Binding Affinity and Specificity of *Escherichia coli* RNase H1: Impact on the Kinetics of Catalysis of Antisense Oligonucleotide−RNA Hybrids

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ABSTRACT: In this study we report for the first time the binding affinity of RNase H1 for oligonucleotide duplexes. We used a previously described 17-mer antisense sequence [Monia, B. P., Johnston, J. F., Ecker, D. J., Zounes, M. A., Lima, W. F., & Freier, S. M. (1992) *J. Biol. Chem. 267*, 19954−19962] hybridized to a complementary oligoribonucleotide to evaluate both the binding affinity and the catalytic rate of RNase H1. The dissociation constants (K_d) of RNase H1 for the various substrates tested were determined by inhibition analysis using chemically modified noncleavable oligonucleotide heteroduplexes. Catalytic rates were determined using heteroduplex substrates containing chimeric antisense oligonucleotides composed of a five-base deoxynucleotide sequence flanked on either side by chemically modified nucleotides. We find that the enzyme preferentially binds A-form duplexes: RNase H bound A-form duplexes (RNA:RNA and DNA:RNA) approximately 60-fold tighter than B-form duplexes (DNA:DNA) and approximately 300-fold tighter than single-strand oligonucleotides. The enzyme exhibited equal affinity for both the wild type (RNA:DNA) oligonucleotide substrate and heteroduplexes containing various 2′-sugar modifications, while the cleavage rates for these chemically modified substrates were without exception slower than for the wild type substrate. The introduction of a single positively charged 2′-propoxyamine modification into the chimeric antisense oligonucleotide portion of the heteroduplex substrate resulted in both decreased binding affinity and a slower rate of catalysis by RNase H. The cleavage rates for heteroduplexes containing single-base mismatch sequences within the chimeric oligonucleotide portion varied depending on the position of the mismatch but had no effect on the binding affinity of the enzyme. These results offer further insights into the physical binding properties of the RNase H−substrate interaction as well as the design of effective antisense oligonucleotides.

RNase H hydrolyzes RNA in RNA−DNA hybrids. This enzyme, first described by Hausen and Stein (1970) in calf thymus, has subsequently been described in a variety of organisms. RNase H activity appears to be ubiquitous in eukaryotes and bacteria (Itaya & Kono, 1991; Itaya et al., 1991; Kanaya & Ktaya, 1992; Busen, 1980; Rong & Carl, 1990; Edar et al., 1993). Although RNase H's constitute a family of proteins of varying molecular weight, nucleolytic activity and substrate requirements appear to be similar for the various isotypes. For example, RNase H functions as an endonuclease exhibiting limited sequence specificity and requiring divalent cations (e.g., Mg^{2+}, Mn^{2+}) to produce 5′-phosphate and 3′-hydroxyl ends (Crouch & Dirkensen, 1982).

Because eukaryotic RNase H remains poorly characterized, primarily due to the lack of a cloned enzyme, RNase H1 from *Escherichia coli* is the best characterized. The 3-dimensional structure of *E. coli* RNase H1 has been determined by X-ray crystallography, and the key amino acids involved in binding and catalysis have been identified by site-directed mutagenesis (Nakamura et al, 1991; Katayangi et al., 1990; Yang et al., 1990; Kanaya et al., 1991). The enzyme has two distinct structural domains. The major domain consists of four α helices and one large β sheet composed of three antiparallel β strands. The Mg^{2+} binding site is located in the β sheet and consists of three amino acids, Asp-10, Glu-48, and Gly-11 (Katayanagi et al., 1993). This structural motif of the Mg^{2+} binding site surrounded by β strands is similar to that in DNase I (Suck & Oefner, 1986). The minor domain is believed to constitute the predominant binding region of the enzyme and is composed of an α helix terminating with a loop. The loop region is composed of a cluster of positively charged amino acids that are believed to bind electrostatically to the minor groove of the DNA:RNA heteroduplex substrate. Although the conformation of the RNA:DNA substrate can vary from A-form to B-form depending on the sequence composition, in general RNA:DNA heteroduplexes adopt an A-like geometry (Pardi et al., 1981; Hall & Mclaughlin, 1991; Lane et al., 1993). The entire binding interaction appears to comprise a single helical turn of the substrate duplex.

RNase H activity is often implicated in antisense oligodeoxynucleotide mediated degradation of RNA (Monia et al., 1993; Mirabelli & Crooke, 1993; Chiang et al., 1991). To date the best characterized class of antisense oligonucleotides are phosphorothioate oligodeoxynucleotides [for review see Crooke (1995)]. These modified oligonucleotides are able to form stable hybrids with their complementary RNA and have repeatedly been shown to be effective modulators of cellular and viral gene expression (Stein et al., 1988; Toulme & Helene, 1988; Crooke, 1992; Monia et al., 1992). These analogs have been shown to serve as substrates for RNase H when bound to their complementary

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[1] Abbreviations: DTT, dithiothreitol; gap, deoxynucleotide portion of chimeric oligonucleotide; CV, coefficient of variance.

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RNA targets (Gao et al., 1991). In fact, kinetic studies have shown that phosphorothioate oligodeoxynucleotides form heteroduplexes that are cleaved with a slightly lower K_m by *E. coli* RNase H1 than the phosphodiester analog (Crooke et al., 1995). However at higher concentrations, phosphorothioate oligodeoxynucleotides have been shown to inhibit RNase H1 activity (Toulme & Helene, 1988; Gao et al., 1991). More recently other types of nucleotide modifications have been designed with the intent of not only improving the metabolic stability of the oligonucleotide but also increasing the affinity of the oligonucleotide for the target RNA. It appears that more often than not the same properties that enhance the affinity of these analogs for RNA result in loss of RNase H activity. For example, while 2′-methoxy and other 2′-modified oligonucleotides have been shown to exhibit greater metabolic stability and affinity for their RNA targets these compounds do not support RNase H activity (Monia et al., 1993; Cook, 1993; Inoue et al., 1987a,b; Kogoma et al., 1985; Sproat & Lamond, 1993).

Recent antisense strategies have focused on chimeric, or splint, oligonucleotides that intermix deoxy and modified nucleotides within the oligomer in order to achieve the desired increases in metabolic stability and binding affinity while at the same time supporting RNase H activity (Monia et al., 1993; Inoue et al., 1987b; Furdon et al., 1989; Tidd et al., 1988, 1989; Quartin et al., 1989; Agrawal et al., 1990; Hayase et al., 1990; Dagle et al., 1990; Baker et al., 1990). Many different iterations of these oligonucleotides have been designed and tested. Popular design motifs include capping either one or both ends of the oligonucleotide with a contiguous string of modified nucleotides, i.e., wings, to enhance affinity and protect the compound from exonuclease degradation. The remaining sequence or "gap" is then a phosphorothioate oligodeoxynucleotide and thus serves as the substrate for the enzyme.

Kinetic analyses of RNase H digestion of chimeric oligonucleotides indicate the rate of catalysis decreases with diminishing number of contiguous deoxynucleotides (Monia et al., 1993; Crooke et al., 1995; Furdon et al., 1989; Agrawal et al., 1990). Studies of chimeric oligonucleotides with a deoxynucleotide portion centered within the oligonucleotide (the gap) and flanked by the modified nucleotides (the wings) demonstrate that the rates of catalysis for chimeric oligonucleotides with five-deoxynucleotide gaps are approximately 3-fold slower than the unmodified substrate (Crooke et al., 1995). Chimeric oligonucleotides with gaps ≤ 4 deoxynucleotides were determined not to support RNase H1 activity (Crooke et al., 1995). Furthermore, different 2′-analogs, (e.g., 2′-methoxy or 2′-propoxy) placed in the wings resulted in an equivalent effect on the cleavage rate of RNase H cleavage. Similar findings were shown with both cellular based assays and *in vitro* assays using partially purified mammalian RNase H (Monia et al., 1993). Evaluation of antisense activity within the mammalian cells revealed that chimeric oligonucleotides with a gap <5 deoxynucleotides in length were ineffective. Antisense activity was restored with chimeric oligonucleotides containing a gap ≥ 5 deoxynucleotides in length. This minimum deoxynucleotide length correlated perfectly with the minimum length required for efficient RNase H activation in the *in vitro* system. Furthermore, these data suggest that the substrate requirements for both *E. coli* and mammalian RNase H are similar.

Also, it has been previously demonstrated that the initial site of cleavage of RNA bound to a chimeric oligonucleotide containing a deoxy gap is adjacent to the 2′-modified deoxy nucleotide junction closest the 3′-end on the RNA (Crooke et al., 1995). Then with apparent processivity, cleavage of adjacent nucleotides on the RNA bound to the deoxynucleotide portion is effected. This pattern of cleavage can be disrupted by inserting base or sugar modifications in various positions within the gap (Crooke et al., 1995).

While it has been demonstrated that the rate of catalysis for chimeric oligonucleotide substrates with ≤ 7 contiguous deoxynucleotides is slower than unmodified substrates, the mechanism by which the enzymatic activity is curtailed remains unclear. Specifically it is unknown whether the reduction in rate that is observed is due simply to the diminished size of the substrate portion of the chimeric oligonucleotide or are the nucleotide analogs somehow interfering with the enzyme. Although no cocrystal structure for RNase H with the substrate exists, modeling studies based on the crystal structure of the enzyme alone suggest that the binding interaction between the enzyme and substrate comprises approximately 10 base pairs on the heteroduplex substrate (Kanaya et al., 1991). Therefore in the case of the five-deoxynucleotide chimera, the enzyme is likely interacting with both the modified and deoxynucleotide portions of the antisense strand of the chimeric substrate. The relative extent to which either catalysis and/or binding is affected by the modified nucleotides remains to be answered. The binding affinity of RNase H for either modified or unmodified substrates has not previously been reported.

In this study we explore the impact of chemically modified oligonucleotides on both the binding affinity of RNase H for the various substrates as well as the rate of catalysis of the enzyme. The modifications studied included internucleotide linkages and 2′-sugar modifications. These oligonucleotides were designed as either fully modified, i.e., modifications at all sequence positions, or as chimeras. Non cleavable substrate analogs, i.e., fully modified oligonucleotides, were used to determine the binding affinity of the enzyme and the chimeric oligonucleotides were used to measure the rate of catalysis in the context of these modifications. The modifications used were designed to examine four specific properties of the enzyme substrate interaction: (1) the importance and overall contribution of the electrostatic interactions; (2) the role of the helical conformation of the heteroduplex; (3) the steric relationship between the enzyme and the minor groove of the substrate; (4) and the ability of the enzyme to recognize structural defects in the heteroduplex substrate.

MATERIALS AND METHODS

Materials. *E. coli* RNase H (5 units/μL) was purchased from USB (Cleveland, OH). T4 Polynucleotide Kinase was from Promega (Madison, WI). [γ-^{32}P]ATP (7000 Ci/mmol) was purchased from ICN (Irvine, CA). Inhibit-ACE was 5′ → 3′ (Boulder, CO). Yeast tRNA was from Gibco BRL (Grand Island, NY). Trichloroacetic acid (0.6 N) was purchased from Sigma (St. Louis, MO). RNase T1 from *Aspergillus oryzae* was purchased from Boehringer Mannheim (Indianapolis, IN).

Oligonucleotide Synthesis. 2′-Alkoxy and 2′-fluoro monomers were synthesized as previously described (Kawasaki

et al., 1993; Guinosso et al., 1991; R. H. Griffery, unpublished). Synthesis of phosphorothioate (2′-modified and deoxy) and phosphodiester oligonucleotides and oligoribonucleotides were performed using an Applied Biosystems 380B automated DNA synthesizer as previously described (Kawasaki et al., 1993; Guinosso et al., 1991). Synthesis of methylene methylimino (MMI) oligonucleotides was performed as previously described (Morvan et al., 1996). Purification of oligonucleotide products was also as previously described (Kawasaki et al., 1993; Guinosso et al., 1991; R. H. Griffery, unpublished; Morvan et al., 1996). Purified products were greater than 90% full-length material as determined by polyacrylamide gel electrophoresis analysis.

^{32}P Labeling of Oligonucleotides. The oligoribonucleotide (sense strand) was 5′-end labeled with ^{32}P using [γ-^{32}P]ATP, T4 polynucleotide kinase, and standard procedures (Ausubel et al., 1989). The labeled RNA was purified by electrophoresis on 12% denaturing PAGE (Sambrook et al., 1989). The specific activity of the labeled oligonucleotide was approximately 6000 cpm/fmol.

Determination of Initial Rates. Hybridization reactions were prepared in 120 μL of reaction buffer [20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT] containing 750 nM antisense oligonucleotide, 500 nM sense oligoribonucleotide, and 100 000 cpm ^{32}P-labeled sense oligoribonucleotide. Reactions were heated at 90 °C for 5 min and 1 unit of Inhibit-ACE was added. Samples were incubated overnight at 37 °C. Hybridization reactions were incubated at 37 °C with 1.5×10^{-8} mg of *E. coli* RNase H enzyme for initial rate determinations and then quenched at specific time points. Samples were analyzed by trichloroacetic acid (TCA) assay or by denaturing polyacrylamide gel electrophoresis as previously described (Crooke et al., 1995).

Determination of Binding Affinity. Binding affinities were determined by inhibition analysis. Hybridization reactions for the wild type substrate, i.e., oligodeoxynucleotide: oligoribonucleotide hybrid, were prepared as described above except in a final volume of 60 μL. Wild type substrate concentrations ($n > 5$) were varied between 10 and 500 nM. The competing substrate analog was prepared in 60 μL reaction buffer containing equimolar concentrations of the modified sense and antisense oligonucleotides in excess of the wild type substrate concentration. Following overnight incubation at 37 °C, the competing substrate analog was added to the wild type substrate reaction. The mixture was incubated with *E. coli* RNase H for initial rate determinations of the wild type substrate in the presence of the competing substrate, as described above. The samples were analyzed by TCA assay or denaturing PAGE analysis as previously described. These data were analyzed by both the Lineweaver−Burk and Augustinsson methods in order to determine the inhibitory constant (K_i) for the competing substrate, also as previously described (Piszkiewicz, 1977).

RESULTS

Substrate Structure. The substrates used in this study are divided into four categories: single-strand oligonucleotides, fully modified oligonucleotide duplexes, chimeric oligonucleotide duplexes, and "structurally modified" chimeric oligonucleotide duplexes (Table 1A−D and Figure 1A). The fully modified oligonucleotide duplexes contain nucleotide analogs at every sequence position within either the antisense and/or sense oligonucleotides. The chimeric oligonucleotides contain modified residues at positions 1−6 and 12−17, counting 5′ to 3′ on the antisense oligonucleotide. The remaining five deoxynucleotide residues are centered on the oligonucleotide and constitute positions 7−11 (Figure 1A). The "structurally modified" duplexes contain single mismatch base pairs within the context of the 2′-methoxy chimeric oligonucleotide design. The mismatch base pairs are positioned at either the center of the oligodeoxynucleotide gap or at the 5′ junction between the deoxynucleotide gap and the flanking 2′-methoxy modified portion.

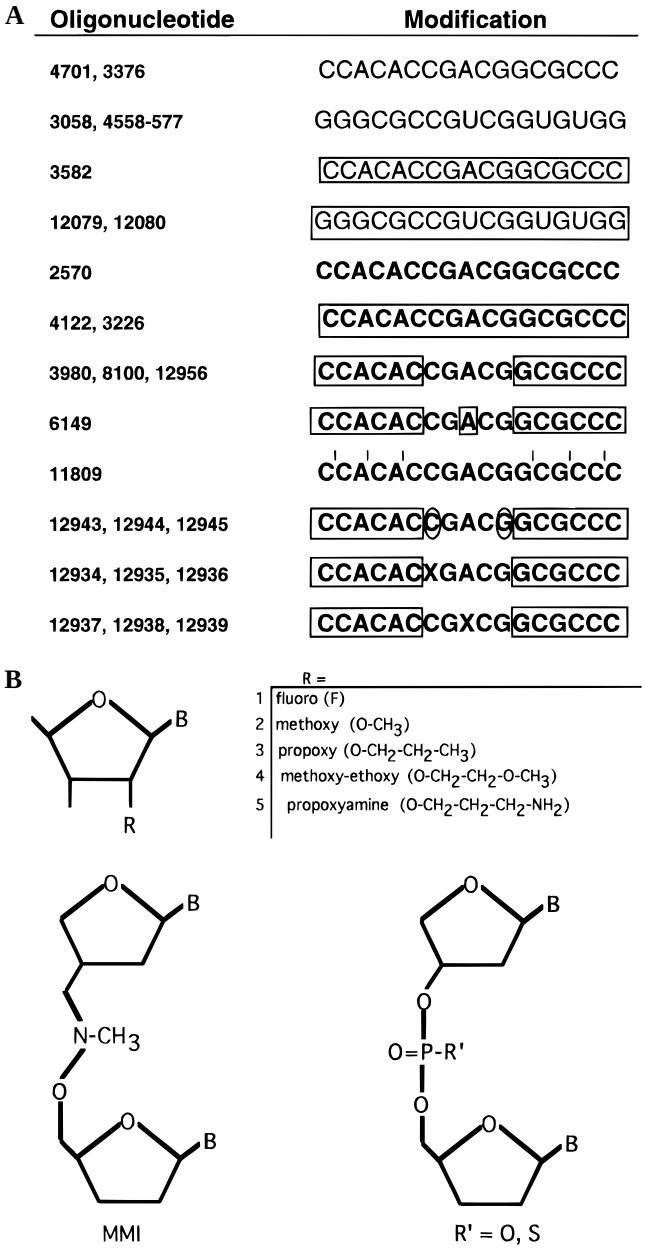

FIGURE 1: Position and structure of internucleotide and 2′-alkyoxy modifications. (A) Phosphorotioate and phosphodiester linkages are shown respectively in bold and plain lettering; boxed sequences represent 2′-alkyoxy modifications; sequences enclosed within circles represent 2′-propoxyamine substitutions; the position of the mismatch nucleotide is represented by ×; and MMI internucleotide modifications (11809) are located at the hash marks. (B) Structure of the 2′-sugar (R) and internucleotide (R′) modifications.

The nucleotide modifications used can be divided into two categories: modification of the internucleotide linkage and

Table 1: Binding Affinity and Rate of Catalysis of RNase H for Oligonucleotide Substrates[a]

antisense[b]	sense[b]	K_d[e] (μM)	V_0[d] (pmol L^{-1} min^{-1})	antisense[b]	sense[b]	K_d[e] (μM)	V_0[d] (pmol L^{-1} min^{-1})
			(A) Single-Strand Substrates				
4701[c] (DNA/P═O)	−	942	0	2570 (DNA/P═S)	−	14	0
3582 (2′OMe/P═O)	−	118	0				
			(B) Fully Modified Oligonucleotide Heteroduplex Substrates				
4701[c] (DNA)	3058[c] (RNA)	−	3250	4122 (2′OMe/P═S)	12080 (2′F/P═O)	2.0	0
4701[c] (DNA)	12079[c] (2′OMe/P═O)	3.4	0	4122 (2′OMe/P═S)	12079 (2′OMe/P═O)	1.0	0
2570 (DNA/P═S)	3058 (RNA)	−	2950	3226 (2′F/P═S)	3058 (RNA)	0.8	0
2570 (DNA/P═S)	12079 (2′OMe/P═O)	0.5	0	3376 (RNA)	3058 (RNA)	1.6	0
3582 2′OMe/P═O	3058 (RNA)	3.1	0	4701 (DNA)	4558−577 (DNA)	176	0
4122 (2′OMe/P═S)	3058 (RNA)	0.6	0				
			(C) Chimeric Oligonucleotide Heteroduplex Substrates				
3980[c] (2′OMe/P═S)	3058[c] (RNA)	−	1075	12943 (2′OPrNH 6, 12)[f]	3058 (RNA)	−	163
3980[c] (2′OMe/P═S)	12079[c] (2′OMe/P═O)	2.5	0	12943 (2′OPrNH 6, 12)[f]	12079 (2′OMe/P═O)	13.1	0
6149 (2′OMe/P═S)	3058 (RNA)	1.0	0	12944 (2′OPrNH 6)[f]	3058 (RNA)	−	204
6149 (2′OMe/P═S)	12079 (2′OMe/P═O)	2.3	0	12944 (2′OPrNH 6)[f]	12079 (2′OMe/P═O)	8.9	0
8100 (2′OPr/P═S)	3058 (RNA)	−	600	12945 (2′OPrNH 12)[f]	3058 (RNA)	−	290
12956 (2′OMe-OEt/P═S)	3058 (RNA)	=	850	12945 (2′OPrNH 12)[f]	12079 (2′OMe/P═O)	9.4	0
11809 (MMI/P═S)	3058 (RNA)	−	1000				
			(D) Chimeric Oligonucleotide Heteroduplex Substrates with Single-Base Mismatch				
3980[c] (2′OMe/P═S)	3058[c] (RNA)	−	1075	12936 (mismatch T, 7)[f]	3058 (RNA)	−	235
3980[c] (2′OMe/P═S)	12079[c] (2′OMe/P═O)	2.5	0	12937 (mismatch C, 9)[f]	3058 (RNA)	−	600
12934 (mismatch G, 7)[f]	3058 (RNA)	−	360	12938 (mismatch G, 9)[f]	3058 (RNA)	−	685
12934 (mismatch G, 7)[f]	12079 (2′OMe/P═O)	3.8	0	12939 (mismatch T, 9)[f]	3058 (RNA)	−	732
12935 (mismatch A, 7)[f]	3058 (RNA)	−	358				

[a] Initial rate and K_d measurements were determined as described in Materials and Methods. [b] The position and structure of the modifications used are shown in Figure 1. [c] Parent oligonucleotide for each substrate category. [d] The initial rates are an average of $n \geq 3$ measurements with estimated errors of CV < 10%. [e] Dissociation constants are derived from ≥3 slopes of Lineweaver−Burk and/or Augustisson analysis. Estimated errors for the dissociation constants are ±2-fold. [f] Numerical notations indicate the position of the modified or mismatch nucleotide, counting 5′ to 3′ on the antisense oligonucleotide.

modifications of the 2′-position (Figure 1B). The former includes phosphorothioate (P═S) and methylene methylimino (MMI) substitution of the phosphodiester (P═O) linkage. The latter consists of fluoro (F), methoxy (OMe), propoxy (OPr), methoxyethoxy (OMe−OEt), and propoxyamine (OPrNH) substitutions at the 2′-position. All phosphorothioate oligonucleotides contain the phosphorothioate linkages at each internucleotide position with the exception of those containing MMI linkages at the designated positions (Figure 1A).

Enzyme Kinetics. The rate of catalysis was determined to vary widely with substrate concentration for the heteroduplex substrates tested, primarily due to differences in K_m. To accurately compare the various heteroduplex substrates independent of the influence of substrate concentration, initial rate measurements were performed under substrate saturating conditions. Saturating substrate levels were defined for each substrate using Michaelis−Menten analyses, as previously described (Piszkiewicz, 1977). The ability of phosphorothioate oligonucleotides to inhibit RNase H has been previously demonstrated (Gao et al., 1991; Crooke et al., 1995). As a result, assays using phosphorothioate oligonucleotide substrates were performed at concentrations determined to be above those necessary for enzyme saturation and below those that inhibit the enzyme. The enzyme concentration was kept constant for all the substrates tested.

Cleavage rates for single-strand oligonucleotides and fully modified heteroduplexes are listed in Table 1A and 1B. Consistent with previous observations, neither the single-strand oligonucleotides nor the heteroduplexes containing full 2′-modified oligonucleotides supported RNase H activity (Monia et al., 1993; Crooke et al., 1995). The P═S modified antisense oligodeoxynucleotide substrate (2570+3058) was cleaved at approximately the same rate as the wild type substrate (4701+3058) (Table 1B).

Cleavage rates for the heteroduplexes containing the chimeric five-deoxy gap oligonucleotides are listed in Table

1C. Consistent with earlier studies, the various modified chimeric substrates were without exception cleaved at a slower rate than the wild type substrate (4701+3058, Table 1B) (Crooke et al., 1995). For example, the 2′-methoxy modified chimeric oligonucleotide substrate (3980+3058, Table 1C) was cleaved approximately 3-fold more slowly than the wild type duplex (4710+3058, Table 1B). The RNA in the various 2′-modified chimeric oligonucleotide substrates (e.g., F, OMe, OPr, OMe-OEt) was digested by the enzyme at approximately the same rate, again consistent with previous reports. Placement of a single 2′-methoxy analog at the center of the five-deoxynucleotide gap chimeric oligonucleotide (6149+3058, Table 1C) rendered the heteroduplex substrate uncleavable. Intermittent replacement of the phosphorothioate internucleotide linkage with MMI in the oligodeoxynucleotide (11809+3058, Table 1C) resulted in a rate of cleavage similar to that observed for the 2′-methoxy/phosphorothioate chimeric substrate (3980+3058, Table 1C) and approximately 3-fold slower than the wild type dulex (4701+3058, Table 1B).

The most significant reduction in activity was observed with the 2′-propoxyamine modified chimeric oligonucleotides (12943, 12944, and 12945, Table 1C). These oligonucleotides were designed with either a single 2′-propoxyamine substitution at either end of the deoxynucleotide gap or a double substitution at both ends of the gap (Figure 1A). Regardless of the position or number of 2′-propoxyamine substitutions these substrates were cleaved approximately 10−20 fold more slowly than the wild type (Table 1B) and 5-fold more slowly than the parent 2′-methoxy chimeric duplex (3980+3058, Table 1C).

The position and sequence of the single-base sequence mismatches introduced into the 2′-methoxy chimeric oligonucleotide are described in Figure 1A. The mismatched base pairs are located at the center of the deoxynucleotide gap (nucleotide position 9) and adjacent to the cleavage site (nucleotide position 7) on the chimeric oligonucleotide. For each location, all three base mismatches were incorporated. Cleavage rates for these structurally modified oligonucleotides are listed in Table 1D. The effect of the single-base mismatch on the rate of catalysis was dependent on the position relative to the gap of the mismatch and independent of the mismatch base sequence. Mismatches introduced immediately adjacent to the 2′-methoxy−deoxy junction (12934+3058, 12935+3058, and 12936+3058, Table 1D) resulted in approximately 3-fold reduction in activity with respect to the parent 2′-methoxy chimeric duplex (3980+3058, Table 1C). Introduction of a mismatch at the center of the gap, i.e., two base pairs downstream of the 2′-methoxy−deoxy nucleotide junction (12937+3058, 12938+3058, and 12939+3058, Table 1D) resulted in only a slight reduction in the cleavage rate when compared to the parent 2′-methoxy chimeric substrate.

Polyacrylamide gel electrophoretic analyses of the cleavage products from these chimeric oligonucleotide substrates are shown in Figure 2. Consistent with earlier studies, the five-deoxynucleotide gap chimeric substrates produced a single cleavage site at the nucleotide adjacent to the 2′-modified-deoxynucleotide junction closest to the 3′ end of the RNA substrate (Crooke et al., 1995). This pattern of cleavage was consistent for all the 2′-modified chimeric substrates with the exception of those incorporating mismatch sequences at the center of the deoxynucleotide gap (12937, 12938, and 12939, Figure 2, lane 12). Digestion of these substrates resulted in an additional cleavage site immediately upstream of the primary cleavage site. The MMI-modified 2′-methoxy chimeric substrate also resulted in two cleavage sites (Figure 2, lane 7). This cleavage pattern is likely the result of the relative placement of the MMI modifications in the oligonucleotide which resulted in a six-deoxynucleotide gap.

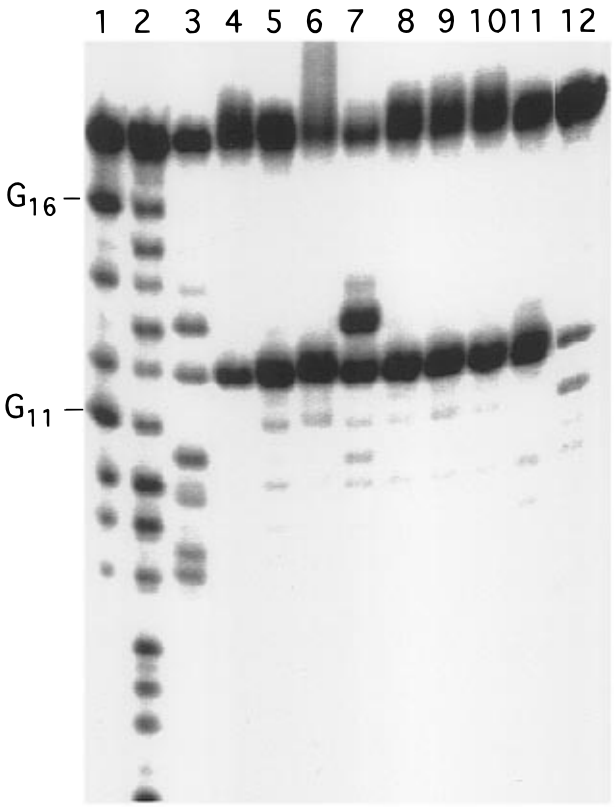

FIGURE 2: Effects of oligonucleotide modifications on the *E. coli* RNase H1 cleavage pattern. Antisense and sense RNA oligonucleotides were preannealed and digested with RNase H as described in Materials and Methods. Polyacrylamide gel analysis of the digestion products was performed as described in Materials and Methods. RNase T1 digestion of ^{32}P-labeled RNA oligonucleotide (3058) was performed in 10 μL containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 3 μM tRNA, and 10^4 cpm RNA. Reactions were incubated at 37 °C for 5 min (lane 1). The base hydrolysis ladder was prepared by incubation of the ^{32}P-labeled RNA at 90 °C for 5 min in 10 μL containing 100 mM sodium carbonate, pH 9.0 (lane 2). The digestion patterns for the various antisense/RNA oligonucleotide heteroduplexes (lanes 3−12) are as follows: DNA/P═S (2570+3058) (lane 3); 2′-methoxy/P═S (3980+3058) (lane 4); 2′-propoxy/P═S (8100+3058) (lane 5); 2′-methoxyethoxy/P═S (12956+3058) (lane 6); MMI/P═S (11809+3058) (lane 7); 2′-propoxyamine at positions 6 and 12 (12943+3058) (lane 8); 2′-propoxyamine at position 6 (12944+3058) (lane 9); 2′-propoxyamine at position 12 (12945+3058) (lane 10); mismatch G at position 7 (12934+3058) (lane 11); mismatch C at position 9 (12937+3058) (lane 12).

Binding Affinity of RNase H for Modified Heteroduplexes. The dissociation constants (K_d) of RNase H for the various oligonucleotides and heteroduplexes were determined indirectly using a competition assay. Specifically, the cleavage rate of the wild type DNA:RNA heteroduplex was determined at a variety of substrate concentrations in both the presence and absence of the competing noncleavable heteroduplex substrate analog. Lineweaver−Burk and Augustinsson analysis of the data were used to determine the inhibitory constant (K_i) for the competing substrate analog

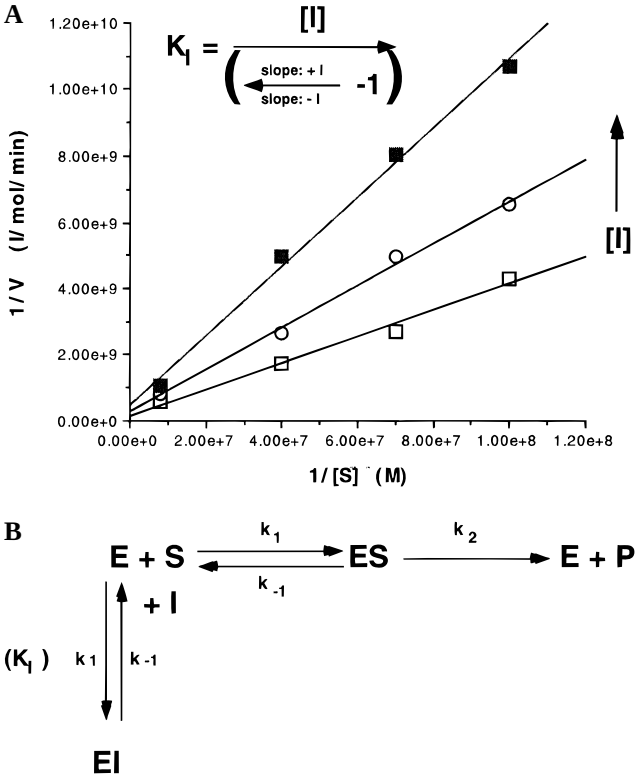

FIGURE 3: Lineweaver−Burk analysis of wild type (RNA/DNA) substrate with increasing modified substrate. (A) Initial rates were determined and Lineweaver−Burk analysis was applied as described in Materials and Methods.The initial rates for the wild type substrate are shown with open boxes. Open circles represent the initial rates for the wild type substrate in the presence of 1.0 μM 2′-methoxy chimeric oligonucleotide/full 2′-methoxy oligonucleotide duplex (3980+12079). Closed boxes represent the initial rates for the wild type substrate in the presence of 2.5 μM (3980+12079). (B) Kinetic pathway for cleavable substrates in the presence of noncleavable inhibitor.

(Figure 3A). The K_i is equivalent to the K_d of the enzyme when a noncleavable heteroduplex inhibitor is used (Figure 3B). Lineweaver−Burk analysis was also used to assess the type of inhibition involved. All of the substrates assayed by this method were determined to function as competitive inhibitors.

The binding affinity of RNase H to single-strand oligonucleotides is shown in Table 1A. RNase H exhibits lower affinity for single-strand oligonucleotides than for duplexed oligonucleotides. The dissociation constant (K_d) for RNase H binding to a single-strand phosphodiester oligodeoxynucleotide (4701, Table 1A) was approximately 300-fold greater than the K_d for the enzyme binding to the phosphodiester heteroduplex (4701+3058, Table 1B). The affinity of RNase H for a 2′-modified single-strand oligonucleotide (3582, Table 1A) was approximately 40-fold weaker than for the parent 2′-modified heteroduplex (3582+3058, Table 1B). Although RNase H binds approximately 70-fold tighter to a phosphorothioate-modified oligodeoxynucleotide (2570, Table 1A) than to a phosphodiester oligodeoxynucleotide (4701, Table 1A), when compared to the parent phosphorothioate heteroduplex (2570+12079, Table 1B) the K_d for single-strand phosphorothioate oligodeoxynucleotide is approximately 30-fold greater than for the double-strand phosphorothioate heteroduplex.

The dissociation constants for RNase H binding to full 2′-modified oligonucleotide heteroduplexes are shown in Table 1B. The enzyme appears to exhibit similar binding affinities for the various 2′-modified heteroduplexes tested. The K_d range between the various 2′-modified substrates falls within the error range of the assay, approximately ±2-fold. Placement of the 2′-modifications within either the antisense or the sense strand had equal impact on the K_d. The K_d for RNase H binding to the double-strand oligodeoxynucleotide (4701+4558−557, Table 1B) was approximately 50-fold weaker than the K_d for RNase H binding to the oligodeoxynucleotide:2′-methoxy oligonucleotide heteroduplex (4701+12079).

Results from the RNase H binding affinity assay for the five-deoxy gap chimeric substrates are listed in Table 1C and 1D. The binding affinity of RNase H to the 2′-methoxy chimeric oligonucleotides was approximately equal to the binding affinities measured for the full 2′-modified heteroduplexes. For example, a K_d of 3.4 μM was measured for the oligodeoxynucleotide:2′-methoxy oligonucleotide heteroduplex (4701+12079, Table 1B) and a K_d of 2.5 μM was determined for the 2′-methoxy chimeric oligonucleotide:2′-methoxy oligonucleotide heteroduplex (3980+12079, Table 1C). As was the case with the enzyme kinetics determinations, the most significant impact on the binding affinity was observed with the 2′-chimeric substrates incorporating the 2′-propoxyamine analog. RNase H bound to these substrates approximately 4−5-fold more weakly than to the parent 2′-methoxy chimeric heteroduplex. The chimeric substrates containing the mismatched sequences bound to the enzyme with similar affinity, irrespective of the position or sequence of the mismatch, as compared to the parent 2′-methoxy chimeric heteroduplex (Table 1D).

DISCUSSION

Structural Properties of Chemically Modified Heteroduplexes. The effects nucleotide analogs exhibit on the interaction between RNase H and the modified substrates are presumably determined by the physical properties of these analogs. Therefore a brief description of the known thermodynamic and structural properties of these modified oligonucleotides is appropriate. In general, many of the nucleotide modifications used in this study share similar physical characteristics with respect to duplex formation. For example oligonucleotides containing these modifications hybridize to RNA with affinity that is approximately equal or greater than for hybridization of an oligoribonucleotide (Kogoma et al., 1985; Inoue et al., 1987b). In addition, NMR and circular dichromism (CD) studies indicate that many of the modifications (e.g., MMI, 2′-F, 2′-OMe, and 2′-OPr) shift the sugar conformation into a 3′-*endo* pucker characteristic of RNA (Nakamura et al., 1991; R. H. Griffery, unpublished; Morvan et al., 1996; Itoh & Tomizawa, 1980). When hybridized to RNA the resulting duplex conformation is A-form. In addition the relatively high purine content in the RNA strand for this sequence suggests an energetic propensity for the DNA:RNA heteroduplex to base stack in the A-form conformation (Ratmeyer et al., 1994). These structural studies also suggest that the 2′-modifications in this study are positioned within the minor groove (Itoh & Tomizawa, 1980; R. H. Griffey, unpublished).

Binding Specificity of RNase H. The purpose of this study was to improve our understanding of the binding interaction between the enzyme and chemically modified substrates and

determine to what extent this interaction affects enzymatic activity. Although the binding affinity of RNase H for heteroduplex substrates has not been reported, structural and mutational studies of the enzyme have been used to speculate on the enzyme/substrate binding interaction. The crystal structure of *E. coli* RNase H1 suggests that the reactive site is nestled within a cluster of lysines. These positively charged amino acids are believed to bind electrostatically to the heteroduplex and thus are believed to define the binding surface of the enzyme (Kanaya et al., 1991). Alanine-scanning mutagenesis of the enzyme showed that reduced enzymatic activity corresponded with a reduction in the number of lysines. The interaction between the binding surface of the enzyme and the phosphate groups on the substrate is believed to take place within the minor groove of the heteroduplex.

We chose inhibition analysis as the method for determining the binding affinity of RNase H for the various substrates for several reasons. First, efforts to measure the binding interaction directly (e.g., nitrocellulose filter binding) proved unsuccessful, presumably due to the weak binding affinity of the enzyme for the substrate. Second, it is possible to formally separate the binding process from the catalytic process with the inhibition assay by the use of noncleavable substrate analogs. Third, this method provides information about the nature of the enzyme/substrate binding interaction. For example, the type of inhibition (e.g., competitive, uncompetitive, non-competitive) gives an indication of whether the inhibitor is binding to the reactive site on the enzyme, modifying the substrate, or causing an allosteric effect resulting in a conformational change in the enzyme.

Our data support the predicted binding interaction based on previous structural and mutational studies of RNase H. Lineweaver−Burk and Augustinsson analyses of the heteroduplexes assayed indicate that without exception these substrates act as competitive inhibitors. Therefore it appears that oligonucleotides binding to the enzyme effectively block the active site and that the predominant binding region for polyanions includes this active site. However, the enzyme/substrate interaction appears to be more specific than mere electrostatic interaction between a positively charged protein surface and polyanions. The binding affinities of RNase H for single strand oligonucleotides were between 30- and 300-fold lower than for the oligoribonucleotide homoduplex. These results suggest that the enzyme binds preferentially to phosphate groups which are spatially oriented within a helical structure as opposed to a random coil. In addition, the binding affinity of RNase H for the double-strand oligodeoxynucleotide was approximately 60-fold lower than for the oligoribonucleotide homoduplex. Therefore it is not only a helix, but also the appropriate helical conformation that appears to be important with greater binding affinity exhibited for A-form like helices than for B-form helices. The dissociation constants for RNase H binding to both the 2′-sugar modifications and modifications of the internucleotide linkage appear to support this observation. Thus we conclude that RNase H specifically binds A-form like helices which include double-strand RNA and DNA:RNA hybrids.

Studies on 2′-modified substrates confirm the notion that RNase H is a dsRNA binding protein. RNase H exhibited the greatest affinity for the various 2′-modified oligonucleotides substrates. These 2′-modified oligonucleotide substrates have either been shown or are predicted to exhibit the preferred A-form helical substrate structure when hybridized to RNA. Interestingly, while these 2′-modifications appear to adopt a preferred conformation with respect to RNase H binding, their size and predicted position within the heteroduplex would suggest possible steric interference with the enzyme. These modifications are predicted to be positioned within the minor groove of the heteroduplex substrate, a region also predicted to be the binding site for the enzyme. Yet, binding affinities for the various 2′-modifications were similar, suggesting that these 2′-alkoxy groups do not, in fact, interfere with the binding interaction.

Binding affinities similar to those measured for the 2′-modified substrates were observed for the phosphorothioate and MMI modified substrates. Phosphorothioate internucleotide linkages have been shown to exert a negligible effect on the helical structure of the substrate. Therefore phosphorothioate-modified substrates are expected to form the appropriate helical structure based on the 2′-modification and/or complementary oligoribonucleotide sequence composition. The MMI modification is a neutral and achiral internucleotide linkage. NMR and modeling studies of MMI modified oligonucleotides indicate that the 3′-CH_2 group of the MMI linkage shifts the sugar conformation to an RNA-like pucker, thus helping the oligonucleotide to preorganize into the preferred A-geometry for duplex formation (Morvan et al., 1996). Again, these results indicate that the higher binding affinity observed with these internucleotide modified substrates is due to the fact that these heteroduplex sequences form the preferred A-form geometry. These data also suggest that replacement of approximately 25% of the negatively charged phosphate groups on the antisense strand of the heteroduplex substrate with neutral MMI linkages was insufficient to affect binding.

While the removal of as many as seven negative charges from the antisense strand of the heteroduplex substrate had no effect on RNase H binding, the introduction of a single 2′-propoxyamine resulted in a significant loss in binding affinity. Similar to the above 2′-modifications, 2′-propoxyamine modifications are predicted to lie within the minor groove of the heteroduplex substrate (R. H. Griffey, unpublished). The size and position within the heteroduplex of the 2′-propoxyamine modification are predicted to be comparable to the size and position of the 2′-methoxyethoxy modification which when incorporated into the chimeric substrate resulted in no loss in activity. Thus the reduction in the binding affinity of RNase H for the 2′-propoxyamine-modified substrate is likely not due to steric interference between the enzyme and the substrate but is probably the result of the positive charge of the modification. Therefore electrostatic repulsion between the positively charged enzyme and this positively charged 2′-modification is likely responsible for the reduction in binding affinity. In contrast, in a previous study we showed that positively charged substituents lying in the major groove are reasonably well tolerated. These results further support the notion that the binding interaction between the enzyme and substrate is predominantly an electrostatic interaction and that this interaction occurs within the minor groove (Crooke et al., 1995).

Impact of Binding Affinity on Enzymatic Activity. Comparisons between the binding affinity and the catalytic rate of RNase H for the chimeric and unmodified heteroduplex substrates suggest that for most analogs the differences observed in activity are not the result of changes in the

binding affinity of the enzyme for these substrates. For example, RNase H digested the 2′-methoxy chimeric substrate (3980+3058) approximately three times slower than the wild type heteroduplex substrate (4710+3058). In contrast, the binding affinities of RNase H for both chimeric and wild type heteroduplex substrates were approximately equal. These data suggest that the slower cleavage rate for the chimeric heteroduplex substrate is not due to a corresponding loss in the binding affinity of the enzyme but is likely the result of the reduction of the size of the catalytic site, i.e., deoxynucleotide portion of the chimeric oligonucleotide. Previous studies have shown that decreasing the size of the deoxynucleotide gap results in a decrease in the rate of cleavage by RNase H (Crooke et al., 1995). Although the enzyme is capable of binding to various regions within the chimeric heteroduplex substrate with equal affinity, only binding to the deoxynucleotide region of the substrate will result in hydrolysis of the RNA. Therefore smaller sites for catalytic action presumably cause a reduction in the rate because a smaller proportion of binding interactions result in hydrolysis of the substrate.

The chimeric substrates incorporating the 2′-propoxyamine modification appear to be the exception. In this particular case there appears to be a direct correlation between the reduction in binding affinity of the enzyme for these substrates and the rates of cleavage. These results suggest that the loss in catalytic activity with the introduction of the 2′-propoxyamine modification is directly due to a corresponding loss in the binding affinity of the enzyme for the substrate which is likely due to the putative electrostatic repulsion between the positively charged modification and the enzyme.

The rate and site of cleavage for the chimeric heteroduplexes containing mismatch sequences varied depending on the position of the mismatch. The cleavage rates for the substrates containing the mismatch adjacent to the initial cleavage site (nucleotide position 7 on the chimeric oligonucleotide) were approximately 3-fold slower than for the fully complementary chimeric substrate. Although the cleavage rates for the heteroduplex substrates with the mismatch positioned at the center of the deoxynucleotide gap (nucleotide position 9 on the chimeric oligonucleotide) were only slightly slower than that observed for the fully complementary substrate, the cleavage pattern was altered with an additional cleavage product immediately upstream of the initial cleavage site. In contrast, RNase H exhibited equal affinity for both the mismatch and fully complementary substrates. These differences between the binding affinity, cleavage rate and cleavage pattern suggest that the binding interaction is not as sensitive to these types of structural defects as the catalytic process. If for example the mismatch bases were stacked inward, structural perturbation of the heteroduplex would likely be minimal and therefore undetectable by the binding process. On the other hand the catalytic process appears to be sensitive to subtle structural modifications within the backbone of the heteroduplex substrate and may therefore be affected by subtle changes in the secondary structure of the substrate at or near the cleavage site.

Biological Implications of the Binding Specificity and Affinity of RNase H. RNase H hydrolyzes RNA in an RNA/DNA heteroduplex. The specificity of this enzyme appears to be due to both the binding interaction and the catalytic process. RNase H appears to specifically bind A-form-like helices enabling the enzyme to selectively bind RNA homoduplexes and RNA/DNA heteroduplexes in the presence of excess double-strand DNA, single-strand random nucleotide coils and other polyanions. The cleavage specificity of the enzyme for RNA/DNA heteroduplexes appears to be due to the catalytic process which has been shown to be sensitive to steric and/or structural alterations within the nucleotide backbone of the RNA/DNA duplex.

The binding affinity of RNase H for the heteroduplex may provide additional information on the biological activity of the enzyme. The micromolar dissociation constant of RNase H for the heteroduplex substrate appears to be relatively high for this interaction. A weak binding affinity may under certain circumstances serve to improve the function of the enzyme. This phenomenon was previously demonstrated with the *Tetrahymena* ribozyme (Young et al., 1991). Mutations were introduced into the ribozyme which effectively destabilized the ribozyme/substrate interaction. The decrease in affinity resulting from this destabilization in turn served to increased the turnover rate of the enzyme. With respect to RNase H, improved turnover due to a weak binding affinity is particularly desirable if the enzyme concentration is limiting with respect to the substrate concentration. Thus under these conditions, improved turnover would allow fewer enzyme molecules to target a greater number of substrate molecules. Additionally, a weak binding affinity may improve the activity of the enzyme if the binding specificity of the enzyme is low. For example, while the binding specificity of the enzyme appears to include the A-form family of duplexes, only RNA/DNA heteroduplexes serve as substrates for the enzyme. This would suggest that many binding interactions between the enzyme and the various A-form like duplexes occur prior to each productive binding interaction, i.e., between the enzyme and the heteroduplex substrate. Therefore a weak binding affinity in this particular case may improve the rate at which the enzyme scans A-form duplexes for the intended heteroduplex substrate. Similar mechanisms have been described for the activities of Endonuclease III and T4 Endonuclease V, enzymes involved in DNA repair (Dodsen et al 1994; Kuo et al., 1992). These enzymes bind nonspecifically to double-strand DNA by electrostatic interactions, scanning the nontarget DNA sequences to search for the damaged sites and subsequently specifically recognize and remove the damaged sites.

With regard to the design of improved antisense drugs, these studies provide additional insights. First, if the biological termination event is suspected to be the result of an RNase H mechanism then a compromise between enhancing affinity of an oligonucleotide for its RNA target and loss of catalytic efficiency must be made. For example, increasing the number of 2′-modifications within an antisense oligonucleotides will in most cases increase the affinity of the oligonucleotide for its target RNA while at the same time result in a decrease in RNase H activity. Second, chemical and/or structural modifications at or near the cleavage site are poorly tolerated. Therefore, specificity may be enhanced by limiting the site available for cleavage, particularly with respect to mismatch base pairing. In this case, mismatch base pairing within an antisense oligonucleotide hybrid not only improves the specificity of the antisense oligonucleotide by reducing its affinity for the target RNA but if positioned within the deoxynucleotide gap also results in a reduction

in the RNase H activity. Consequently, the ideal size of the deoxynucleotide gap is dependent upon a number of factors but as a general rule a gap size of seven deoxynucleotides is perhaps an optimal compromise. Third, although RNase H activity does not appear to be dependent upon the heteroduplex sequence, the binding specificity of the enzyme suggests that sequences which exhibit a propensity to form the preferred A-form duplex may serve as better substrates for RNase H. RNA sequences containing high purine content are predicted to base stack in the A-form conformation (Ratmeyer et al., 1994), thus RNase H activity may be improved with antisense oligonucleotides containing pyrimidine rich sequences. Fourth, with the exception of positively charged groups, all of the 2′-modifications tested were equally tolerated by the enzyme suggesting that regions flanking the deoxynucleotide gap may be optimized for improved hybridization affinity and metabolic stability without affecting RNase H activity.

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REFERENCES

Agrawal, S., Mayrarrd, S. H., Zamecnik, P. C., & Pederson, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1401−1405.
Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) in *Current Protocols in Molecular Biology*, John Wiley, New York.
Baker, C., Holland, D., Edge, M., & Colman, A. (1990) *Nucleic Acids Res. 18*, 3537−3543.
Busen, W. (1980) *J. Biol. Chem. 255*, 9434−9443.
Chiang, M.-Y., Chan, H., Zounes, M. A., Freier, S. M., Lima, W., & Bennett, C. F. (1991) *J. Biol. Chem. 266*, 18162−18171.
Cook, P. D. (1993) in *Antisense Research and Applications* (Crooke, S. T., & Lebleu, B., Eds.) CRC Press, Inc., Boca Raton, FL.
Crooke, S. (1992) *Annu. Rev. Pharmacol. Toxicol. 32*, 329−376.
Crooke, S. T. (1995) in *Therapeutic Applications of Oligonucleotides*, R. G. Landes Co., Austin, TX.
Crooke, S. T., Lemonidis, K. M., Neilson, L., Griffey, R., Lesnik, E. A., & Monia, B. P. (1995) *Biochem J. 312*, 599−608.
Crouch, R. J., & Dirksen, M. L. (1982) in *Nuclease* (Linn, S. M., & Roberts, R. J., Eds.) pp 211−241, Cold Spring Harbor Laboratory Press, Plainview, NY.
Dagle, J. M., Walder, J. A., & Weeks, D. L. (1990) *Nucleic Acids Res. 18*, 4751−4757.
Dodson, M. L., Michaels, M. L., & Lloyd, R. S. (1994) *J. Biol. Chem. 269*, 32709−32711.
Edar, P. S., Walder, R. Y., & Walder, J. A. (1993) *Biochimie 75*, 123−126.
Furdon, P. J., Dominski, Z., & Kole, R. (1989) *Nucleic Acids Res. 17*, 9193−9203.
Gao, W.-Y., Han, F.-S., Storm, C., Egan, W., & Cheng, Y.-C. (1991) *Mol. Pharmacol. 41*, 223−229.
Guinosso, C. J., Hoke, G. D., Freier, S. M., Martin, J. F., Ecker, D. J., Mirabelli, C. K., Crooke, S. T., & Cook, P. D. (1991) *Nucleosides Nucleotides 10*, 259−262.
Hall, K. B., & Mclaughlin, L. W. (1991) *Biochemistry 30*, 10606−10613.
Hausen, P., & Stein, H. (1970) *Eur. J. Biochem. 14*, 279.
Hayase, Y., Inoue, H., & Ohtsuka, E. (1990) *Biochemisry 29*, 8793−8797.
Inoue, H., Hayase, Y., Iwai, S., Miura, K., & Ohtsuka, E. (1987a) *Nucleic Acids Res. 15*, 6131−6148.
Inoue, H., Hayase, Y., Iwai, S., & Ohtsuka, E. (1987b) *FEBS Lett. 215*, 327−330.
Itaya, M., & Kono, K. (1991) *Nucleic Acids Res. 19*, 4443−4449.
Itaya, M., Mcelvin, D., Chatterjie, S. K., & Crouch, R. J. (1991) *Mol. Gen. Genet. 227*, 438−445.
Itoh, T., & Tomizawa, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2450−2445.
Kanaya, S., & Ktaya, M. (1992) *J. Biol. Chem. 267*, 10184−10192.
Kanaya, S., Katsuda-Kakai, C., & Ikehara, M. (1991) *J. Biol. Chem. 266*, 11621−11627.
Katayangi, K., Miyagawa, M., Matsushima, M., Ishkiawa, M., Kanaya, S., Ikehara, M., Matsuzaki, T., & Morikawa, K. (1990) *Nature 347*, 306−309.
Katayanagi, K., Okumura, M., Morikawa, K. (1993) *Proteins: Struct., Funct., Genet. 17*, 337−346.
Kawasaki, A. M., Casper, M. D., Freier, S. M., Lesnik, E. A., Zounes, M. C., Cummins, L. L., Gonzalez, C., & Cook, P. D. (1993) *J. Med. Chem. 36*, 831−841.
Kogoma, T., Subia, N. L., & Meyenburg, K. (1985) *Mol. Gen. Genet. 200*, 103−109.
Kuo, C-F., McRee, D. E., Fisher, C. L., O'Handley, S. F., Cunningham, R. P., & Tainer, J. A. (1992) *Science 258*, 434−440.
Lane, A. N., Ebel, S., & Brown, T. (1993) *Eur. J. Biochem. 215*, 297−306.
Mirabelli, C. K., & Crooke, S. T. (1993) in *Antisense Research and Applications* (Crooke, S. T., & Lebleu, B., Eds.) pp. 15−35, CRC Press, Inc., Boca Raton, FL.
Monia, B. P., Johnson, J. F., Ecker, D. J., Zounes, M. A., Lima, W. F., & Freier, S. M. (1992) *J. Biol. Chem. 267*, 19954−19962.
Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., & Freier, S. M. (1993) *J. Biol. Chem. 268*, 14514−14522.
Morvan, F., Sanghvi, Y. S., Perbost, M., Vasseur, J. J., & Bellon, L. (1996) *J. Am. Chem. Soc. 118*, 225−226.
Nakamura, H., Oda, Y., Iwai, S., Inoue, H., Ohtsuka, E., Kanaya, S., Kimura, S., Katsuda, C., Katayanagi, K., Morikawa, K., Miyashiro, H., & Ikehara, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11535−11539.
Pardi, A., Martin, F. H., & Tinoco, I. (1981) *Biochemistry 20*, 3986−3996.
Piszkiewicz, D. (1977) in *Kinetics of Chemical and Enzyme-Catalyzed Reactions*, Oxford University Press, New York.
Quartin, R. S., Brakel, C. L., & Wetmur, J. G. (1989) *Nucleic Acids Res. 17*, 7253−7262.
Ratmeyer, L., Vinayak, R., Zhong, Y. Y., Zon, G., & Wilson, W., D. (1994) *Biochemistry 33*, 5298−5304.
Rong, Y. W., & Carl, P. I. (1990) *Biochemistry 29*, 383−389.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
Sproat, B. S., & Lamond, A. I. (1993) in *Antisense Research and Applications* (Crooke, S. T., & Lebleu, B., Eds.) CRC Press, Inc., Boca Raton, FL.
Stein, C. A., Subasinghe, C., Shinozuka, K., & Cohen, J. S. (1988) *Nucleic Acids Res. 16*, 3209−3221.
Suck, D., & Oefner, C. (1986) *Nature 321*, 620−625.
Tidd, D. M., & Warenius, H. M. (1989) *Br. J. Cancer 60*, 343.
Tidd, D. M., Hawley, P., Wernius, H. M., & Gibson, I. (1988) *Anti-Cancer Drug Des. 3*, 117.
Toulme, J. J., & Helene, C. (1988) *Gene (Amsterdam) 72*, 51−58.
Yang, W., Hendrickson, W. A., Crouch, R. J., & Satow, Y. (1990) *Science 249*, 1398−1405.
Young, B., Herschlag, D., & Cech, T. R. (1991) *Cell 67*, 1007−1019.

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