-mer vs. 11-mer substrate

To study the contributions of positions −1 to −4 to the cleavage reaction, we designed a 14-mer substrate whose 5′-end was extended by 3 nt (i.e., GGA) (Fig. 1). The 5′-end extension reduced slightly the overall ability of the 14-mer substrate to be cleaved, as compared with the original 11-mer substrate (Ananvoranich and Perreault, 1998). However, this cleavage activity can be rescued and, in several cases, increased by altering the sequence of the −1 to −4 region. According to the delta ribozyme kinetic pathway, the first step is the binding of the substrate to the ribozyme (formation of the PI stem), followed by a relatively slow internal conformational rearrangement yielding the active ES′ complex (Mercure et al., 1998). It seems reasonable to suggest that the extension at the 5′-end slows this rearrangement. Cross-linking analysis shows that the 2′-hydroxyl group of the ribose at position −3 is in close proximity to the cytosine at position 47 of the J4/2 junction (Bravo et al., 1996). Furthermore, binding shift assays have indicated a potential interaction between the 2′-hydroxyl group of the ribose at position −1 and C_{47} (Mercure et al., 1998). Therefore, it is likely that the 5′-end extension limits the conformational transition through a steric effect, which should be and is, in fact, observed to be more pronounced in the presence of a hairpin or double-stranded region. This hypothesis of a steric effect is supported by comparison of the kinetic parameters obtained for the cleavages of similar sequences located on mRNA (Roy et al., 1999). More specifically, the HDAg mRNA showed a higher K_m' (>70-fold) and slower k_2 (<30-fold) compared with cleavage of 11-mer substrates of similar sequence under single turnover conditions.

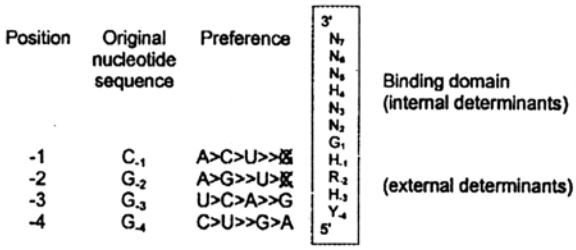

FIG. 6. Summary of the sequence preference. The data obtained with the singly mutated substrates for positions −1 to −4 are summarized at left. A nucleotide marked by an X is one that produces an uncleavable substrate. The global consensus sequence specificity for delta ribozyme cleavage is shown in the box.

Nucleotide preference at positions −1 to −4

We synthesized a collection of singly and multiply mutated substrates and tested their cleavability. Some were uncleavable, whereas other show a >60-fold difference in relative specificity (0.12 to 7.01) between the most and less efficiently cleaved substrates. Clearly, this collection shows that the base identity at each position from −1 to −4 contributes differently to the ability of a substrate to be cleaved (summarized in Fig. 6). For these positions, we derived $_{-1}$HRHY$_{-4}$ (where H indicates U, C, or A) as a consensus sequence to be cleaved. This sequence differed from that in our previous report, where a preference for the presence of a purine at all four positions was suggested (Roy et al., 1999). However, it should be noted that this study was performed with a limited number of substrates.

For position −1, which is adjacent to the cleavage site, the order of reactivity was A > C > U, with G giving an uncleavable substrate. This order differs slightly from previous reports with another delta ribozyme that proposes U > A > C (Nishikawa et al., 1997). This report was simply based on maximum cleavage percentages and used a genomic version of the ribozyme. In agreement with our results, Nishikawa et al. (1997) showed that the presence of a G in position −1 results in an uncleavable substrate. Using an 8-nt long (positions −1 to 7) substrate it was also shown that a G in position −1 has a detrimental effect on cleavage (Perrotta and Been, 1992). Most likely the presence of a G in position −1 significantly modifies the chemical environment near the scissile phosphate. For position −2, the order of reactivity was A > G >> U, with C giving an uncleavable substrate. However, the reactivity of the base at position −2 was also dependent on the adjacent base in position −1. In the presence of a pyrimidine at position −1, a pyrimdine at position −2 gave either a poorly cleaved substrate (i.e., with a U) or an uncleavable substrate (i.e., with a C). We believe that the two consecutive pyrimidines interact by base pairing to the guanosines of the J1/4 junction (i.e., $_{27}GG_{28}$). As a result, it prevents formation of the P1.1 stem involving $_{27}GG_{28}$ and $_{11}CC_{12}$ of the L3 loop (Fig. 1). Both the genomic and antigenomic self-cleaving delta sequences include the P1.1 stem involving the perfectly conserved guanosine of the J1/4 junction. To confirm that any pyrimidines in positions −1 and −2 base paired with $_{27}GG_{28}$, we performed a series of nuclease mapping experiments. A trace amount of 5′-end-labeled ribozyme was preincubated with an excess of either the 14-mer substrate with cytosines at positions −1 and −2 or the 11-mer RNA analog identical to the substrate except for the presence of a deoxyribose residue at position 4 (the cleavage site), namely SdC4. The 14-mer substrate is uncleavable, whereas SdC4 is a competitive inhibitor (Mercure et al., 1998). Consequently, both molecules permit nuclease mapping under reaction conditions. Ribonuclease T_1, which specifically cleaves 3′ of guanosine residue located in single-stranded regions, was used to probe both complexes. The accessibility of ribonuclease T_1 to $_{27}GG_{28}$ was reduced in the presence of the 14-mer substrate compared with accessibility in the presence of the SdC4 analog (data not shown), supporting the model that includes the formation of base pairs between $_{-1}CC_{-2}$ of the substrate and $_{27}GG_{28}$ of the ribozyme. However, the difference in accessibility was not significant enough to state this conclusively. The lack of a significant difference may result from the fact that P1.1 stem seems to be adopted only in the active ribozyme-substrate complex. Kinetic intermediates observed in the folding of *trans*-acting delta ribozyme using oligonucleotide hybridization assays coupled with RNAse H activity have shown that the P1.1 stem forms after the formation of an appropriate P1 stem and does so only in the presence of Mg^{2+} (S. Ananvoranich and J.P. Perreault, personal communication). Furthermore, it appears that P1.1 stem, which is composed of only 2 bp, is relatively unstable. Consequently, it is difficult to detect in solution even though it is essential for cleavage. Regardless of the composition of the P1.1 stem, the substrate specificity of delta ribozyme cleavage will always depend at least on 11 nt (positions −4 to 7).

Sequence specificity for delta ribozyme cleavage

The results presented here shed light on new features that contribute to the substrate requirement of delta ribozyme cleavage (Fig. 6). These features are of primary importance in the design of efficient delta ribozyme therapeutic tools. In summary, at least 11 substrate nucleotides contribute to determining the ability of a substrate to be cleaved efficiently. In addition, it seems preferable that those 11 nt be in a single-stranded region, as (1) the binding of the 7 nt that form the P1 stem (i.e., the recognition domain) is relatively easy if they are single stranded because there is no competition with formation of an intrinsic secondary structure of the RNA target, and (2) we show that it is advantageous for a substrate to have no hairpin immediately upstream of the cleavage site (i.e., positions −1 to −4). Although this portion of the substrate is not a part of the recognition domain, it plays a crucial role as an external determinant of the ability of a substrate to be cleaved, and it could impose a steric effect limiting the cleavage activity. This general preference agrees with all results obtained in the present work, as well as with those from cleavage of the HDAg mRNA using delta ribozyme (Roy et al., 1999). In the latter case, kinetics performed under both single-turnover and multiple-turnover conditions were in agreement. Therefore, it seems that the sequence specificity defined here would be significant within the context of an mRNA.

Generally, hammerhead and hairpin ribozymes are designed so as to bind their substrate through formation of two small helices of 5–6 bp each, therefore involving 10–12 nt defining the substrate specificity. Here, we demonstrate that 11 contiguous nucleotides of the substrate contribute to determining the ability an RNA molecule to be cleaved by delta ribozyme. Therefore, this specificity is as strict as that of any other ribozyme.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council (MRC) of Canada to J.-P.P., who is an MRC scholar. D.A.L. and P.D. are the recipients of predoctoral fellowships from the Fonds de la Recherche en Santé du Québec (FRSQ).

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Received October 8, 1999; accepted in revised form November 22, 1999.