Structural Requirements at the Catalytic Site of the Heteroduplex Substrate for Human RNase H1 Catalysis*

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Human RNase H1 cleaves RNA exclusively in an RNA/DNA duplex; neither double-strand DNA nor double-strand RNA is a viable substrate. Previous studies suggest that the helical geometry and sugar conformation of the DNA and RNA may play a role in the selective recognition of the heteroduplex substrate by the enzyme. We systematically evaluated the influence of sugar conformation, minor groove bulk, and conformational flexibility of the heteroduplex on enzyme efficiency. Modified nucleotides were introduced into the oligodeoxynucleotidylolnucleotide at the catalytic site of the heteroduplex and consisted of southern, northern, and eastern biased sugars with and without 2′-substituents, non-hydrogen-bonding base modifications, abasic deoxyribonucleotides, intranucleotide hydrocarbon linkers, and a ganciclovir-modified deoxyribonucleotide. Heteroduplexes containing modifications exhibiting strong northern or southern conformational biases with and without 2′-substituents were cleaved at a significantly slower rate than the unmodified substrate. Modifications imparting the greatest degree of conformational flexibility were the poorest substrates, resulting in dramatically slower cleavage rates for the ribonucleotide opposing the modification and the surrounding ribonucleotides. Finally, modified heteroduplexes containing modifications predicted to mimic the sugar pucker and conformational flexibility of the deoxyribonucleotide exhibited cleavage rates comparable with those of the unmodified substrate. These data suggest that sugar conformation, minor groove width, and the relative positions of the intra- and internucleotide phosphates are the crucial determinants in the selective recognition of the heteroduplex substrate by human RNase H1 and offer immediate steps to improve the performance of DNA-like antisense oligonucleotides.

RNase H hydrolyzes RNA in RNA-DNA hybrids (1). RNase H activity appears to be ubiquitous in eukaryotes and bacteria (2–7). Although RNases H constitute a family of proteins of varying molecular mass, the nucleolytic activity and substrate requirements appear to be similar for the various isotypes. For example, all RNases H studied to date function as endonucleases, exhibiting limited sequence specificity and requiring divalent cations (e.g. Mg2+ and Mn2+) to produce cleavage products with 5′-phosphate and 3′-hydroxyl termini (8).

Recently, two human RNase H genes have been cloned and expressed (9–11). RNase H1 is a 286-amino acid protein and is expressed ubiquitously in human cells and tissues (9). The amino acid sequence of human RNase H1 displays strong homology to RNase H1 from yeast, chicken, Caenorhabditis elegans, yeast, and E. coli (9). Although the biological roles for the human enzymes are not fully understood, RNase H2 appears to be involved in de novo DNA replication, and RNase H1 has been shown in mice to be important for mitochondrial DNA replication (12).

Human RNase H1 has been shown to play a dominant role in the activity of DNA-like antisense oligonucleotides (13). Human RNase H1 protein is overexpressed in both several cell lines and mouse liver, and the level of human RNase H1 is reduced by using DNA-like antisense oligonucleotides (ASOs) and small interfering RNAs targeting the enzyme. The effects of these manipulations on the potencies of a number of DNA-like ASOs to several different target RNAs showed that increasing the level and activity of human RNase H1 increases the potency of the ASOs (13). Moreover, overexpression of human RNase H1 in mouse liver increases the potency of a DNA-like ASO targeting Fas after intravenous administration. Finally, reducing the level and activity of RNase H1 reduces the potencies of the ASOs (13).

The structure of human RNase H1 was shown to consist of a 73-amino acid region homologous to the RNA-binding domain of yeast RNase H1 at the N terminus of the protein and separated from the catalytic domain by a 62-amino acid spacer region (14–16). The catalytic domain is highly conserved in the amino acid sequences of other RNase H1 proteins and contains the key catalytic and substrate-binding residues required for activity (14, 17–20). Site-directed mutagenesis of human RNase H1 revealed that the spacer region is required for RNase H activity (14). Although the RNA-binding domain was shown not to be required for RNase H activity, this region is responsible for the enhanced binding affinity of the human enzyme for the heteroduplex substrate as well as the strong positional preference for cleavage exhibited by the enzyme (14, 21). The RNA-binding domain of human RNase H1 is conserved in other eukaryotic RNases H1, and the highly conserved lysines at positions 59 and 60 of human RNase H1 have been shown to be important for binding to the heteroduplex substrate.

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1 H. Wu, unpublished data.

2 The abbreviations used are: ASOs, antisense oligonucleotides; HPLC, high performance liquid chromatography.

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strate (21). The conserved tryptophan at position 43 is responsible for properly positioning the enzyme on the substrate for catalysis (21).

Human RNase H1 exhibits a strong positional preference for cleavage, i.e. human RNase H1 cleaves the heteroduplex substrate between 7 and 12 nucleotides from the 3' -DNA/5' -RNA terminus (14 and 21). Based on site-directed mutagenesis of both human RNase H1 and the heteroduplex substrate, the RNA-binding domain was shown to be responsible for the observed positional preference for cleavage (21). The RNA-binding domain of human RNase H1 appears to bind to the 3'-DNA/5'-RNA pole of the heteroduplex substrate, with the catalytic site of the enzyme positioned slightly less than one helical turn from the RNA-binding domain (21). Substitution of either the terminal 3'-DNA with a single ribonucleotide or 5'-RNA with a 2'-methoxythioxy deoxyribonucleotide was shown to cause a concomitant 3'-shift of the first 5'-cleavage site on the RNA, suggesting that altering duplex geometry interferes with proper positioning of the enzyme on the heteroduplex substrate (21). Although the interaction between the RNA-binding domain and the heteroduplex substrate has been characterized, the mechanism by which the catalytic domain of RNase H1 recognizes the substrate has not been fully elucidated.

Human RNase H1 is a nuclease that cleaves RNA exclusively in an RNA/DNA duplex via a double-strand DNA cleavage mechanism. Neither double-strand RNA nor double-strand DNA duplexes support RNase H1 activity (22, 23). The observed structural differences between the RNA/DNA heteroduplex and double-strand RNA and double-strand DNA duplexes suggest a possible role for the helical geometry and sugar conformation of the DNA and RNA in the selective cleavage of the heteroduplex substrate by human RNase H1 (24–26). Specifically, the deoxyribonucleotides within double-strand DNA form a southern C'-endo sugar conformation, resulting in a B-form helical conformation, whereas ribonucleotides within double-strand RNA form a northern C'-endo pucker and an A-form helical geometry (26). In contrast, the deoxyribonucleotides of the RNA/DNA heteroduplex have been shown to adopt an eastern O'-4'-endo sugar pucker, resulting in a helical conformation in which the RNA strand adopts A-form geometry and the DNA strand shares both the A- and B-form helical conformations. The conformational diversity observed for the DNA strand is likely a function of the intrinsic flexibility of the deoxyribonucleotide compared with RNA and may also be important for human RNase H1 activity. DNA also differs from RNA in that the furanose ring of deoxynucleotide is much more flexible, i.e. it exhibits a near-symmetrical potential energy barrier for both southern and northern sugar conformations (26).

Consistent with these observations, heteroduplexes containing 2'-fluoro deoxyribonucleotides, which have been shown to exhibit a sugar conformation compatible with DNA when hybridized to RNA, have also been shown to support RNase H1 cleavage, the observed positional preference for cleavage by human RNase H1 and the RNA/DNA heteroduplex substrate in two instances, and in the catalytic activity (27). Modified nucleosides that have been introduced into the oligodeoxyribonucleotides at the human RNase H1 preferred cleavage sites on the heteroduplex and consisted of the DNA-like southern C'-2'-endo, RNA-like northern C'-3'-endo, and eastern O'-4'-endo biased sugars with and without 2'-substituents (see Fig. 1A). In addition, varying degrees of conformational flexibility were introduced into the heteroduplex substrate by incorporating modified deoxyribonucleotides that II-stack with the adjacent deoxyribonucleotides but do not form hydrogen bonds with the bases of the RNA strand, abasic deoxyribonucleotides, hydrocarbon intranucleotide linkers, and the ganciclovir-modified deoxyribonucleotide (see Fig. 1B). The initial cleavage rates (V0) and the site-specific cleavage rates of the modified heteroduplexes were compared with those of the wild-type RNA/DNA heteroduplex.

**MATERIALS AND METHODS**

**Preparation of Human RNase H1**—Human RNase H1 containing an N-terminal His tag was expressed and purified as described previously (29). Briefly, the plasmids were transfected into E. coli BL21(DE3) (Novagen). The bacteria were grown in Terrific Broth medium (Bio 101, Inc.) at 37°C and harvested at A600 = 1.2. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 2 h. The cells were lysed in 6 mM guanidine hydrochloride, 100 mM sodium phosphate, and 10 mM Tris (pH 8.0) for 16–20 h at 24°C. The recombinant proteins were incubated for 1 h with nickel-nitrilotriacetic acid superflow beads (1 ml/50 ml of lysate; Qiagen Inc.). The nickel-nitrilotriacetic acid medium was packed into a fast protein liquid chromatography column, and the RNase H1 proteins were partially purified at a flow rate of 5 ml/min with sequential gradients (buffer A = 100 mM sodium phosphate, 10 mM Tris-HCl, and 8 mM urea (pH 6.3); buffer B = 100 mM sodium phosphate, 10 mM Tris-HCl, and 2 mM urea (pH 6.3); and buffer C = 100 mM sodium phosphate, 10 mM Tris-HCl, 2 mM urea, and 100 mM EDTA (pH 7.0)). The eluent was further purified by ion-exchange fast protein liquid chromatography on a Mono S column at a flow rate of 1 ml/min (buffer A = 20 mM sodium phosphate, 2 mM urea, and 200 mM NaCl (pH 7.0) and buffer B = 20 mM sodium phosphate, 2 mM urea, and 2 mM NaCl (pH 7.0)). Fractions containing RNase H1 were pooled and concentrated. The concentrated protein was purified by reverse phase fast protein liquid chromatography on a Resource RPC column at a flow rate of 1 ml/min (buffer A = 2% acetonitrile in distilled H2O and 0.065% trifluoroacetic acid and buffer B = 80% acetonitrile in distilled H2O and 0.05% trifluoroacetic acid). Fractions were lyophilized, resuspended in distilled H2O, and analyzed by SDS-PAGE.

**Synthesis of Oligonucleotides**—The oligoribonucleotides were synthesized on a PE-ABI 380B synthesizer using 5'-O-silyl-2'-O-bis(2-cyanoethyl) methyl ribonucleoside phosphorodiamidites and procedures described elsewhere (30). The oligoribonucleotides were purified by reverse-phase HPLC. The oligodeoxyribonucleotides were synthesized on a PE-ABI 380B automated DNA synthesizer using standard phosphodiester chemistry. The oligodeoxyribonucleotides were purified by precipitation two times out of 0.5 M NaCl with 2.5 volumes of 95% ethanol.

**Synthesis of Modified Oligonucleotides**—1,4-Anhydro-5-O-(4,4'-dimethoxytrityl)-2-deoxy-β-D-erythrose-4,6-pentenol-3-[2-cyanoethyl-N,N-diisopropyl)phosphoramidite, 5'-O-(4,4'-dimethoxytrityl)-3'-4'-methyleneylidinocytidine-2'-thiophosphoramidite, 5'-O-(4,4'-dimethoxytrityl)-3'-4'-methyleneyli dine-2'-thiophosphoramidite, and 5'-O-(4,4'-dimethoxytrityl)-3'-4'-methyleneylidinocytidine-2'-thiophosphoramidite were generated from QIAcore Inc. 1-[2-Deoxy-2-fluoro-5-O-(4,4'-dimethoxytrityl)]-3-[2-cyanoethyl-N,N-diisopropyl]phosphoramidite, 5'-O-(4,4'-dimethoxytrityl)-2'-deoxy-2'-fluorothymidine-3'-[2-cyanoethyl-N,N-diisopropyl]phosphoramidite, 2-fluoro-6-methylbenzo-
imidaizole deoxyribonucleotide, 4-methylbenzimidazole deoxyribonucleotide, hydrocarbon linkers, and 5'-O-(4,4'-dimethoxysteroyl)-2'S-methyl-2'-thio-5'-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] were synthesized as described (31–33). The nucleoside 3'-β-C-methylthymidine was synthesized from 1,2-O-isoproplidene-α-xyloforanose and converted to the 5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-acyctoethylisopropylimidazole)phosphoramidite as described previously (34, 52). The 4'-α-C-methylthymidine nucleotide was synthesized in 12 steps, starting from commercially available 1,2,5,6-di-O-isoproplidene-α-D-glucorafanose (Pfanstiehl Laboratories Inc., Waukegan, IL). The alternative synthesis of this nucleoside has been reported (35, 36). It was converted to 5'-O(4,4'-dimethoxytrityl)-4'-α-C-methylthymidine-3-O-(2-acyctoethylisopropyrimidazole)phosphoramidite following the procedure described for similar compounds (37, 38). Standard phosphoramidites and solid supports were used for incorporation of A, T, G, and C residues. A 0.1M solution of each amide in anhydrous acetonitrile was used for the synthesis of modified oligonucleotides. The oligonucleotides were synthesized on functionalized Controlled-Pure Glass on an automated solid-phase DNA synthesizer with the final dimethoxymethyl group retained at the 5'-end. For incorporation of modified amides, 6 eq of phosphoramidite solutions were delivered in two portions, each followed by a 3-min coupling wait time. All other steps in the protocol supplied by the manufacturer were used without modification. Oxidation of the internucleotide phosphate to the phosphoramidite was carried out using a 0.1 M solution of iodine in 20:1 (v/v) pyridine:water with a 10-min oxidation wait time. The coupling efficiencies were >97%. To deprotect oligonucleotides containing 2'-deoxy-2'-fluorothymidine and 2'-deoxy-2'-fluororibonafuranosyluridine, the solid supports bearing the oligonucleotides were suspended in aqueous ammonia (28–30 weight %)/ethanol (3:1; 3 ml for 2-μmol scale synthesis) and heated at 55 °C for 6 h. For all other modified oligonucleotides after deprotection of the synthesis, the solid supports bearing the oligonucleotides were suspended in aqueous ammonium hydroxide (28–30 weight %; 2 ml for 2-μmol scale synthesis) and kept at room temperature for 2 h. The solid support was filtered, and the filtrate was heated at 55 °C for 6 h to complete the removal of all protecting groups. Crude oligonucleotides were purified on a Waters HPLC C18 7.8 × 300-mm column (buffer A 0.1 M ammonium acetate (pH 6.5–7); buffer B = acetonitrile; 5–60% of buffer B in 55 min at a flow rate of 2.5 ml/min (λmax = 260 nm). Detrylation was achieved by adjusting the pH of the solution to 3.8 with acetic acid and by keeping at room temperature until complete removal of the trityl group, as monitored by HPLC analysis. The oligonucleotides were then desalted by HPLC to yield modified oligonucleotides in 30–40% isolated yield calculated based on the load- ing of the 3'-base onto the solid support (39). The oligonucleotides were characterized by electrospray mass spectroscopy, and their purity was assessed by HPLC and capillary gel electrophoresis. The purity of the oligonucleotides was >90%.

Oligonucleotides with abasic sites were conveniently generated by the use of uracil-DNA glycosylase (40). Oligonucleotides containing deoxyuridine residues were synthesized as described above. The HPLC-purified oligonucleotides (0.32 mg) were dissolved in uracil-DNA glycosylase (149 μl; 1 unit in 1 ml dissolved in 30 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.05% Tween 20, and 50% glycerol) and incubated at 37 °C for 4 h. The reaction was terminated by filtering the enzyme using a low binding membrane filter (0.22 μm; Millipore Corp., Bedford, MA). The release of uracil was observed upon HPLC analysis of the reaction mixture using a Waters C18 3.9 × 300-mm column (Delta Pack, 15 μm, 300 Å) (buffer A = 100 mM ammonium acetate; buffer B = acetonitrile; 0–25% buffer B in 55 min at a flow rate of 2.5 ml/min (λmax = 260 nm). The reaction mixture was directly injected into the analytical HPLC. The oligonucleotides were purified by HPLC as described above. The purity (>90%) of the oligonucleotides was assessed by HPLC analysis.

**Preparation of 32P-Labeled Substrate**—The RNA substrate was 5'-end-labeled with 32P using 20 units of T4 polynucleotide kinase (Promega Corporation, Madison, WI) and 32P-ATP (ICN), 400 μM final concentration. The reaction mixture was incubated at 37 °C for 30 min. The labeled oligonucleotide was purified by electrophoresis on a 12% denaturing polyacrylamide gel (41). The specific activity of the labeled oligonucleotide is 3000–8000 cpm/μmol.

**Preparation of the Heteroduplex**—The heteroduplex substrate was prepared in 100 μl containing 100–1000 μM unlabeled oligonucleo-
FIG. 1. Positions and structures of the deoxyribonucleotide and intranucleotide linker modifications. A, modified nucleotides containing conformationally biased sugar puckers. The northern biased modifications include 2-thiouridine (S\(^2\)U) and 2'-fluorothymidine (2'-Fluro). The southern biased modifications include 2'-methylthymidine (2'-S-methyl), 4'-methylthymidine (4'-methyl), 3'-methylthymidine (3'-methyl), and pseudouridine (pseudo-U). 2'-Arafluoropyrimidine (2'-ara-Fluro) represents the eastern biased sugar modification. B, structures of the modifications designed to introduce conformational flexibility into the heteroduplex. These modifications include the propyl (C\(_3\)), butyl (C\(_4\)), and pentyl (C\(_5\)) hydrocarbon linkers; the tetrahydrofuran (THF), abasic, and ganciclovir (GV) modifications; and the 5'-stacking 2-fluoro-6-methylbenzoimidazole (2-F-6-Me-ben), 4-methylbenzoimidazole (4-Me-ben), and 2,4-difluorotoluyl (2,4-F-toluyl) deoxyribonucleotides. C, \(x\) indicates the position of the modification for the respective oligodeoxyribonucleotide. The positions are numbered 5' \(\rightarrow\) 3' on the oligodeoxyribonucleotide.
The initial cleavage rates \( (V_0) \) observed for the modified heteroduplexes were predominantly dependent on the class of modification tested rather than the position of the specific modification within the oligodeoxynucleotide (Table I). For example, several modified heteroduplexes (e.g. 2'-arafluoropyrimidine, pseudouridine, and II-stacking deoxyribonucleotides) exhibited initial cleavage rates 2–3-fold slower than the \( V_0 \) of the unmodified substrate, whereas other modified heteroduplexes (2'-arafluoropyrimidine, pseudouridine, and II-stacking deoxyribonucleotides (e.g. 2-fluoro-6-methylbenzoimidazole, 4-methylbenzoimidazole, and 2,4-difluorotoluyl deoxyribonucleotides)) exhibited initial cleavage rates comparable with the rate observed for the unmodified substrate (Table I). It is important to note that a 2-fold reduction in the initial cleavage rate due to a single nucleotide modification is significant considering that human RNase H1 cleaves the substrate at multiple positions within the heteroduplex. In contrast, the heteroduplexes containing the same modification at different positions within the substrate

**Table I**

Relative initial and site-specific cleavage rates for the modified heteroduplex substrates

| Modification and position | Ratio site-specific cleavage rates (modified/unmodified) | Ratio \( V_0 \) (modified/unmodified) |
|---------------------------|---------------------------------------------------|----------------------------------|
|                           | \(-2\) | \(-1\) | \(0^1\) | \(+1\) | \(+2\) | \(-2\) |
| **Part A. 2'-Arafluoro**  |         |        |        |        |        |        |
| T7                        | 0.9    | 1.1    | 0.9    | 1.0    | 0.9    | 1.1    |
| T8                        | 1.3    | 1.5    | 0.8    | 1.5    | 1.5    | 1.1    |
| T9                        | 1.0    | 1.1    | 1.5    | 0.6    | 0.4    | 1.1    |
| C10                       | 0.7    | 1.1    | 0.8    | 1.0    | 0.6    | 1.2    |
| C11                       | 1.1    | 0.7    | 0.6    | 0.8    | 0.6    | 1.1    |
| C15                       | –      | –      | 1.1    | 1.3    | 0.4    | 1.1    |
| **Pseudouridine**         |         |        |        |        |        |        |
| T7                        | 0.8    | 0.7    | 0.6    | 0.6    | 0.6    | 0.9    |
| T8                        | 1.4    | 1.3    | 0.7    | 0.2    | 0.3    | 1.1    |
| T9                        | 1.0    | 0.8    | 1.0    | 0.2    | 0.2    | 1.2    |
| **2'-Fluoro**             |         |        |        |        |        |        |
| T7                        | 0.5    | 0.2    | 0.2    | 0.1    | 0.1    | 0.6    |
| T8                        | 0.8    | 0.6    | 0.2    | 0.3    | 0.6    | 0.7    |
| T9                        | 1.1    | 0.9    | 0.2    | 0.3    | 0.6    | 0.7    |
| **2-Thiouridine**         |         |        |        |        |        |        |
| T7                        | 0.4    | 0.4    | 0.2    | 0.1    | 0.1    | 0.5    |
| T8                        | 1.1    | 0.6    | 0.0    | 0.0    | 0.7    | 0.7    |
| T9                        | 1.3    | 1.0    | 0.3    | 0.3    | 0.9    | 0.7    |
| **2'-S-Methyl**           |         |        |        |        |        |        |
| T7                        | 0.6    | 0.6    | 0.1    | 0      | 0      | 0.5    |
| T8                        | 0.5    | 0.6    | 0.1    | 0      | 0      | 0.4    |
| T9                        | 0.7    | 0.6    | 0.2    | 0.1    | 0.3    | 0.4    |
| **4'-Methyl**             |         |        |        |        |        |        |
| T7                        | 0.5    | 0.3    | 0      | 0      | 0      | 0.5    |
| T8                        | 0.5    | 0.3    | 0      | 0      | 0      | 0.6    |
| T9                        | 0.7    | 0.6    | 0.2    | 0.1    | 0.3    | 0.4    |
| **3'-Methyl**             |         |        |        |        |        |        |
| T7                        | 0.3    | 0.7    | 1.0    | 0.8    | 0      | 0.8    |
| T8                        | 1.6    | 0.8    | 0.7    | 0.1    | 0.1    | 0.8    |
| T9                        | 1.3    | 0.7    | 0.6    | 0.2    | 0      | 0.7    |
| **Part B. Propyl linker** |         |        |        |        |        |        |
| A12                       | 0.6    | 0      | 0      | 0      | 0      | 0.5    |
| G14                       | –      | 0      | 0      | 0      | 0      | 0.6    |
| C15                       | –      | –      | 0      | 0      | 0      | 0.5    |
| **Butyl linker**          |         |        |        |        |        |        |
| A12                       | 0.5    | 0.1    | 0      | 0      | 0      | 0.5    |
| G14                       | –      | 0.1    | 0      | 0      | 0      | 0.6    |
| C15                       | –      | –      | 0.1    | 0      | 0      | 0.5    |
| **Pentyl linker**         |         |        |        |        |        |        |
| A12                       | 0.1    | 0.1    | 0      | 0.5    | 0.9    | 0.6    |
| G14                       | –      | –      | 0.1    | 0      | 0      | 0.5    |
| C15                       | –      | –      | 0.1    | 0      | 0      | 0.5    |
| **Tetrahydrofuran**       |         |        |        |        |        |        |
| A12                       | 0      | 0      | 0      | 0.6    | 0.5    | 0.4    |
| G14                       | –      | 0.5    | 0      | 0.1    | 0      | 0.4    |
| Abasic                    |         |        |        |        |        |        |
| T8                        | 0.6    | 0.3    | 0.2    | 0.3    | 0.2    | 0.6    |
| C10                       | 0.5    | 0.4    | 0.5    | 0.3    | 0.1    | 0.6    |
| C13                       | 0.6    | 0.3    | 0.2    | 0.3    | 0.2    | 0.5    |
| **Ganciclovir**           |         |        |        |        |        |        |
| G14                       | –      | 0.1    | 0.5    | 0.3    | 0.4    | 0.4    |
| 2-Fluoro-6-methylbenzoimidazole | 0      | 0.8    | 0.8    | 0.9    | 0.9    | 0.8    |
| 4-Methylbenzoimidazole    |         |        |        |        |        |        |
| A12                       | 0.7    | 0.7    | 0.9    | 0.9    | 0.5    | 0.8    |
| G14                       | 0.9    | 1.0    | 1.3    | 0.8    | 0.2    | 0.8    |
| C10                       | 1.3    | 1.5    | 1.0    | 1.0    | 0.1    | 0.9    |
| C13                       | 1.3    | 1.1    | 0.8    | 0.7    | 0.5    | 1.1    |
showed only a ±10% difference in initial cleavage rates (Table I), and the effects did not vary as a function of the specific nucleotide modified. For example, the cleavage rates for the ribonucleotide opposing an abasic site were 0.2, 0.5, and 0.2 of those for the control at positions 9–11, respectively. The relative cleavage rates at positions 9 and 11 were comparable even though positions 9 and 11 in the natural substrates were thymidine and cytosine. Moreover, the effects on the cleavage at adjacent sites were comparable. Similar results were observed for other modifications that produced dramatic reductions in the cleavage rates (e.g. 2'-fluoro) and modifications that had little to no effect on the cleavage rates (2'-arafluoro).

The modifications that exhibited the greatest impact on the site-specific cleavage rate for the ribonucleotide opposing the modification also exhibited the broadest effect on the site-specific cleavage rates for surrounding ribonucleotides. The tetrahydrofuran, hydrocarbon linker, 4'-methylthymidine, and abasic deoxyribo nucleotide modifications, which significantly reduced or ablated the site-specific cleavage rates for the ribonucleotide opposing the modification, also showed significantly slower site-specific cleavage rates for the surrounding 3' and 5' ribonucleotides (e.g. positions -2 to +2) that for the unmodified substrate (Table I). Interestingly, with the exception of 4'-methylthymidine, these modifications were predicted to impart the greatest degree of conformational flexibility at the site of the modification. The heteroduplexes containing the pseudouridine, 2'-arafluoropyrimidine, 3'-methylthymidine, and II-stacking deoxyribo nucleotide modifications, which exhibited little to no reduction in the site-specific cleavage rate for the ribonucleotide opposing the modification, also showed only a modest reduction in the site-specific cleavage rates for the surrounding ribonucleotides (Table I). For a majority of the modified deoxyribo nucleotides tested, the influence on the human RNase H1 activity of the adjacent ribonucleotides appeared to be unidirectional (Fig. 1A). For example, 2'-methylthiothymidine, 3'-methylthymidine, 2-thiouridine, 2'-fluorothymidine, and pseudouridine reduced the site-specific cleavage rates for the adjacent 3'-ribonucleotides more significantly than for the 5'-ribonucleotides.

Although there was generally quite a good correlation between the effects on the cleavage rate for the ribonucleotide opposing the modification and the overall cleavage rate, there were interesting exceptions. Consider 2'-thiouridine; this modification at position 8 ablated the cleavage at the opposing site and reduced the relative overall rate to 0.7, whereas the same modification at position 7 reduced the relative site-specific rate to 0.2 of the control and the overall relative rate to 0.5. These results can be explained by different effects on the site-specific cleavage rates for the unmodified substrate and heteroduplexes containing the 4'-methylthymidine modification at position T9 and the 2-thiouridine (S2U) at position T8. The substrates were incubated in the absence and presence of human RNase H1 for 5 min (lanes 2, 8, and 14), 10 min (lanes 3, 9, and 15), 15 min (lanes 4, 10, and 16), 30 min (lanes 5, 11, and 17), and 60 min (lanes 6, 12, and 18). The ribonucleotide positions are numbered accordingly: the ribonucleotide opposing the modification (position 0), the first (position +1) and second (position -2) ribonucleotides 5' to the modification, and the first (position +1) and second (position +2) ribonucleotides 3' to the modification.
specific cleavage rates for adjacent ribonucleotides. In contrast, the effects of the 4'-methylthymidine modifications on the overall rates were less significant than on the site-specific rates for the opposing and surrounding ribonucleotides, and this is due to the fact that the ablated cleavage sites account for approximately half of the total site-specific cleavage rates. These results emphasize the importance of performing both overall and site-specific measurements.

**DISCUSSION**

In this study, we performed a complementary mutational analysis on the structure of the substrate at the catalytic site for human RNase H1. We designed a series of modified heteroduplexes with the modifications positioned within the catalytic site of the substrate (Fig. 1). The modifications consisted of nucleotides exhibiting northern, southern, and eastern sugar conformations; base modifications that II-stack with adjacent nucleotides but do not form hydrogen bonds; abasic deoxynucleotides; internucleotide hydrocarbon linkers ranging from three to five residues; and ganciclovir substitution of the deoxycytosine to determine the role of helical geometry, sugar conformation, bulk in the minor groove, and conformational flexibility within the heteroduplex in human RNase H1 activity.

**Role of Sugar Conformation in the Catalytic Site of the Heteroduplex**—The northern biased deoxyribonucleotides selected for this study included the 2'-fluorothymidine and 2-thiouridine modifications (Fig. 1A). Both modifications lacked bulky 2'-substituents to avoid possible steric interactions with the enzyme. These modifications were determined to influence sugar conformation through distinctly different mechanisms. For example, the highly electronegative fluorine of 2'-fluorothymidine acts in conjunction with the gauche effect to strongly stabilize the sugar in the northern pucker (31). In the case of 2-thiouridine, it has been shown at the dinucleotide level that the highly polarizable sulfur stabilizes the C-3'-endo sugar conformation as well as the stacking interactions with the neighboring nucleotides and imparts stronger hydrogen bonding due to the increased acidity of the N-3 imino proton (44–46). The heteroduplexes containing the northern biased modifications showed significantly slower site-specific cleavage rates for the ribonucleotide opposing the modification (position 0) (Table I, part A). Furthermore, these modified heteroduplexes also exhibited significantly slower site-specific cleavage rates for the adjacent 3'-ribonucleotide, i.e. position +1, whereas little to no reduction for the site-specific cleavage rates was observed for the adjacent 5'-ribonucleotide, i.e. position −1; thus, these modifications appear to influence the structure of the adjacent base pairs in a unidirectional manner (Table I, part A).

In contrast to northern biased deoxyribonucleotides, modified deoxyribonucleotides exhibiting a southern biased sugar pucker more closely mimicked the sugar conformation of native deoxyribonucleotides. Surprisingly, the effects of southern biased deoxyribonucleotides modifications on RNase H1 activity have not been previously investigated. Heteroduplexes containing the 2'-methylthiothymidine modification were poor substrates for human RNase H1, exhibiting significantly slower initial cleavage rates (V0) and site-specific cleavage rates for both the opposing and adjacent ribonucleotides (Table I, part A). The 2'-methylthiothymidine nucleoside was highly southern biased as a result of the electronegativity and steric bulk of the 2'-substituent. The 2'-methylthio substituent potentially posed a similar steric problem for the enzyme as the 2'-alkoxy moieties and may account for the observed loss in human RNase H1 activity. In contrast, the pseudouridine modification had little to no effect on cleavage rates.

Site-directed mutagenesis of the E. coli and human RNase H1 enzymes combined with molecular modeling of the enzyme-substrate complex suggest that the enzyme binds to the minor groove of the heteroduplex substrate via the 2'-hydroxyls of the RNA strand and the phosphate of the DNA strand surrounding the scissile phosphate (Fig. 3) (13, 41, 42). These data suggest that, consistent with the predicted binding model, human RNase H1 likely interacts with the minor groove of the heteroduplex substrate and that a low energy barrier for interconversion of the sugar conformation is preferred.

The nucleotide modifications predicted to mimic the sugar pucker of the deoxyribonucleotide of an RNA/DNA heteroduplex (e.g. heteroduplexes containing the 2'-arafluoropyrimidine and pseudouridine modifications) exhibited cleavage rates comparable with the rates observed for the unmodified substrate (Table I, part A). The 2'-arafluoro modification has been shown by NMR to form the eastern O-4'-endo sugar conformation similar to DNA when hybridized to RNA (27). In addition, the size and position of the 2'-ara substituent, i.e. the fluorine is directed upward and away from the minor groove, are predicted not to sterically interfere with the enzyme. In the case of pseudouridine, the NMR structure indicated a modestly higher southern biased sugar pucker population for the nucleoside due to the influence of the torsion angle of the C–C glycosyl bond and the C-1' and C-2' bond (47). Furthermore, the CD spectra for RNA/ pseudouridine and DNA/pseudouridine heteroduplexes showed an RNA-like C-3'-endo and a DNA-like C-2'-endo sugar pucker for the pseudouridine deoxyribonucleotides, respectively, suggesting that pseudouridine exhibits a conformational flexibility comparable with DNA (48, 49). Apparently, both the eastern sugar pucker and conformational flexibility of the deoxyribonucleotide furanose ring are favored by the enzyme, i.e. locking the sugar north or south resulted in slower cleavage rates.

**Role of the Deoxyribonucleotide Phosphate Groups**—Heteroduplexes containing the 4'-methylthymidine modifications were also poor substrates for human RNase H1, exhibiting initial cleavage rates 2–3-fold slower than those of the unmodified heteroduplex (Table I, part A). 4'-Methylthymidine inhibited the human RNase H1 cleavage of the ribonucleotide opposing the modification and the adjacent 3'-ribonucleotides. The site-specific rates for the adjacent 5'-ribonucleotides were significantly lower than those for the unmodified substrate. In contrast, the heteroduplexes containing the 3'-methyl-modified deoxyribonucleotide, which have been shown to exhibit a sugar conformation similar to that of the 4'-methyl nucleosides, were significantly better substrates for human RNase H1. The observed differences in the human RNase H1 activity for the 4'-methylthymidine and 3'-methylthymidine heteroduplexes may be a function of the position of the 4'-methyl moiety on the furanose ring, which is predicted to contribute bulk in the minor groove, potentially interfering with enzyme binding (38). Furthermore, the loss in human RNase H1 activity observed for the 4'-methylthymidine heteroduplexes suggests that proper orientation of the phosphate group on the deoxyribonucleotide opposing the scissile ribonucleotide is important for human RNase H1 activity.

**Role of Conformational Flexibility in the Catalytic Site of the Heteroduplex**—Conformational flexibility was introduced at the catalytic site of the heteroduplex substrate, with modifications exhibiting incrementally increasing flexibility with the hydrocarbon linkers predicted to exhibit the greatest degree of conformational flexibility, followed by abasic and ganciclovir-modified deoxyribonucleotides and II-stacking deoxyribonucleotides (e.g. 2-fluoro-6-methylbenzimidazole, 4-methylbenzimidazole, and 2,4-difluorotoluyl deoxyribonucleotides) (Fig. 4). W. F. Lima, unpublished data.
Table I (part B) shows that with increased conformational flexibility at the catalytic site, the initial cleavage rates \((V_0)\) and site-specific cleavage rates decreased. For example, the hydrocarbon linkers were predicted to exhibit the greatest degree of conformational flexibility and were among the poorest substrates for RNase H1 activity. The site-specific rates for the ribonucleotide opposing these modifications and the surrounding 3'- and 5'-ribonucleotides were either significantly reduced or ablated, resulting in -2-fold slower initial cleavage rates \((V_0)\) compared with those for the unmodified substrate (Table I). The broad effect on the site-specific rates for the ribonucleotides surrounding the opposing ribonucleotide is likely due to the fact that the conformationally flexible linkers bridge both the 3'- and 5'-deoxyribonucleotides. Interestingly, similar effects on the cleavage rates were observed for all three hydrocarbon linkers even through the linkers ranged in length from three to five carbons, with the propyl and pentyl linkers predicted to be shorter and longer than the length of the native deoxyribonucleotide linkage, respectively, and the butyl linker predicted to most closely approximate the intraphosphate distance of the deoxyribonucleotide. The ganciclovir-modified, abasic, and tetrahydrofuran-modified deoxyribonucleotides were also poor substrates for human RNase H1, although the site-specific cleavage rates of these heteroduplexes were slightly faster than the rates observed for the heteroduplexes containing the hydrocarbon linkers (Table I, part B). Taken together, these data suggest that a conformationally rigid phosphate backbone is required for human RNase H1 activity.

Furthermore, the slight improvement in the cleavage rates observed for the ganciclovir and abasic modifications compared with the hydrocarbon linkers suggests that the furanose ring of the abasic deoxyribonucleotide and the hydrogen bond base pair formation of the ganciclovir modification likely offer modest conformational rigidity to the substrate.

In contrast, the II-stacking deoxyribonucleotides (e.g., 2-fluoro-6-methylbenzoimidazole, 4-methylbenzoimidazole, and 2,4-difluorotoluyl deoxyribonucleotides) better supported human RNase H1 activity (Table I, part B). Comparable initial cleavage rates and site-specific cleavage rates were observed for these heteroduplexes and the unmodified substrate. Interestingly, the site-specific cleavage rates for the second 3'-ribonucleotide were significantly slower, suggesting that a stable hydrogen bond base pair is required 2 bp 5' to the scissile phosphate. The fact that the II-stacking deoxyribonucleotides exhibited site-specific cleavage rates for the ribonucleotide opposing the modification that were comparable with those of the unmodified substrate suggests that these modifications likely form favorable stacking interactions, resulting in a stable helical conformation. In fact, previous studies have shown that the 2-fluoro-6-methylbenzoimidazole deoxyribonucleotide is an effective substitute of native deoxyribonucleotides, and this modification was shown to act as an efficient template for replicating DNA with KF exopolymerase (50).

**Fig. 3. Model for the interaction of RNase H1 with the heteroduplex substrate at the catalytic site.** The designations for ribonucleotide positions are described in the legend to Fig. 2. The positions of the amino acids shown are for *E. coli* RNase H1, and the positions of the conserved amino acids in the human enzyme are shown in parentheses.

Interactions between Enzyme and Substrate at the Catalytic Site—The interactions between RNase H1 and the heteroduplex substrate at the catalytic site have been inferred by molecular modeling and site-directed mutagenesis of *E. coli* and human RNase H1 (Fig. 3) as well as by the crystal structure of the *E. coli* enzyme (42). Specifically, site-directed mutagenesis suggests that the glutamine at position 72 of the *E. coli* enzyme
forms a hydrogen bond with the 2'-hydroxyl of the ribonucleotide at position −1 and that the backbone imino and carbonyl groups of Cys13 function as proton donor and acceptor, respectively, in the hydrogen-bonding interaction with the 2'-hydroxyl of the ribonucleotide at position +1 (42). Asp44 and Asp44 of the enzyme were suggested to bind electrostatically with the phosphates of the deoxyribonucleotides opposing the scissile ribonucleotide and the ribonucleotide at position −1, respectively. The aspartic acid residues at positions 10 and 70 were predicted to bind the 2'-hydroxyl of the scissile ribonucleotide via Mg2+ ion coordination (42). Finally, these amino acid residues are conserved in human RNase H1 and have been shown by site-directed mutagenesis to be required for activity (14, 43).

The loss of human RNase H1 activity observed for the heteroduplexes containing a southern biased 2'-methylthioymidine modification and the lack of cleavage observed for the northern biased 2'-alkoxy-modified heteroduplexes are consistent with the predicted binding site for the enzyme (22, 23, 28). Together, these data suggest that, irrespective of sugar conformation, bulky 2'-substituents positioned in the minor groove of the heteroduplex substrate interfere with human RNase H1 cleavage. Similarly, the 2-thio substitution of 2-thiouridine is predicted to be situated within the minor groove of the heteroduplex, and the slower cleavage rates observed for these heteroduplexes may be the result of either sulfur steric interference with the enzyme or its strong electronegative properties.

The modified heteroduplexes examined here suggest that the width of the minor groove of the heteroduplex substrate is important for RNase H1 catalysis and that variations in minor groove width as a function of sugar pucker appear to obliterate the proper positioning of the enzyme on the heteroduplex substrate. RNA/DNA heteroduplexes are unique in that these duplexes exhibit a minor groove width of −9 Å, which is approximately midway between the minor groove widths for the A-form (∼11 Å) and B-form (∼6 Å) duplexes (24–26). Heteroduplexes containing the 2'-arafluoro deoxyribonucleotides, which produce a minor groove width comparable with that of the RNA/DNA heteroduplex, exhibited comparable human RNase H1 cleavage rates. On the other hand, deoxyribonucleotide modifications exhibiting a northern biased sugar conformation (e.g. 2'-thiothymidine) reduced human RNase H1 activity. Consequently, the wider minor groove generated by these modifications likely precludes the associated metal ion coordination of the enzyme with the 2'-hydroxyl of the scissile ribonucleotide and electrostatic interaction with the phosphate of the modified deoxyribonucleotide (Fig. 3) (42). Similarly, a wider minor groove could account for the observed reduction in the site-specific rates for the adjacent 3'-ribonucleotide by preventing the formation of the putative hydrogen bond between the enzyme and the 2'-hydroxyl of the ribonucleotide at position +1 and the electrostatic interaction with the phosphate of the opposing deoxyribonucleotide (Fig. 3). Consistent with these observations and the proposed model for the interaction of the enzyme with the heteroduplex substrate at the catalytic site, the significantly slower cleavage rates observed for the 4'-methylthymidine heteroduplexes suggest that proper positioning of the deoxyribonucleotide phosphate opposing the scissile ribonucleotide is critical for human RNase H1 cleavage. In addition, these observations suggest that the human RNase H1 activity associated with the deoxyribonucleotides exhibiting the northern versus southern biased sugar conformations is likely the result of differences in the relative positions of the inter- and intranucleotide phosphates on the heteroduplex substrate. Last, the cleavage rates observed for the 3'-methylthymidine-modified heteroduplexes suggest minor groove widths that are narrower than those of the RNA/DNA heteroduplex are tolerated better compared with wider minor grooves by human RNase H1.

Conformational flexibility of the deoxyribose also appears to be an important structural feature of the heteroduplex substrate for human RNase H1 activity. The preferred eastern O-4-endo sugar pucker observed for the DNA strand of the heteroduplex is the result of the nearly symmetrical potential energy barrier for both southern and northern sugar conformations exhibited by deoxyribonucleotides. Both the pseudouridine and 2'-arafluoro deoxyribonucleotides exhibited conformational flexibility in the sugar, and the heteroduplexes containing these modifications showed cleavage rates comparable with those of the unmodified substrate. Furthermore, modifications exhibiting strong conformationally biased sugars (e.g. 2'-fluoro deoxyribonucleotides) were less efficiently cleaved by the enzyme.

 Whereas conformational flexibility of the deoxyribose was preferred, flexibility in the phosphate backbone of the DNA strand inhibited human RNase H1 activity. Modifications such as the hydrocarbon linkers and abasic deoxyribonucleotides that permit free rotation of the phosphate moiety were shown to inhibit human RNase H1 activity. Again, these data suggest that proper positioning of the phosphate groups of the deoxyribonucleotide, presumably for electrostatic contact with the enzyme, is essential for human RNase H1 catalysis. The cleavage rates observed for the II-stacking deoxyribonucleotides suggest that stable base stacking independent of hydrogen bond formation between the bases at the catalytic site offers sufficient rigidity to the phosphate backbone. Taken together, these data suggest that variation in sugar conformation is significantly better tolerated by human RNase H1 than conformational flexibility in the phosphate backbone.

Previous studies have shown that nucleotides exhibiting conformationally biased sugars can bias the sugar conformation of the surrounding deoxyribonucleotides. For example, the NMR structures of chimeric RNA-DNA/RNA heteroduplexes show that the deoxyribonucleotides adjacent to the RNA of the chimeric strand adopt the northern pucker of the RNA (51). The transmission the northern sugar conformation of the RNA into the adjacent deoxyribonucleotides is likely due the intrinsically flexible nature of the deoxyribose sugar. Furthermore, these data suggest that modifications resulting in higher conformationally biased sugar populations would have a greater influence on the structure of the surrounding deoxyribonucleotides and consequently a greater impact on human RNase H1 activity. The impact of the highly northern biased 2'-fluoro deoxyribonucleotide modification on the human RNase H1 activity of the surrounding ribonucleotides shown here suggests that conformational transmission exhibits a modest influence on human RNase H1 activity compared with other factors such as steric bulk in the minor groove and conformational flexibility within the phosphate backbone. It is important to note that the heteroduplexes examined here contained single nucleotide substitutions with a conformationally biased sugar. We have observed that substituting contiguous stretches of modified nucleotides with conformationally biased sugars exhibited a greater influence on the human RNase H1 activity against adjacent deoxyribonucleotides (data not shown).

The structure of human RNase H1 shows that, in addition to the catalytic domain shared with the E. coli homolog, human RNase H1 contains an RNA-binding domain at the N terminus of the protein (9). Human RNase H1 appears to identify the first 3'-DNA/5'-RNA base pair to achieve the proper positioning of the catalytic domain slightly less than one helical turn from the RNA-binding domain (21). Only when the enzyme is
bound at the correct site and the helical geometry is appropriate will the catalytic unit be positioned appropriately to cleave the RNA. As a result, altering the local helical geometry (e.g. altering the minor groove width or the inter- and intranucleotide phosphate distances) at the catalytic site on the heteroduplex may have a global effect on the precise positioning of the catalytic region with respect to the RNA-binding domain of human RNase H1 required for catalysis. Because the enzyme is predicted to position the catalytic domain 3' on the RNA relative to the RNA-binding domain, consistent with the results presented here, a local change in duplex geometry at the catalytic site on the substrate would impair the human RNase H1 activity.

**Implications for the Design of Antisense Oligonucleotides**

The demonstration that human RNase H1 plays a dominant role in the activities of DNA-like ASOs suggests that additional studies exploring the substrate preferences, enzymology, and regulatory processes for RNase H1 should support improved design of antisense agents. The demonstration that increases in RNase H1 activity correlated with increases in potency suggests that recruitment of RNase H1 to the ASO/RNA duplex and/or cleavage of the RNA by the enzyme is limiting for ASO activity. Any strategy that would improve these processes should improve ASO potency (13).

In this regard, the results presented here suggest that the preferred properties for the modified oligodeoxyribonucleotide include 1) a conformationally flexible sugar producing an O-4'-endo pucker when hybridized to RNA, 2) no sterically bulky 2'-substituents, and 3) a conformationally rigid phosphate backbone. Clearly, the 2'-arafuoro-modified, pseudouridine-modified, 3'-methyl-modified, and H-stacking deoxyribonucleotides exhibit many of these qualities. In light of the fact that none of the modifications tested were shown to enhance human RNase H1 activity compared with native deoxyribonucleotides and that these modifications offer no clear advantage over native deoxyribonucleotides with respect to either duplex stability or nuclease resistance, other strategies to improve the potency of ASOs should be considered. For example, the calculated placement of these modifications in chimeric ASOs may be an effective means to improve human RNase H1 activity by potentially blocking the conformational transmission of 2'-alkoxy deoxyribonucleotide into the deoxyribonucleotide region of the chimeric ASO. Work is in progress to test these ideas.

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Structural Requirements at the Catalytic Site of the Heteroduplex Substrate for Human RNase H1 Catalysis
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