Human RNase H1 Uses One Tryptophan and Two Lysines to Position the Enzyme at the 3'-DNA/5'-RNA Terminus of the Heteroduplex Substrate*

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In a previous study, we showed that the RNA-binding domain of human RNase H1 is responsible for the positional preference for cleavage exhibited by the enzyme (Wu, H., Lima, W. F., and Crooke, S. T. (2001) J. Biol. Chem. 276, 23547–23553). Here, we identify the substituents on the heteroduplex substrate and the amino acid residues within the RNA-binding domain of human RNase H1 involved in positioning of the enzyme. The human RNase H1 cleavage patterns observed for heteroduplexes with various 3'-DNA/5'-RNA and 5'-DNA/3'-RNA termini indicate that the 5'-most cleavage site on the oligoribonucleotide is positioned 7 bp from the first 3'-DNA/5'-RNA base pair. The presence or absence of phosphate or hydroxyl groups at either the 3'-DNA or 5'-RNA terminus had no effect on the human RNase H1 cleavage pattern. Substitution of the 3'-deoxynucleotide with a ribonucleotide, 2'-methoxyethyl nucleotide, or mismatched deoxyribonucleotide resulted in the ablation of the 5'-most cleavage site on the oligoribonucleotide. Mutants in which Trp^{43} and Lys^{59}-Lys^{60} of the RNA-binding domain were substituted with alanine showed a loss of the positional preference for cleavage. Comparison of the $k_{cat}$, $K_m$, and $K_r$ for the alanine-substituted mutants with those for human RNase H1 suggests that Lys^{59} and Lys^{60} are involved in binding to the heteroduplex and that Trp^{43} is responsible for properly positioning the enzyme on the substrate for catalysis. These data suggest that Trp^{43}, Lys^{59}, and Lys^{60} constitute an extended nucleic binding surface for the RNA-binding domain of human RNase H1, with the entire interaction taking place at the 3'-DNA/5'-RNA pole of the heteroduplex. These results offer further insights into the interaction between human RNase H1 and the heteroduplex substrate as well as approaches to enhance the design of effective 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. Mg^{2+} and Mn^{2+}) 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, Escherichia coli, and mouse (9). The human RNase H2 enzyme is a 299-amino acid protein with a calculated mass of 33.4 kDa and has also been shown to be ubiquitously expressed in human cells and tissues (10). Human RNase H2 shares strong amino acid sequence homology with RNase H2 from Caenorhabditis elegans, yeast, and E. coli (10).

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 amino terminus of the protein and separated from the conserved catalytic domain by a 62-amino acid spacer region (12–14). 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 (12, 15–18). Site-directed mutagenesis of human RNase H1 revealed that the spacer region is required for RNase H activity, whereas the RNA-binding domain is not required for RNase H activity, i.e. RNA-binding domain deletion mutants exhibit RNase H activities comparable to those of the wild-type enzyme (12).

The RNA-binding domain of human RNase H1 is conserved in other eukaryotic RNases H1 (Fig. 1A) (9, 14). The highly conserved lysines at positions 59 and 60 of the human enzyme have been shown in a 46-amino acid peptide of Saccaromyces cerevisiae RNase H1 to be important for binding to the heteroduplex substrate (13). The NMR structure of the RNA-binding domain of S. cerevisiae RNase H1 consists of a triple-stranded antiparallel β-sheet sandwiched between two α-helices and shares strong structural similarities with the N-terminal domain of the ribosomal RNA-binding protein L9 (14, 19). The conserved lysine residues are located within the third β-strand. In addition, a highly conserved tryptophan at position 22 was shown to project outwards. A solvent-exposed aromatic amino acid at this position was also observed in the L9 protein and has been shown to be important for binding to 23 S ribosomal RNA (19).

The role of the RNA-binding domain of human RNase H1 is not known. 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, i.e. human RNase

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1 H. Wu, unpublished data.
H1 cleaves the heteroduplex substrate between 7 and 12 nucleotides from the 3′-DNA/5′-RNA terminus (12). The positional preference for cleavage exhibited by the enzyme is consistent with the proposed biological role for RNase H1, viz. the generation and/or removal of RNA primers during lagging-strand DNA replication (20–21). The average length of the RNA primers (e.g. 7–14 ribonucleotides) correlates well with the observed cleavage specificity exhibited by human RNase H1 and suggests one possible role for the RNA-binding domain (22). Clearly, understanding how the RNA-binding domain recognizes the 5′-RNA/3′-DNA terminus of the heteroduplex substrate and positions the enzyme to cleave 7–12 nucleotides downstream may provide further insights into the role of the RNA-binding domain with respect to RNase H1 activity, the proposed biological role, and the design of antisense oligonucleotides.

In this study we prepared heteroduplex substrates with various 3′-DNA/5′-RNA and 5′-DNA/3′-RNA termini as well as modified 3′-DNA/5′-RNA termini to identify the substituents on the substrate important for positioning of human RNase H1. In addition, mutants of the highly conserved Trp43, Lys59, and Lys60 residues of the RNA-binding domain of human RNase H1 were substituted with alanine to determine the role of these amino acids with respect to the positional preference for cleavage exhibited by the enzyme.

MATERIALS AND METHODS

Construction of Human RNase H1 Mutants—The mutagenesis of human RNase H1 was performed using a PCR-based technique derived from Landt et al. (23). Briefly, two separate PCRs were performed using a set of site-directed mutagenic primers and two vector-specific primers (24). The RNase H1(W43A) mutant, the 5′-oligodeoxynucleotide used for PCR was TTTTGACCTTACGATGCTCCACAGGACAC, and the 3′-oligodeoxynucleotide used was CACTCATTTTGATCCTGGCAGAAAGACCCG. The PCR primers for the RNase H1(K59A,K60A) mutant were 5′-CCAGATTTGCTGCTTTTGCCACAGAGGATG and 3′-AGCAGCAAATCTGGCAGCAGGA. For the RNase H1(W43A,K59A,K60A) mutant, the PCR product from the RNase H1(W43A) mutant was used as a template for a second round of PCR with the primers used for the RNase H1(K59A,K60A) mutant. Two rounds of PCR were performed as previously described (24). The final PCR product was purified and cloned into the expression vector pET17b (Novagen), resulting in His-tagged fusion proteins (24). The incorporation of the desired mutation was confirmed by DNA sequencing.

Preparation of Human RNase H1 Proteins—the plasmids were transfected into E. coli BL21 (DE3) (Novagen). The bacteria were grown in Terrific Broth (Bio 101, Inc.) at 37 °C and harvested at A660n = 1.2. The cells were induced with 1 mM isopropanol-β-d-thiogalactopyranoside at 37 °C for 2 h. The cells were lysed in 6 M 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 1 ml of nickel-nitrilotriacetic acid Super Flow beads (QIAGEN Inc.), 50 ml of lysisate.

The nickel-nitrilotriacetic acid medium was packed into an FPLC column, and the RNase H1 proteins were partially purified with sequential gradients (flow rate, 5 ml/min; buffer A, 100 mM sodium phosphate, 10 mM Tris-HCl, and 8 M urea (pH 6.3); buffer B, 100 mM sodium phosphate, 10 mM Tris-HCl, 2 mM urea, and 200 mM NaCl (pH 7.0)). The eluent was further purified by ion exchange FPLC (Mono S column; flow rate, 1 ml/min; buffer A, 20 mM sodium phosphate, 2 mM urea, and 200 mM NaCl (pH 7.0)); 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 FPLC (Resource RPC column; flow rate, 1 ml/min; buffer A, 2% acetonitrile in deionized H2O and 0.05% trifluoroacetic acid; buffer B, 80% acetonitrile in deionized H2O and 0.05% trifluoroacetic acid). Fractions were lyophilized, resuspended in deionized H2O, and analyzed by SDS-PAGE (25).

Synthesis of Oligonucleotides—The oligoribonucleotides were synthesized on a PE-ABI 380B synthesizer using 5′-O-silyl-2′-O-bis(2-ace- toxyethoxy)methyl ribonucleoside phosphoramidites and procedures described elsewhere (26). The oligoribonucleotides were purified by reverse-phase high pressure liquid chromatography. The DNA oligonucleotides were synthesized on a PE-ABI 380B automated DNA synthesizer by standard phosphoramidite chemistry. The DNA oligonucleotides were purified by precipitation twice from 0.5 M NaCl with 2.5 volumes of ethyl alcohol. The 3′-modified oligonucleotides were synthesized with controlled pore glass (Glen Research Corp., Sterling, VA) containing either the 3′-deoxyoligonucleotide or 2′,3′-dideoxyoligonucleotide.

Preparation of 32P-Labeled Substrate—The RNA substrate was 5′-end-labeled with 32P using 20 units of T4 polynucleotide kinase (Promega), 120 pmol of [γ-32P]ATP (7000 Ci/mmol; ICN), 40 pmol of RNA, 70 mM Tris (pH 7.6), 10 mM MgCl2, and 50 mM dithiothreitol. The kinase reaction was incubated at 37 °C for 30 min. The labeled oligoribonucleotide was purified by electrophoresis on a 12% denaturing polyacrylamide gel (27). The specific activity of the labeled oligoribonucleotide is ~3000–8000 cpm/fmol.

Preparation of the Heteroduplex—The heteroduplex substrate was prepared in 100 µl containing unlabeled oligoribonucleotide ranging from 100 to 1000 nM, 105 cpm/µl labeled oligoribonucleotide, and 2-fold excess complementary oligodeoxynucleotide, and hybridization buffer (20 mM Tris (pH 7.5) and 20 mM KCl). Reactions were heated at 90 °C for 5 min and cooled to 37 °C, and 60 units of Prime RNase inhibitor (5′ Prime Inc., Boulder, CO) and MgCl2 at a final concentration of 1 mM were added. Hybridization reactions were incubated for 2–16 h at 37 °C, and 1 mM tris(2-carboxyethyl)phosphine was added.

Multiple-turnover Kinetics—The human RNase H1 proteins were incubated with dilution buffer (50 mM Tris, 50 mM NaCl, and 100 µM tris(2-carboxyethyl)phosphine (pH 7.5)) for 1 h at 24 °C. The heteroduplex substrate was digested with 0.4 ng of enzyme at 37 °C. A 10-µl aliquot of the cleavage reaction was removed at 30 s intervals ranging from 2 to 120 min and quenched by adding 5 µl of stop solution (8 M urea and 120 mM EDTA). The aliquots were heated at 90 °C for 2 min and resolved on a 12% denaturing polyacrylamide gel, and the substrate...
and product bands were quantitated on an Amersham Biosciences PhosphorImager. The concentration of the converted product was plotted as a function of time. The initial cleavage rate was obtained from the slope (moles of RNA cleaved per min) of the best fit line for the linear portion of the plot, which comprises, in general, <10% of the total reaction and data from at least five time points. The initial cleavage rates were plotted as a function of the substrate concentration (n ≥ 4), and the data were fit to the Michaelis-Menten equation using the program Ultrafit (Biosoft). The $K_m$ corresponds to the heteroduplex substrate concentration at half-maximum rate, and $V_{max} = V_{cat}/$[total RNase H1], where $V_{max}$ corresponds to the horizontal asymptote of the hyperbolic curve.

**Single-turnover Experiments**—Reactions were prepared and analyzed as described for the multiple-turnover kinetics with the exception that excess human RNase H1 proteins ranging in concentration from 100 to 500 nM were used. The reactions were quenched, analyzed, and quantitated from 100 to 500 nM. The competing non-cleavable substrate analog oligoribonucleotide and oligodeoxyribonucleotide ranging in concentration from 250 to 500 nM were used. The percent RNA cleaved for each site corresponds to the fraction of converted product for each site multiplied by 100.

**Determination of Dissociation Constants ($K_d$)**—Binding affinities were determined by inhibition analysis (28). Here, the cleavage rate was determined for the heteroduplex substrate at a variety of concentrations in both the presence and absence of a competing non-cleavable substrate analog. The heteroduplex substrate was prepared as described above, except in a final volume of 50 μl and with equimolar oligoribonucleotide and oligodeoxyribonucleotide ranging in concentration from 100 to 500 nM. The competing non-cleavable substrate analog was prepared in 50 μl of hybridization buffer containing equimolar oligoribonucleotide and complementary 2'-fluoro-modified oligo-nucleotide. The concentration of the non-cleavable substrate analog was in excess of the heteroduplex substrate and ranged from 0.5 to 1 μM. Reactions were heated at 90 °C for 5 min and cooled to 37 °C. Prime RNase inhibitor and MgCl₂ were added to the reactions as described above. Reactions were incubated at 37 °C for 2–16 h, and the non-cleavable substrate analog was added to the heteroduplex substrate. Tris(2-carboxyethyl)phosphine was added at a final concentration of 100 μM, and the combined reaction was digested with 0.4 ng of human RNase H1 proteins. The reactions were quenched, analyzed, and quantitated as described for multiple-turnover kinetics.

**RESULTS**

The human RNase H1 site-specific cleavage rates for the heteroduplexes containing a 25-mer oligoribonucleotide and complementary oligodeoxyribonucleotides ranging in length from 19 to 26 nucleotides are shown in Fig. 2. These heteroduplexes contained single-strand RNA overhangs at either the 3′-terminus (Fig. 2A) or 5′-terminus (Fig. 2B). Consistent with previous observations (29), the predominant human RNase H1 site-specific cleavages were observed between 7 and 10 nucleotides from the 5′ terminus of the oligoribonucleotide (Fig. 2). Varying the length of the heteroduplex by shortening the 5′ terminus of the oligodeoxyribonucleotide and effectively increasing the length of the 3′-RNA overhang had no effect on the either the positions or rates of the site-specific cleavages (Fig. 2A). In contrast, shortening the length of the heteroduplex from the 3′ terminus of the oligodeoxyribonucleotide affected both the positions and rates of the site-specific cleavages (Fig. 2B). For example, removing a nucleotide at the 3′ terminus of the oligodeoxyribonucleotide resulted in the ablation of the 5′-site-specific cleavage on the oligoribonucleotide and a 3′-shift in the cleavage pattern. The elimination of additional terminal 3′-nucleotides from the oligodeoxyribonucleotide resulted in similar 3′-shifts in the cleavage pattern. As a result, a fixed distance of 7 bp was observed between the 5′-RNA/3′-DNA terminus and the 5′-site-specific cleavage.

Heteroduplexes in which the 3′-DNA/5′-RNA termini contained either a phosphate or hydroxyl group on either the oligodeoxyribonucleotide or oligoribonucleotide exhibited similar cleavage patterns (Fig. 3, substrates A–E). Substitution of the terminal 3′-nucleotide of the oligodeoxyribonucleotide with either a ribonucleotide or 2′-methoxyethyl-modified nucleotide resulted in the ablation of the 5′-site-specific cleavage on the oligoribonucleotide (Fig. 3, substrates F–I). A mismatch at the 3′-DNA/5′-RNA terminus also resulted in the loss of the 5′-site-specific cleavage on the oligoribonucleotide (Fig. 3, substrate J). These data suggest that the 5′-site-specific cleavage by human RNase H1 is determined by the first deoxyribonucleotide/ribonucleotide base pair on the 3′-DNA/5′-RNA terminus of the heteroduplex. Furthermore, these data show that the enzyme recognizes a DNA/RNA base pair as different from an RNA/RNA or 2′-modified nucleotide/RNA base pair and that neither phosphate nor hydroxyl groups at the 3′-DNA/5′-RNA terminus are used to position the enzyme.

To identify the amino acids responsible for the strong positional preference for cleavage exhibited by human RNase H1, three mutants were prepared in which alanine was substituted for the conserved lysine residues at positions 59 and 60 (RNase H1(K59A,K60A)), the conserved tryptophan at position 43 (RNase H1(W43A)), and all three conserved amino acids (RNase H1(W43A,K59A,K60A)) (Fig. 1B). The cleavage patterns for the wild-type enzyme as well as the three RNase H1 mutants were determined by measuring the percent RNA cleaved for each position on the heteroduplex. To ensure that the observed cleavage pattern was the result of primary cleavages, the assay was performed under single-turnover kinetics with the enzyme concentration in excess of the substrate concentration. The positions of the cleavage sites for wild-type human RNase H1 under single-turnover kinetics were consistent with the positions of the cleavage sites observed for the wild-type enzyme under multiple-turnover kinetics (Fig. 4). Furthermore, as would be expected, the positional preference for cleavage was more pronounced under single-turnover kinetics (e.g. the majority of RNase H1 cleavages occurred within 7–8 nucleotides from the 5′ terminus of the RNA). All three human RNase H1 mutants exhibited broader cleavage patterns than the wild-type human enzyme (Fig. 4). The RNase H1(W43A,K59A,K60A) mutant (Fig. 4d) exhibited the broadest cleavage pattern, with cleavage sites ranging from 6 to 16 nucleotides from the 5′ terminus of the oligoribonucleotide. In fact, the RNase H1(W43A,K59A,K60A) mutant did not appear to exhibit a positional preference for cleavage (e.g. the cleavage rates at 15 and 17 nucleotides from the 5′ terminus of the oligoribonucleotide were comparable to the cleavage rates at the preferred sites 7–9 nucleotides from the 5′ terminus of the oligoribonucleotide). Thus, its behavior is comparable to the behavior of a human RNase H1 mutant in which the RNA-binding domain is deleted (12).

The kinetic constants for wild-type and mutant human RNase H1 enzymes are shown in Table I. Both the RNase H1(K59A,K60A) and RNase H1(W43A,K59A,K60A) mutants exhibited $K_m$ values 2-fold higher than the $K_m$ for human RNase H1. Comparably increases in the dissociation constants ($K_d$) for the heteroduplex were observed for the RNase H1(K59A,K60A) and RNase H1(W43A,K59A,K60A) mutants compared with the wild-type enzyme. A modest reduction in the catalytic rate ($k_{cat}$) was observed for both mutants, resulting in an ~3-fold reduction in the bimolecular rate constant ($k_{cat}/K_m$) compared with the wild-type enzyme (Table I). Conversely, the RNase H1(W43A) mutant exhibited a significantly lower $K_m$ and $k_{cat}$ compared with human RNase H1 (Table I). Finally, the RNase H1(W43A) mutant appeared to bind to the heteroduplex with an affinity comparable to that of the wild-type enzyme (Table I).

**DISCUSSION**

Human RNase H1 has been shown to exhibit a strong positional preference for cleavage in comparison with *E. coli* RNase H1 (29). The human enzyme predominately cleaved the heteroduplex substrate 7–10 nucleotides from the 5′-RNA/3′-DNA terminus, whereas *E. coli* RNase H1 exhibits a broader cleavage pattern. The RNA-binding domain of human RNase H1...
Fig. 2. Human RNase H1 site-specific cleavage rates for heteroduplex substrates containing various 3'-DNA/5'-RNA and 5'-DNA/3'-RNA termini. The abscissas report the sequence positions of RNase H cleavage sites. Oligoribonucleotide sequences are reported 5' to 3'. Underlined sequences correspond to the positions of the complementary oligodeoxyribonucleotides. A and B, initial cleavage rates ($V_0$) for human RNase H1 (solid bars) at each position on heteroduplexes with various 5'-DNA/3'-RNA and 3'-DNA/5'-RNA termini, respectively. Arrows indicate the position of each additional 5'-cleavage site on the RNA as a result of the addition of a 3'-deoxynucleotide to the complementary oligodeoxyribonucleotide. Initial rate measurements ($V_0$) for each site on the heteroduplex were determined as described for multiple-turnover kinetics under "Material and Methods." The $V_0$ values are an average of three measurements with estimated errors of coefficient of variation < 10%.
was shown to be responsible for the positional preference for cleavage exhibited by the human enzyme, as deletion of this domain resulted in an *E. coli* RNase H1 cleavage pattern (12). These data also suggest that, based on the positional preference for cleavage exhibited by the human enzyme, the RNA-binding domain likely interacts with the 3′/DNA/5′-RNA minus of the heteroduplex.

Consistent with these observations, we have shown, under multiple-turnover kinetics, that human RNase H1 exhibited a similar positional preference for cleavage for a variety of heteroduplex sequences (Fig. 2). The positions of the 3′-DNA/5′-RNA and 5′-DNA/3′-RNA termini were varied on a 25-nucleotide oligoribonucleotide by truncating the 3′ or 5′ terminus, respectively, of the complementary oligodeoxyribonucleotide. The resulting heteroduplexes ranged in length from 19 to 25 nucleotides and contained either 3′- or 5′-RNA overhangs. In addition, a 26-mer oligodeoxyribonucleotide was used to generate a 25-mer heteroduplex with a single 3′-deoxynucleotide overhang (Fig. 2B). The human RNase H1 cleavage patterns observed for these heteroduplexes indicate that the 5′-site-specific cleavage on the oligoribonucleotide is positioned 7 bp from the 3′-DNA/5′-RNA base pair. The position of the 5′-DNA/3′-RNA terminus appeared to have no affect on the positional preference for cleavage, as the fixed 7-bp distance

| Substrate | X       | Y       | B       | R       | R       | R       | B       |
|-----------|---------|---------|---------|---------|---------|---------|---------|
| A         | OH      | H       | T       | OH      | OH      | OH      | A       |
| B         | OH      | H       | T       | P=O     | OH      | OH      | A       |
| C         | P=O     | H       | T       | OH      | OH      | OH      | A       |
| D         | P=O     | H       | T       | P=O     | OH      | OH      | A       |
| E         | H       | H       | T       | P=O     | OH      | OH      | A       |
| F         | OH      | OH      | U       | P=O     | OH      | OH      | A       |
| G         | P=O     | OH      | U       | P=O     | OH      | OH      | A       |
| H         | H       | OH      | U       | P=O     | OH      | OH      | A       |
| I         | P=O     | MOE     | T       | P=O     | OH      | OH      | A       |
| J         | OH      | H       | A       | P=O     | OH      | OH      | A       |

![Diagram](image_url)

**Fig. 3.** Positions of human RNase H1 cleavage sites on heteroduplex substrates with modified 3′-DNA/5′-RNA termini. A, the 3′-DNA terminus (X) includes modifications at the 3′-position (R1) and 2′-position (R2) on the sugar as well as the base (B). The 5′-RNA terminus (Y) includes modifications at the 5′-position (R3) and 2′-position (R3) on the sugar. B, the arrows indicate the sites of human RNase H1 cleavage, and the size of the arrows reflects the relative cleavage intensities. Substrates A–E resulted in a cleavage pattern with the 5′-cleavage site on the oligoribonucleotide 7 bp from the 3′-DNA/5′-RNA terminus of the heteroduplex. Substrates F–J resulted in a cleavage pattern with the 5′-cleavage site on the oligoribonucleotide 8 bp from the 3′-DNA/5′-RNA terminus of the heteroduplex. The numbers above the first arrows indicate the number of nucleotides cleaved, counting from the 5′ terminus of the oligoribonucleotide.
from the 3′-DNA/5′-RNA terminus to the 5′-site-specific cleavage was maintained. The presence of 5′-RNA overhangs had no effect on the position of the 5′-site-specific cleavage. In contrast, a single 3′-DNA overhang appeared to result in an additional 5′-cleavage site, although the initial rate for this site was slow relative to the rates observed for other cleavage sites within this heteroduplex.

Under single-turnover kinetics, a pronounced positional preference for cleavage was observed (Fig. 4). The single-turnover reactions were performed with the enzyme in excess of the heteroduplex and therefore measured a single interaction between the enzyme and substrate. The fact that the positional preference was more pronounced under these conditions suggests that, given a single interaction between human RNase H1 and the heteroduplex, the majority of the RNase H1 proteins bound to the heteroduplex in such a manner as to cleave the substrate 7–10 nucleotides from the 5′-RNA.

The substitution of the 3′-deoxyribonucleotide with either a ribonucleotide or 2′-methoxyethyl nucleotide resulted in the ablation of the 5′-site-specific cleavage (Fig. 3, substrates F–I). Both the ribonucleotide and 2′-methoxyethyl nucleotide result in a C4′-endo-sugar conformation, which produces an A-form helical conformation when hybridized to RNA. This conformation differs from the helical conformation observed for RNA/DNA heteroduplexes (30). The deoxyribonucleotides of the RNA/DNA heteroduplex form predominantly an O4′-endo-sugar conformation, resulting in a narrower minor groove and a wider major groove compared with the A-form duplex (30). Therefore, the loss of the 5′-site-specific cleavage on the oligoribonucleotide resulting from the substitution of the 3′-deoxyribonucleotide with either a ribonucleotide or 2′-methoxyethyl nucleotide may be due to a loss of enzyme binding at the

![Graphs showing site-specific cleavages for human RNase H1 and RNA-binding domain mutants.](image-url)
The observed positional preference for cleavage exhibited by human RNase H1 correlates well with the proposed biological role for this enzyme. A recent study clearly demonstrated that the homologous mouse RNase H1 enzyme is required for mitochondrial DNA replication and plays a critical role in embryonic development (20). The mouse enzyme is believed to participate in the generation and/or removal of RNA primers during lagging-strand DNA synthesis. These RNA primers form chimeric structures consisting of a 7–14-ribonucleotide region at the 5′ terminus and contiguous stretches of DNA extending in the 3′ direction (22). Based on the positional preference for cleavage exhibited by human RNase H1, cleavage of the chimeric structure would occur at or near the RNA/DNA junction, effectively removing the RNA primer. Alternatively, human RNase H1 cleavage of the RNA/DNA heteroduplex formed during DNA replication would produce the observed 7–14-ribonucleotide primers for lagging-strand DNA synthesis.

The RNA-binding domain of human RNase H1 is highly conserved in eukaryotic RNases H1 (Fig. 1A) (9, 14). This domain contains three highly conserved amino acid residues (Trp43, Lys59, and Lys60), which are likely to have a functional role (Fig. 1B). Mutants were prepared in which Trp43, Lys59, and Lys60, and all three conserved amino acid residues were substituted with alanine (RNase H1(W43A), RNase H1(K59A, K60A), and RNase H1(W43A,K59A,K60A), respectively) (Fig. 1B). The cleavage patterns for the three mutants show an attenuation of the positional preference for cleavage exhibited by the wild-type enzyme (Fig. 4). The RNase H1(W43A,K59A, K60A) mutant resulted in a broader cleavage pattern compared with the wild-type enzyme (Table I). These observations are consistent with the proposed role of the lysine residues of yeast RNase H1, which have been shown to be involved in binding to the heteroduplex (13).

The NMR structure for the 46-amino acid peptide of the RNA-binding domain of S. cerevisiae RNase H1 shows the conserved lysine residues (Lys38 and Lys39) positioned within the third β-strand of the triple-stranded β-sheet (14). The side chain amine groups of Lys38 and Lys39 were shown to project in an opposite direction on the β-strand. Furthermore, the distance spanning the side chain nitrogens is ~14 Å, and this distance correlates well with the width of the minor groove of the heteroduplex, a region predicted to be the binding site for the enzyme (31). The interstrand phosphate–phosphate distance across the minor groove is estimated at ~19 Å, placing the non-bridging oxygens of the phosphates within hydrogen bond distances of the conserved lysine residues. Therefore, the lysine residues likely form electrostatic interactions with the phosphates of the heteroduplex backbone within the minor groove of the substrate, the major groove being too narrow for the span of the amine groups on the conserved lysines. Within the minor groove, several binding modalities are possible, with the lysines interacting with phosphate groups of the same base pair, of adjacent base pairs, or within the same strand.

The tryptophan residue (Trp43) within the RNA-binding domain of human RNase H1 appears to contribute to heteroduplex binding, as the RNase H1(W43A,K60A) and RNase H1(W43A,K59A,K60A) mutants exhibited a 2-fold greater dissociation constant (Kd) for the heteroduplex compared with the human enzyme (Table I). In addition, a 2-fold increase in the Kd was observed for these mutants compared with human RNase H1. The increase in the Kd for the RNase H1(K59A,K60A) and RNase H1(W43A,K59A,K60A) mutants is consistent with the previously reported 2-fold increase in the Kd for the RNA-binding domain deletion mutant of human RNase H1 (12). The 5-fold increase in the Kd reported for the RNA-binding domain deletion mutant of human RNase H1 versus the 2-fold increase in the Kd for the RNase H1(K59A,K60A) and RNase H1(W43A,K59A,K60A) mutants suggests that additional amino acid residues within the RNA-binding domain of the human enzyme may contribute to the positioning of the RNA-binding and catalytic domains of human RNase H1 on the heteroduplex substrate. A, each observed cleavage site on the RNA is coupled to a specific binding interaction between the RNA-binding domain (RNA-BD) and the 3′-DNA/5′-RNA pole of the heteroduplex.

Instead of contributing to substrate binding, as these modifications are predicted to be on the surface of the enzyme binding specifically to a deoxyribonucleotide/ribonucleotide base pair within the 3′-DNA/5′-RNA pole of the heteroduplex.

The alteration in helical geometry or steric interference by the 2′-substituents disrupts the binding interaction between the RNA-binding domain and the heteroduplex, resulting in the observed ablation of catalytic activity at the 5′-most cleavage site on the RNA.

binding interaction (12). Finally, the contribution of the lysine residues appears to be limited to substrate binding, as only a slight loss of catalytic activity (kcat) was observed for the RNase H1(K59A,K60A) and RNase H1(W43A,K59A,K60A) mutants compared with the wild-type enzyme (Table I). These observations are consistent with the proposed role of the lysine residues of yeast RNase H1, which have been shown to be involved in binding to the heteroduplex (13).

Fig. 5. Schematic illustrating the relationship between the positions of the RNA-binding and catalytic domains of human RNase H1 on the heteroduplex substrate. A, each observed cleavage site on the RNA is coupled to a specific binding interaction between the RNA-binding domain (RNA-BD) and the 3′-DNA/5′-RNA pole of the heteroduplex substrate. The distance between the heteroduplex/RNA-binding domain interaction and the catalytic site (cat) is ~7 bp. B, the hatched box represents an RNA/RNA, 2′-methoxyethyl/RNA, or mismatched base pair at the 3′-DNA/5′-RNA terminus of the heteroduplex. The alteration in helical geometry or steric interference by the 2′-substituents disrupts the binding interaction between the RNA-binding domain and the heteroduplex, resulting in the observed ablation of catalytic activity at the 5′-most cleavage site on the RNA.
tive modes that are not conducive to catalysis. The conserved tryptophan residue of *S. cerevisiae* RNase H1 exhibits a unique structure in that, by packing against the side chain of Lys\(^{59}\), the aromatic ring of the tryptophan is solvent-exposed (14). Several RNA-binding proteins use aromatic amino acids to form stacking interactions with the nucleotide bases of the RNA and the outward projection of the tryptophan side chain of *S. cerevisiae* RNase H1 suggest a similar role for this residue (32, 33). Therefore, Trp\(^{43}\) of human RNase H1 may form either stacking interactions with the bases of the heteroduplex or hydrogen bonds with the base (e.g. the N\(_2\) of guanosine) via the minor groove. Again, the narrow major groove of the heteroduplex would preclude the possibility of the tryptophan intercalating within this region.

Together, the conserved Trp\(^{43}\), Lys\(^{59}\), and Lys\(^{60}\) residues constitute an extended nucleic binding surface for the RNA-binding domain of human RNase H1, with the entire interaction taking place at the 3′-DNA/5′-RNA pole of the heteroduplex. In this model, base pairing would be important for intercalation of Trp\(^{43}\), and the interstrand phosphate distance across the minor groove would be critical for the interaction with Lys\(^{59}\) and Lys\(^{60}\). Consistent with the data presented here, alternative helical conformations (e.g. RNA/RNA and 2′-methoxyethyl nucleotide/RNA duplexes) as well as mismatched base pairs would ablate binding of the RNA-binding domain to the substrate at those positions (Fig. 3). In addition, the contribution of the tryptophan residue becomes all the more important considering that several binding modalities are possible between the conserved lysines and the heteroduplex. Here, Trp\(^{43}\) would be used to triangulate the positioning of the lysines such that only one binding modality is possible. Elimination of Trp\(^{43}\), as observed, have little effect on the binding affinity of the enzyme for the heteroduplex, but would result in nonproductive binding interactions.

How does a shift in the site of binding result in a shift in cleavage? Binding appears to take place at the 3′-DNA/5′-RNA pole of the heteroduplex and slightly less than one helical turn from the position of cleavage (Fig. 5). Any shift in the site of binding should shift the cleavage site (Fig. 5A). Similarly, any change in duplex geometry would dramatically affect the precise positioning required at the cleavage site (Fig. 5B). The challenge of positioning enzymes on nucleic acid substrates is a general biological problem. Human RNase H1 appears to identify the first 3′-DNA/5′-RNA base pair to achieve proper positioning. This may be accomplished by positioning the amino acids involved in binding (e.g. Trp\(^{43}\), Lys\(^{59}\), and Lys\(^{60}\)) and separating them from the catalytic site, thus the observed need for the spacer region (12). Only when the enzyme is bound at the correct site and the helical geometry is appropriate will the catalytic unit be properly cleaved to the RNA. This may be a solution that is used by many nucleases. Clearly, these and other concepts will be tested and clarified when x-ray crystal structures are available.

Human RNase H1 has recently been shown to play a critical role in the degradation of targeted RNAs induced by DNA-like antisense oligonucleotides. As numerous antisense oligonucleotides are in clinical development and such drugs are used to validate gene function in *vitro* and *in vivo*, improving the performance of these drugs may have broad value. Antisense oligonucleotides often contain modified nucleotides (e.g. 2′-methoxyethyl or 2′-methoxy) for improved binding affinity for the targeted RNA and metabolic stability. The demonstration that the RNA-binding domain of human RNase H1 recognizes the 3′-DNA/5′-RNA terminus of the heteroduplex and positions the catalytic site approximately one helical turn from the terminus suggests a variety of design motifs that may enhance the efficacy of DNA-like antisense oligonucleotides. For example, the minimum length of the deoxyribonucleotide region of the antisense oligonucleotide should be in the range of 7–10 nucleotides. In addition, introducing modified nucleotides at the 5′-pole of the antisense oligonucleotide is predicted to have little or no effect on human RNase H1 activity, whereas modifications placed within the 3′-pole would result in the ablation of preferred cleavage sites. Similarly, these data suggest that the conjugation of substituents to the antisense oligonucleotide should be performed at the 5′ terminus.