The Influence of Antisense Oligonucleotide-induced RNA Structure on Escherichia coli RNase H1 Activity*

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The ability of Escherichia coli RNase H1 to hydrolyze structured substrates containing antisense oligonucleotides preannealed to a 47-mer RNA was compared with its ability to hydrolyze unstructured substrates containing antisense oligonucleotides duplexed with 13-mer RNA. These results demonstrate that when antisense oligonucleotides were bound to structured RNA, the resultant duplexes were cleaved at rates significantly slower than when the same oligonucleotides were bound to unstructured oligoribonucleotides. Structured substrates exhibited fewer cleavage sites, and each cleavage site was cleaved less rapidly than in unstructured substrates. Furthermore, the enzymatic activity of E. coli RNase H1 for the structured substrates was most affected when the cleavage sites corresponding to the enzymatically most active sites on the unstructured substrates were blocked in the structured substrates. Molecular modeling suggests that the observed ablation of RNase H activity was due to the steric hindrance of the enzyme by the structured RNA, i.e. steric interference of the phosphate groups on the substrate and/or the binding site of the enzyme. When chimeric oligonucleotides composed of a five-base deoxyribonucleotide sequence flanked by chemically modified nucleotides were bound to structured RNA, the resultant duplexes were even worse substrates for RNase H. These results offer further insights into the role of antisense-induced RNA structure on RNase H activity and may facilitate the design of effective antisense oligonucleotides.

Antisense oligonucleotides have been reported to inhibit the expression of numerous viral and mammalian genes in vitro (for review see Refs. 1–3). In addition, a growing number of well controlled studies have been reported showing systemic antisense activities in animals and therapeutic utility in animal models of diseases (4–6). Significant advances have also been reported in the medicinal chemistry of antisense drugs with improvements in potency, pharmacokinetic, and toxicological properties observed (1–2).

One mechanism of action commonly employed by antisense oligonucleotides is the induction of cellular enzymes such as RNase H to degrade the target RNA (7–9). RNase H degrades RNA in DNA-RNA heteroduplexes (10). We are interested in better understanding the structure and function of these enzymes, the binding and cleavage mechanisms and the structure activity relationships of antisense drugs designed to result in cleavage of a target RNA by RNase H.

In a previous study, we reported that Escherichia coli RNase H1 is an endonuclease that can processively cleave RNA in an RNA-DNA duplex in the 3’ → 5’ direction (11). It displays minimal sequence dependence and is quite sensitive to chemical changes in the antisense oligonucleotide. For example, modifications in the 2’ position of the antisense drug that result in A-form-like duplexes and therefore higher $T_m$ values, result in total loss of RNase H activity (7, 12–13). Consequently, chimeric antisense oligonucleotides have been developed with deoxyribonucleotide gaps designed to serve as substrates for RNase H and 2’ modified wings to enhance the nuclease resistance of the drugs as well as improve their affinity for the target RNA (7, 14–17). These approaches have consistently been reported to result in greater antisense potency. More recently, we reported the dissociation constants ($K_d$) for various duplexes and E. coli RNase H1, demonstrating that the enzyme in fact is an RNA binding protein that cleaves RNA only in an RNA-DNA duplex (18).

Measuring the effects of oligonucleotides or chimeric oligonucleotides on cellular target RNAs directly using Northern blot analysis has resulted in the consistent observation that only a relatively few sites in structured RNA are sensitive to antisense oligonucleotide-mediated RNase H degradation (for review, see Refs. 1–3). This has been attributed to the inability of antisense oligonucleotides to bind in regions of significant secondary structure in RNA. However, another factor that could contribute to this observation might be that the structures induced by the binding of the antisense drugs to structured RNA vary in their sensitivity to RNase H. In any event, a key question that remains unanswered is, to what extent does the secondary and tertiary structure of the RNA influence RNase H cleavage?

To evaluate the role of antisense oligonucleotide-induced RNA structure on RNase H activity, we selected a previously described 47-mer RNA corresponding to residues 18–64 of activated Ha-ras mRNA and two unstructured 13-mer RNA oligonucleotides representing segments on either side of the loop region of the structured RNA as the targets for the antisense drugs (19). The secondary structure of the 47-mer RNA has been thoroughly characterized and shown to be a stable hairpin structure consisting of a 12-base pair stem and a 19-nucleotide loop (Fig. 1A). Antisense oligonucleotides were designed to target the 47-mer RNA hairpin on either the 3’ or 5’ side of the loop region immediately adjacent to the stem region (Fig. 1A). The resulting antisense oligonucleotide-induced 47-mer RNA structures consist of a quasi-continuous helix formed by the double-strand RNA stem (homoduplex stem) and the antisense oligonucleotide annealed to the 47-mer RNA (heteroduplex stem). The formation of the two adjacent stem regions

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The abbreviations used are: gap, deoxyribonucleotide portion of chimeric oligonucleotide; wing, chemically modified portion of the chimeric oligonucleotide.

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bridged by a single-strand loop resembles one half of an RNA pseudo-knot structure (20, 21). The heteroduplex stem formed by antisense oligonucleotides annealed to either the 3' or 5' side of the loop region of the 47-mer RNA hairpin produce, respectively, bridging loops analogous to loop 1 or loop 2 of RNA pseudo-knots. Hence, in the current communication the anti-sense oligonucleotide-induced 47-mer RNA structures were designated loop 1 (L1) and loop 2 (L2) pseudo-half-knots (22). The cleavage of these pseudo-half-knot structures by RNase H was compared with the cleavage of "unstructured substrates" consisting of antisense oligonucleotides annealed to complement- 
ary 13-mer RNA targets representing the 13 nucleotides of either the 3' or 5' side of the loop region of the parent 47-mer RNA.

Finally, for the purpose of this study we have chosen to use E. coli RNase H1 because it is pure and well characterized. This enzyme consists of two distinct domains: the catalytic domain and the basic protrusion (23–25). The basic protrusion is believed to constitute the predominant binding domain of the enzyme and is composed of a cluster of basic amino acids. These positively charged amino acids are predicted to bind electrostatically to the negatively charged phosphate groups of the substrate, and this interaction is predicted to occur within the minor groove of the duplex substrate. In addition, structural studies suggest that the enzyme exhibits a selective "binding directionality" with respect to the substrate such that the basic protrusion is positioned upstream of the catalytic site and the enzyme appears to processively cleave the RNA in the direction of the basic protrusion, i.e. 3' → 5' (11).

Our studies demonstrate that the structure of antisense-RNA duplexes, i.e. the ultimate substrate for RNase H, may have substantial influence on the cleavage rates and sites of cleavage by the enzyme. Specifically, the pseudo-half-knot structures induced by the antisense oligonucleotides dramatically reduce RNase H cleavage of the substrate. In addition, steric inhibition by the structured RNA of the sites deemed enzymatically most sensitive on the unstructured substrates resulted in the greatest reduction in enzymatic activity against the pseudo-half-knot substrates.

EXPERIMENTAL PROCEDURES

Materials—E. coli RNase H1 (5 units/ml) was purchased from U. S. Biochemical Corp. T4 polynucleotide kinase was from Promega (Madison, WI). γ-[32P]ATP (7000 Ci/mmol) was purchased from ICN (Irvine, CA). Inhibit-ACE™ was from 5′ to 3′ (Boulder, CO). Calf intestine alkaline phosphatase, G-25 Sephadex Quick Spin™ columns, and RNAses T1, A, and CL3 were purchased from Boehringer Mannheim. Pfu DNA polymerase was from Stratagene (San Diego, CA). The MEGAscript™ T7 transcription kit was purchased from Ambion (Austin, TX). The antisense oligodeoxynucleotides were purchased from Operon (Alameda, CA). The oligodeoxynucleotides were greater than 90% full-length material as determined by capillary gel electrophoresis analysis.

Oligonucleotide Synthesis—2′-Methoxy monomers were synthesized as described previously (26–27). Synthesis of 2′-methoxy and deoxy chimeric oligonucleotides were performed using an Applied Biosystems 380B automated DNA synthesizer as described previously. Purification of oligonucleotide products was also as described previously. Purified products were greater than 90% full-length material as determined by capillary gel electrophoresis analysis.

28P Labeling of RNA Transcripts and Oligoribonucleotides—RNA transcripts and oligoribonucleotides were 5′-end-labeled with 32P using γ-[32P]ATP, T4 polynucleotide kinase, and standard procedures (28). RNA transcripts were 3′-end-labeled using [32P]P post, T4 RNA ligase, and standard procedures. Labeled transcripts and oligonucleotides were purified by electrophoresis on 12% denaturing polyacrylamide gels. The specific activity of the 5′- and 3′-labeled RNA were, respectively, approximately 6000 and 2000 cpm/fmol.

Initial Rate Determinations—Substrate hybrids containing the 47-mer hairpin were prepared in 100 µl of reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 0.1 mM dithiothreitol, 1 unit of Inhibit-ACE™), containing 1–50 µM antisense oligonucleotide, 500 nM transcript, and 107 cpm 32P-labeled transcript. Substrate hybrids containing sense oligoribonucleotide were prepared as above with the exception that 500 nM sense oligoribonucleotide and 107 cpm of 32P-labeled sense oligoribonucleotide were used. Hybridization reactions were incubated for 18 h at 37 °C. Initial rate determinations were performed at 37 °C by addition of 25 nM E. coli RNase H1 to the hybridization reactions. At specific time intervals (0–150 min), 10-µl aliquots of the hybridization reactions were quenched in 10 µl of gel loading buffer (8% urea, 0.25% bromophenol blue, and 0.25% xylene cyanole). Samples were analyzed by denaturing polyacrylamide gel electrophoresis as described previously (11).

Enzymatic Structure Mapping of Oligoribonucleotide-bound Transcripts—Hybrids were prepared in 10 µl of the above reaction buffer containing 500 nM transcript, 107 cpm of 32P transcript and oligoribonucleotide ranging in concentration from 1 to 100 µM. Hybrid mixtures were incubated for 18 h at 37 °C to insure that hybridization was at equilibrium (18). Mixtures were digested with RNases T1, CL3, and A for 5 min at 37 °C and quenched by the addition of 5 µl of 8 M urea. To ensure only primary cleavage sites were detected, the concentration of the enzyme was chosen such that more than 90% of the transcript remained intact. Reactions were resolved using a 12% polyacrylamide sequencing gel. Initial RNase H cleavage rates V0 were calculated from the slope of the linear range of the assay as described previously (11).

Computer Modeling—All the computations were performed on an Indigo2 machine using the MS software (Molecular Simulations Inc., San Diego, CA). The initial structures of the various systems investigated in the present study were model-built. Energy minimizations were performed using the AMBER force field in the DISCOVER module of the software. A distance-dependent dielectric constant employed in the calculations mimicked the effect of explicit solvent. A 10-A cutoff has been used in computing the intermolecular interactions. The total charge on the phosphates was scaled to account for counter-ion effects. Energy minimizations of the model systems involving 300 cycles of steepest descent and at least 1,000 steps of adopted basis Newton-Raphson method (to make sure that the energy tolerance was less than 0.001 kcal/mol) was carried out. AMBER force field has recently been successfully applied in the simulation of nucleic acids. The computed root mean squared deviations (from the initial x-ray structures) support the credibility of the force field parameters (29).

RESULTS

Structure of Antisense Oligonucleotide-induced 47-mer RNA—The structure maps derived from enzymatic probing of the six antisense oligonucleotide-induced RNA structures and the parent 47-mer RNA hairpin are shown in Fig. 1A. These antisense oligonucleotide-induced 47-mer RNA structures are divided into two categories: L1 pseudo-half-knots formed by antisense oligonucleotides targeting the 3′ side of the loop region on the parent 47-mer RNA hairpin (e.g. 26–9, 24–11, and 22–13), and L2 pseudo-half-knots formed by oligoribonucleotides targeting the 5′ side of the loop region on the parent 47-mer RNA hairpin (e.g. 16–9, 16–11, and 16–13). To ensure that only primary cleavage sites were detected, both 3′- and 5′-end-labeled 47-mer RNAs were tested. Only cleavage sites detected with substrates labeled at both termini are reported in Fig. 1A.

Antisense oligonucleotide footprints were determined by the protection of the antisense oligonucleotide binding site on the 47-mer RNA from cleavage by single-strand-specific ribonuclease T1 and A (Fig. 1A). These footprinting data revealed that all six of the oligodeoxynucleotides were annealed to the transcript at the appropriate target sites. In addition, the protection of the antisense oligonucleotide binding sites on the 47-mer RNA from single-strand-specific ribonuclease digestion suggests that, with the exception of the 16–13 pseudo-half-knot, the oligonucleotides were completely base paired with the 47-mer RNA (Fig. 1A). The 16–13 pseudo-half-knot structure map, on the other hand, revealed single-strand-specific ribonuclease digestion of the 47-mer RNA within the putative antisense oligonucleotide binding site (Fig. 1A and B). Specifically, RNase A digestion of the C28 residue on the 47-mer RNA was observed for the 16–13 pseudo-half-knot, suggesting that this
nucleotide was not base paired with the antisense oligonucleotide. In addition, weaker RNase A digestion was observed for the 16–13 pseudo-half-knot at positions C16, U18, and C19 on the 47-mer RNA. The protection of both strands of the homoduplex RNA stem region and the antisense oligonucleotide binding site on the pseudo-half-knot structures from single-strand-specific ribonucleases indicates that these stem regions were double-stranded and thus formed a quasi-contiguous helix.

Enzymatic mapping of the pseudo-half-knot structures shown in Fig. 1 revealed that certain nucleotides on the 47-mer RNA within putative loop regions were protected from single-strand-specific ribonuclease digestion. For example, the U34 residue on the 47-mer RNA of the L2 pseudo-half-knot structures (16–9, 16–11, and 16–13), was not digested by RNase A. In addition, residues G25 and G26 within loop 2 of the 16–9 pseudo-half-knot appear to be protected from RNase T1 digestion.

Catalytic Rates for RNase H—The initial RNase H cleavage rates \( \left( V_0 \right) \) for the various unstructured substrates and pseudo-half-knot substrates are listed in Table I. Without exception, the 47-mer RNA of the antisense oligonucleotide-induced pseudo-half-knot substrates was digested by RNase H at statistically significantly slower rates than the unstructured substrates consisting of the antisense oligonucleotide annealed to the 13-mer RNAs. A slight, but statistically significant increase in the rate of RNase H cleavage with a corresponding increase in oligonucleotide length was observed for the L1 pseudo-half-knots (26–9, 24–11, and 22–13). Conversely, the rates measured for these antisense oligonucleotides annealed to the complementary 13-mer RNA, showed that for the unstructured substrates no significant difference in \( V_0 \) was observed. Comparison of the initial cleavage rates for the L2 pseudo-half-knots shows that increasing the antisense oligonucleotide length from 9 (16–9) to 11 (16–11) nucleotides resulted in a 4-fold increase in the rate \( (p < 0.001) \). No further increase in rate was observed for the 16–13 pseudo-half-knot substrate containing the 13-mer antisense oligonucleotide. The unstructured substrates containing these antisense oligonucleotide (16–9, 16–11, and 16–13) annealed to 13-mer RNA exhibited roughly equal rates. The 13-mer RNA of the unstructured substrates containing the antisense oligonucleotides targeted to the 5′ side of the parent 47-mer RNA loop (16–9, 16–11 and 16–13) was digested at slower rates than the RNA of the substrates composed of the antisense oligonucleotides targeting the 3′ side of the parent 47-mer RNA loop (26–9, 24–11 and 22–13) \( (p < 0.001) \) suggesting that there are rate differences due to differences in the sequence, but that these differences (approximately 8000 versus 4000 pmol liter\(^{-1}\) min\(^{-1}\)) are modest relative to the effects of structure.

Catalytic rates for each RNase H cleavage site on the pseudo-half-knot substrates and unstructured substrates are shown in Fig. 2A. These site-specific cleavage rates \( (V_0) \) were measured by quantitating the amount of RNase H digestion for each individual cleavage site as a function of time. For example, site-specific cleavage rates were determined for residues G26, G27, A29, and G31 of the 22–13 pseudo-half-knot substrate (Fig. 2, A and B). As was observed for the initial RNase H cleavage site.
rates \(V_0\) (Table I), the site-specific cleavage rates for the 47-mer RNA of the pseudo-half-knot substrates were consistently slower than the site-specific cleavage rates measured for the 13-mer RNA of the unstructured substrates (Fig. 2A). Interestingly, the fastest site-specific cleavage rates measured for the 13-mer RNA of the 16–9 and 16–11 unstructured substrates occurred at internucleotide linkages positioned outside of the antisense oligonucleotide binding site (e.g. after residues U24 and G25 of the 16–9 unstructured substrate and residue G28 of the 16–11 unstructured substrate) (Fig. 2A).2

To further evaluate the effects of antisense oligonucleotide-induced structure on the cleavage of specific sites by RNase H, the initial RNase H cleavage rates \(V_0\) for the pseudo-half-knot substrates containing the chimeric oligonucleotide analogs of the 16–9 antisense oligonucleotide annealed to the 47-mer RNA were determined (Table II). These chimeric oligonucleotides consisted of a stretch of five deoxynucleotides flanked by 2′-methoxy nucleotides. Each chimeric oligonucleotide differed by the position of the stretch of deoxynucleotides within the oligomer (Table II). RNase H digestion was observed for only two (14326 and 14327) of the five pseudo-half-knot substrates containing these chimeric antisense oligonucleotides. RNase H digestion of these two pseudo-half-knot substrates occurred at a single site on the 47-mer RNA apposed to the 5′ junction between the deoxynucleotide gap and the 2′-methoxy wing of the chimeric antisense oligonucleotide. These data are consistent with previous findings in which the digestion of RNA annealed to a chimeric antisense oligonucleotide with a five-deoxynucleotide gap resulted in a single cleavage site occurring at the nucleotide apposed to the 5′ junction (11). Cleavage of the pseudo-half-knot substrates containing the chimeric oligonucleotides occurred only when the 5′ junction was positioned at sites observed to be sensitive to RNase H cleavage in the unmodified 16–9 pseudo-half-knot substrate. The rates of cleavage at these sites for the position 14326 and 14327 pseudo-half-knot substrates were approximately equal to the cleavage rate for the unmodified 16–9 pseudo-half-knot substrate.

Cleavage Site Specificity of RNase H—The cleavage pattern resulting from RNase H digestion of the pseudo-half-knot and unstructured substrates containing, respectively, the antisense oligodeoxynucleotides annealed to the 47-mer and 13-mer

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**TABLE I**

*Initial RNase H cleavage rates \(V_0\) for the pseudo-half-knot and unstructured substrates*

<table>
<thead>
<tr>
<th>OLIGONUCLEOTIDE</th>
<th>47-mer (V_0)</th>
<th>13-mer (V_0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16–9</td>
<td>500 ± 60</td>
<td>4150 ± 464</td>
</tr>
<tr>
<td>16–11</td>
<td>1975 ± 225</td>
<td>4550 ± 450</td>
</tr>
<tr>
<td>16–13</td>
<td>1950 ± 350</td>
<td>3950 ± 1750</td>
</tr>
<tr>
<td>L1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26–9</td>
<td>750 ± 13</td>
<td>6100 ± 1006</td>
</tr>
<tr>
<td>24–11</td>
<td>852 ± 2</td>
<td>8117 ± 833</td>
</tr>
<tr>
<td>22–13</td>
<td>925 ± 75</td>
<td>5233 ± 437</td>
</tr>
</tbody>
</table>

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The site-specific cleavage rates were determined as described. The initial rates are an average of at least three measurements. The $V_0$, for the two observed cleavage sites on the 16–9 pseudo-half-knot are listed in the same order as the arrows. Boxed sequences indicate position of 2-methoxy modifications. Arrows show the position of the RNase H digestion site on the 47-mer RNA. The underlined residues indicate the predicted position of the single RNase H cleavage site on the 47-mer RNA based on the position of the $5'$ deoxynucleotide gap. The structure on the right illustrates the secondary structure likely formed by these chimeric antisense oligonucleotides. The chimeric antisense oligonucleotide sequence within the pseudo-half-knot structure on the left are indicated by the boxed sequence.

### Table II

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence</th>
<th>$V_0$ (mmol $^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16–9</td>
<td>$\cdots$GCGCUGG/GG/GGCGCGC$\cdots$</td>
<td>309 ± 6, 135 ± 2</td>
</tr>
<tr>
<td></td>
<td>OCAGCCA</td>
<td>↓</td>
</tr>
<tr>
<td>14326</td>
<td>$\cdots$GCGCUGG/GG/GGCGCGC</td>
<td>573 ± 195</td>
</tr>
<tr>
<td></td>
<td>OCAGCCA</td>
<td>↓</td>
</tr>
<tr>
<td>14327</td>
<td>$\cdots$GCGCUGG/GG/GGCGCGC</td>
<td>365 ± 101</td>
</tr>
<tr>
<td></td>
<td>3′-AGGCCA</td>
<td>$\uparrow$</td>
</tr>
<tr>
<td>14328</td>
<td>$\cdots$GCGCUGG/GG/GGCGCGC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3′-AGGCCA</td>
<td>$\uparrow$</td>
</tr>
<tr>
<td>14329</td>
<td>$\cdots$GCGCUGG/GG/GGCGCGC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3′-AGGCCA</td>
<td>$\uparrow$</td>
</tr>
<tr>
<td>14330</td>
<td>$\cdots$GCGCUGG/GG/GGCGCGC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3′-AGGCCA</td>
<td>$\uparrow$</td>
</tr>
</tbody>
</table>

RNAs was determined by polyacrylamide gel electrophoresis and is shown in Fig. 3. The position of each RNase H cleavage site was determined using ribonucleases T1 and A as sequence markers.

The RNase H digestion patterns show that the 47-mer RNA of the pseudo-half-knot substrates exhibited fewer RNase H cleavage sites than the 13-mer RNA of the unstructured substrates (Fig. 3). For example, two RNase H cleavage sites were observed for the 16–9 pseudo-half-knot substrate as compared with 5 cleavage sites for the 16–9 unstructured substrate. RNase H digestion of residues U22 through G25 on the 47-mer RNA of the 16–9 pseudo-half-knot substrate was not observed, even though the 16–9 antisense oligonucleotide was determined by enzymatic structure mapping to be completely base paired with the 47-mer RNA. Likewise, fewer cleavage sites were observed for the 16–11 pseudo-half-knot substrate as compared with the 13-mer RNA of the unstructured substrate. In this case cleavage of residues U22, G23, and G25 on the 47-mer RNA of the 16–11 pseudo-half-knot substrate was not observed. The 16–13 pseudo-half-knot substrate exhibited no RNase H digestion of residues G25 and G26 on the 47-mer RNA, whereas these positions were digested by the enzyme on the 13-mer RNA of the unstructured substrate. Similarly, RNase H cleavage of residues A22 and A23 on the 13-mer RNA of the 24–11 and 22–13 unstructured substrates was not observed on the 47-mer RNA of the pseudo-half-knot substrates. The RNase H digestion pattern for the 24–11 and 22–13 unstructured substrates showed additional cleavage sites further 3' on the 13-mer RNA when compared with the 47-mer RNA of the 24–11 and 22–13 pseudo-half-knot substrates (e.g. residues A22-G23). Enzymatic structure mapping of these L1 pseudo-half-knots suggests that the A22 and G23 residues were base paired with the antisense oligonucleotides.

### Discussion

Antisense Oligonucleotides Induce Pseudo-half-knot Structures—We used antisense oligonucleotides of varying length and sequence to introduce, in a controlled manner, structural diversity into the ultimate substrate for RNase H; the antisense oligonucleotide-RNA hybrid. These antisense oligonu-
cleotides were designed to target opposite sides of the loop region immediately adjacent to the stem region of the 47-mer RNA hairpin. The resulting pseudo-half-knot structures contain a contiguous helix, presumably coaxially stacked, between the heteroduplex stem formed by the antisense oligonucleotide annealed to the 47-mer RNA and the homoduplex stem of the RNA target (Fig. 1A). Antisense oligonucleotides targeting the 3' side of the loop on the parent 47-mer hairpin (L1 pseudo-half-knots) result in a loop that is predicted to cross the major groove of the heteroduplex stem while antisense oligonucleotides annealed to the 5' side of the loop (L2 pseudo-half-knots) result in a loop that is predicted to cross the minor groove of the heteroduplex stem (22) (Fig. 4, A and B). Finally, structural constraints were placed on the pseudo-half-knot loop by both the position and the length of the heteroduplex stem.

The secondary structure maps of the antisense oligonucleotide-induced 47-mer RNA structures suggest that, with the exception of the 16–13 antisense oligonucleotide-induced 47-mer RNA, oligonucleotides bind to complementary regions on the RNA consistent with the predicted coaxially stacked pseudo-half-knot structures. Furthermore, the protection of certain nucleotides within designated single-strand regions from single-strand-specific ribonuclease degradation need not suggest the formation of additional base pairs. For example, the protection of the 5' terminal residue (U34) of loop 1 and 3' terminal residue of the L2 and 3' terminal residue (C18) of loop 2 from RNase A and CL3 digestion is presumably a function of the structure surrounding the helix junction since these residues do not appear to base pair with other nucleotides. Similar results have been observed for the pseudo-half-knot structures of the trans-activation response (TAR) element of human immunodeficiency virus (22). The G25 and G26 residues of the 16–9 pseudo-half-knot were also protected from single-strand-specific RNase digestion. Again, enzymatic mapping shows that no alternate base pairing is evident. Molecular modeling shows that residues G25 and G26 of the loop region cross the minor groove of the heteroduplex stem between positions U22 and U24 and hence could be protected from RNase T1 digestion (Fig. 4C).

The structure map of the 16–13 pseudo-half-knot shows that the 16–13 antisense oligonucleotide was not completely base paired with the 47-mer RNA. These data suggest that only 9 of a possible 13 base pairs were formed between the 16–13 antisense oligonucleotide and the 47-mer RNA. For the 22–13 pseudo-half-knot on the other hand, the antisense oligonucleotide was completely base paired with the 47-mer RNA. The 22–13 antisense oligonucleotide was formed using 13-mer antisense oligonucleotides, suggesting that the observed differences in the ability of these antisense oligonucleotides to base pair with the 47-mer RNA is a function of the position and not the length of the heteroduplex stem within the pseudo-half-knot structure. The effect of the position of the heteroduplex stem on heteroduplex formation is presumably due to the structure of the loop region. For example, loop 1 of the 22–13 pseudo-half-knot was predicted to cross the major groove of the heteroduplex stem, while loop 2 of the 16–13 pseudo-half-knot was predicted to cross the minor groove of the heteroduplex stem. As a result, the length of the loop region required to span the

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**FIG. 3.** RNase H digestion maps of the pseudo-half-knot and unstructured substrates. Arrows indicate the site of RNase H digestion. Boxed sequences represent antisense oligonucleotide. The hairpin sequence is numbered 5' to 3'. The directionality of RNase H with respect to the L1 and L2 pseudo-half-knot substrates is illustrated by the schematic. The positive symbols (+) and arrow indicate the upstream position of the basic protrusion with respect to the RNA.
Impact of Antisense-induced RNA Structure on RNase H Activity

The structures shown in Fig. 4 illustrate the various boat and twist conformations of the pseudo-half-knots and their impact on RNase H activity. The green and orange regions represent, respectively, the RNA target and the antisense oligodeoxynucleotide. Structure of the 24–11 L1 pseudo-half-knot (A) and 16–11 L2 pseudo-half-knot (B). C, 16–9 pseudo-half-knot structure showing RNA residues G25 and G26 (yellow) crossing the minor groove of the heteroduplex stem between RNA residues U22 and U24 (magenta). D, 16–11 pseudo-half-knot structure showing hydrogen bond formation between the N-4 on the C28 residue and the phosphate group between G25 and G26 (both in magenta). E, 22–13 pseudo-half-knot structure showing possible hydrogen bond formation between the N-4 of C19 and the N-7 of G34 (both in magenta).

heteroduplex stem is dictated by the width of the major and minor grooves. The interphosphate distance across the minor groove for a standard A-form duplex is estimated to be approximately 26 Å for a five-base pair helix, while the distance required to span the narrower major groove is about 8 Å for the same length helix (30). Therefore the loop of L2 pseudo-half-knots would have to span a longer distance per unit length of heteroduplex stem than the loop of L1 pseudo-half-knots. We have calculated the maximum antisense oligonucleotide length for both the L1 and L2 pseudo-half-knots based on the 19 nucleotide length of the parent 47-mer RNA loop. Assuming a nucleotide residue can span a distance of approximately 7 Å of a helical conformation (31), the maximum antisense oligonucleotide length for the L1 and L2 pseudo-half-knots is approximately 15 and 11 nucleotides, respectively. Therefore the 13 base pair heteroduplex stem of the L1 pseudo-half-knot (22–13) falls within the allowable limit for forming a coaxially stacked pseudo-half-knot. Conversely the 13-base pair heteroduplex stem of the L2 pseudo-half-knot (16–13) is too long, causing either a distortion of the coaxially stacked helix or dissociation of the stems in order for the loop to span the required distance. This distortion in the coaxially stacked region of pseudo-knot structures due to the length of the loop region has also been observed for the pseudo-knot structure of mouse mammary tumor virus (32).

**Antisense Oligonucleotide-induced Pseudo-half-knot Structures Inhibit RNase H Activity**—Comparison between the RNase H activity for the pseudo-half-knot substrates containing the 47-mer RNA and the unstructured substrates containing the 13-mer RNA shows that the pseudo-half-knot structures are hydrolyzed less effectively. Specifically, RNase H digestion of the pseudo-half-knot substrates resulted in fewer RNase H cleavage sites and reduced initial cleavage rates ($V_0$) when compared with the unstructured substrates (Table I). Furthermore, the site-specific-cleavage rates ($V_{0a}$) observed for the pseudo-half-knot substrates were consistently slower than the site-specific cleavage rates determined for the unstructured substrates (Fig. 2), except, of course, for those sites in which the 47-mer was cleaved and the 13-mer was not. Therefore it appears that a reduction in the cleavage rate for each site ($V_{0a}$) and/or the number of cleavage sites was responsible for the reduction in the initial cleavage rate ($V_0$) for the pseudo-half-knot substrates. Clearly, this relationship between the number of cleavage sites and the initial cleavage rate is not the only factor responsible for the observed differences in RNase H activity between the pseudo-half-knot substrates and the unstructured substrates. The site-specific cleavage rates for the unstructured substrates show that RNase H cleaves the various sites at different rates and that the initial cleavage rate for the full substrate is predominantly the product of one or two cleavage sites. For example, residue A30 of the 24–11 and 22–13 unstructured substrates was digested at a rate of approximately 5500 and 4000 pmol l$^{-1}$ min$^{-1}$, respectively (Fig. 2). These rates contribute significantly to the initial cleavage rates ($V_0$) of the full substrate of approximately 8000 and 5000 pmol l$^{-1}$ min$^{-1}$, respectively (Table I). The complete loss of RNase H activity at this site in the 24–11 and 22–13 pseudo-half-knot substrates resulted in a significant reduction in the initial cleavage rate of these pseudo-half-knot substrates when compared with the unstructured substrates. Therefore these data suggest that the greatest impact on the RNase H activity for the pseudo-half-knot substrates is likely due to the influence of the antisense oligonucleotide-induced RNA structure on those cleavage sites corresponding to the enzymatically most sensitive sites on the unstructured substrates.

Although all of the pseudo-half-knot structures tested were
determined to inhibit RNase H activity, the influences of these antisense oligonucleotide-induced RNA structures on RNase H activity were different for the L1 and L2 pseudo-half-knot substrates. For example, the L2 pseudo-half-knot substrates exhibited faster rates, with the exception of the 16–9 pseudo-half-knot, than the L1 pseudo-half-knot substrates. These results were surprising since the loop region of L2 pseudo-half-knot substrates was predicted to sterically interfere with the binding site of the enzyme on the substrate, i.e., loop 2 was predicted to cross the minor groove of the heteroduplex stem. The loop region of the L1 pseudo-half-knot substrates on the other hand is predicted to cross the major groove of the heteroduplex stem and consequently should have less impact on the RNase H activity. The initial cleavage rates measured for the unstructured substrates suggest that the difference in RNase H activity between the L1 and L2 pseudo-half-knot substrates is not due to the sequence of the heteroduplex stem since the unstructured substrates corresponding to the heteroduplex stem of the L2 pseudo-half-knots (16–9, 16–11, and 16–13) were digested by RNase H at slower rate than the unstructured substrates corresponding to the heteroduplex stem of the L1 pseudo-half-knots (26–9, 24–11, and 22–13) (Table I). An alternative explanation may be that the predicted position of the loop 2 structure, based on the RNase H digestion pattern, does not accurately reflect the orientation of the loop with respect to the heteroduplex stem in the solution structure.

**Molecular Modeling Suggests That Loop Regions Crossing the Major or Minor Groove of the Heteroduplex Stem Interfere with RNase H**—The reduction in the overall initial cleavage rate observed for the pseudo-half-knot substrates (Vₜₜ) is likely due to the loss of cleavage at specific sites. Computer modeling of the six pseudo-half-knot substrates provides an explanation for these results, as modeling suggests that in every case the reduction and or loss of RNase H activity at specific sites was due to steric interference of the enzyme by the loop region. Specifically, steric hindrance of the enzyme by the loop region appears to be the result of the loop region crossing the RNA backbone of the heteroduplex stem in either the minor groove (L1) or major groove (L2) of the heteroduplex (Fig. 4, A and B). In some cases, the predicted close proximity of the loop region to the heteroduplex stem by the computer models suggested intramolecular interaction between the loop and heteroduplex were possible (Fig. 4, D and E). Therefore, these modeling studies suggest that regardless of whether the loop region crosses the minor or major groove of the heteroduplex stem, the point at which the grooves are crossed places the loop in close proximity to the RNA backbone of the heteroduplex stem and thereby sterically hinders the enzyme.

**Chimeric Oligonucleotides with Modifications That Do Not Support RNase H Activity Exacerbate the Negative Effects of Antisense-induced RNA Structure on RNase H Activity**—The reduction in RNase H activity due to the antisense oligonucleotide-induced RNA structure is compounded when the number of cleavage sites within the substrate is reduced by modifications within the antisense oligonucleotide (e.g., chimeric oligonucleotides). Specifically, the impact of the 16–9 pseudo-half-knot structure on RNase H activity was more dramatic for the chimeric oligonucleotide analogs. These chimeric oligonucleotides were designed with a five-deoxynucleotide gap, which effectively limits cleavage to a single site within the RNA, directly apposing the deoxynucleotide adjacent to the junction between the gap and the modified wing on the 5’ end of the oligonucleotide. Therefore, RNase H digestion of the 14326, 14327, 14328, 14329, and 14330 pseudo-half-knot substrates would be expected to result in cleavage of the RNA at, respectively, residues G20, G21, U22, G23, and U24 (shown underlined, Table II). In fact, the chimeric oligonucleotides with the 5’ gap/wing junction adjacent to positions U22–U24 of the pseudo-half-knot RNA resulted in inactive RNase H substrates. These data are consistent with the RNase H digestion of the 16–9 pseudo-half-knot substrate which showed that the region between residues U22 and U24 was enzymatically inactive. Conversely, residues G20 and G21 on the 16–9 pseudo-half-knot substrate were sensitive to RNase H digestion and consequently, chimeric oligonucleotides designed with the 5’ gap/wing junction adjacent to residues G20 and G21 (e.g., 14326 and 14327), resulted in active RNase H substrates. Thus to be an effective substrate for RNase H, a chimeric oligonucleotide with 2’ modifications that do not serve as substrates for RNase H must be positionned such that the enzymatically sensitive sites are base paired with deoxynucleotides within the gap. In the case of a chimeric oligonucleotide with a five-base gap, the only site of enzymatic sensitivity is apposed to the 5’ junction and therefore cleavage of these substrates only occurs when the 5’ junction is exposed to a sensitive site on the RNA.

**The Binding Directionality of RNase H Contributes to the Variable Effects of Secondary Structure on RNase H Activity**—These pseudo-half-knot structures place the heteroduplex stems in diametrically opposite directions with respect to the adjacent RNA stem regions. As the enzyme is predicted to bind the substrate in such a manner as to orient the binding region, i.e., basic protrusion, in the 5’ direction on the RNA relative to the catalytic site, RNase H catalysis of the L1 and L2 pseudo-half-knots occurs, respectively, with the binding region of the enzyme adjacent to either the single-strand loop region or the RNA stem (see schematic in Fig. 3). The RNA at the 5’ end of the heteroduplex stem and closest to the basic protrusion of the enzyme exhibited little to no effect on the RNase H cleavage pattern between the L1 and L2 pseudo-half-knot substrates, i.e., the first 5’ cleavage site on the RNA was 5–6 nucleotides from the 5’ end of the heteroduplex stem. Conversely, the RNA at the 3’ end of the heteroduplex stem and closest to the catalytic region of the enzyme, with the exception of the 16–9 and 26–9 pseudo-half-knot, exhibited fewer RNase H cleavage sites for the L1 pseudo-half-knot substrates than the L2 pseudo-half-knot substrates. These undigested nucleotides within the L1 pseudo-half-knots are adjacent to the junction between the coaxially stacked stems. The structure created by the intersection of this loop region with the coaxially stacked stems is likely responsible for the loss in RNase H activity. These data suggest that the catalytic region of the enzyme is more sensitive to variations in substrate structure than the binding region. The sensitivity of the catalytic region to structural variations within the substrate is consistent with previous studies (18). Clearly, to draw generalizations about the effects of substrate structures on the directionality of E. coli RNase H1 would require the examination of a larger group of structured substrates.

**Implications for Antisense Oligonucleotide Design**—To a significant extent, medicinal chemical strategies designed to increase the potency of antisense oligonucleotides have focused on increasing the affinity of the antisense oligonucleotides for their cognate RNA targets (for review, see Ref. 2). This approach has resulted in improved potency, particularly when chimeric oligonucleotides designed to enhance the affinity for the target RNA and serve as substrates for RNase H have been employed. Nevertheless, in our experience, the increases in potency observed have been less than predicted based on the increased affinities for the target RNAs. Moreover, for chimeric oligonucleotides designed to bind to many sites in RNA, we have not observed the expected increases in potency, and in some cases we have observed complete loss of antisense activ-
ity. This study provides an explanation for these observations. First, the rate of cleavage on both structured and unstructured heteroduplexes by RNase H tends to be defined by one or two sites that are especially sensitive. Any chimeric oligonucleotide that ablates the potential to cleave a hyper-sensitive site will result in a dramatic loss in cleavage rate and therefore a negative effect on potency. Second, the cleavage of structured RNA-antisense oligonucleotide duplexes is considerably slower than unstructured RNA. Thus in many cases, the binding of the antisense oligonucleotide to the RNA, while necessary, may not be rate-limiting as RNase H cleavage may be quite slow. Thus, at those sites increasing affinity would be expected have little effect on potency.

Considering the observations we have reported with regard to the effect of RNA structure on binding of antisense oligonucleotides to target RNAs (19, 22) in concert with the results reported here, it is obvious that much more attention must be paid to RNA structure and the structure of the heteroduplexes formed by the antisense drug and the targeted RNA. Clearly, as this study focused on E. coli RNase H, results may differ quantitatively for mammalian RNase H, but the basic principles will surely apply.

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REFERENCES


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