We have characterized cloned His-tag human RNase H1. The activity of the enzyme exhibited a bell-shaped response to divalent cations and pH. The optimum conditions for catalysis consisted of 1 mM Mg$^{2+}$ and pH 7–8. In the presence of Mg$^{2+}$, Mn$^{2+}$ was inhibitory. Human RNase H1 shares many enzymatic properties with Escherichia coli RNase H1. The human enzyme cleaves RNA in a DNA-RNA duplex resulting in products with 5′-phosphate and 3′-hydroxy termini, can cleave overhanging single strand RNA adjacent to a DNA-RNA duplex, and is unable to cleave substrates in which either the RNA or DNA strand has 2′ modifications at the cleavage site. Human RNase H1 binds selectively to "A-form"-type duplexes with approximately 10–20-fold greater affinity than that observed for E. coli RNase H1. The human enzyme displays a greater initial rate of cleavage of a heteroduplex-containing RNA-phosphorothioate DNA than an RNA-DNA duplex. Unlike the E. coli enzyme, human RNase H1 displays a strong positional preference for cleavage, i.e., it cleaves between 8 and 12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex. Within the preferred cleavage site, the enzyme displays modest sequence preference with GU being a preferred dinucleotide. The enzyme is inhibited by single-strand phosphorothioate oligonucleotides and displays no evidence of processivity. The minimum RNA-DNA duplex length that supports cleavage is 6 base pairs, and the minimum RNA-DNA "gap size" that supports cleavage is 5 base pairs.

RNase H1 hydrolyzes RNA in RNA-DNA duplexes (1). Proteins with RNase H activity have been isolated from numerous organisms ranging from viruses to mammalian cells and tissues (2–7). Although RNase H isotypes vary substantially in molecular weight and associated functions, the nuclease properties of the enzymes are similar. All RNase H enzymes, for example, function as endonucleases, specifically cleave RNA in RNA-DNA duplexes, require divalent cations, and generate products with 5′-phosphate and 3′-hydroxyl termini (7).

In prokaryotes, three classes of RNase H enzymes, RNase H1, H2, and H3, have been identified. RNase H2 and H3 share significant sequence homology, whereas RNase H3 and RNase H1 share similar divalent cation preference and cleavage properties. Of the three classes, RNase H2 appears to be the most ubiquitous (8). To date no organism has been shown to express active forms of all three classes of RNase H. The best characterized of the prokaryotic enzymes is Escherichia coli RNase H1 (9–13). This enzyme is believed to be involved in DNA replication (14). The key amino acids involved in metal binding, substrate binding, and catalysis have been identified and are highly conserved in the RNase H family (12, 15–17). Furthermore, the enzyme-substrate interaction has been elucidated by the three-dimensional structure of the enzyme as well as chemical and structural modification of the heteroduplex substrate (10, 13, 18–21).

RNase H has also been shown to be involved in viral replication. RNase H domains have been identified in viral reverse transcriptases, and these typically share homology with E. coli RNase H1 (15). The RNase H portion of the enzyme has been shown to cleave the viral RNA strand producing RNA primers for second strand DNA synthesis, thereby converting the viral RNA into double strand DNA (22).

Two classes of RNase H enzymes have been identified in mammalian cells (2–6). They were reported to differ with respect to co-factor requirements and activity. For example, RNase H type 1 has been shown to be activated by both Mg$^{2+}$ and Mn$^{2+}$ and was active in the presence of sulfhydryl reagents, whereas RNase H type 2 was shown to be activated by only Mg$^{2+}$ and inhibited by Mn$^{2+}$ and sulfhydryl reagents (6). Although the biological roles of the mammalian enzymes are not fully understood, it has been suggested that mammalian RNase H type 1 may be involved in replication and that the type 2 enzyme may be involved in transcription (25, 26).

Recently both mammalian RNase H genes have been cloned and expressed (16, 17, 27). In a previous study we have reported the cloning and expression of a His-tag-labeled RNase H from human cells (16). The human enzyme was homologous to E. coli RNase H1. However, its biochemical properties were similar to those reported for the partially purified RNase H type 2. Because it was the first human enzyme to be cloned, it is referred to as human RNase H1. Additionally, a second human RNase H has been cloned (27) but not yet been expressed in an active form. It was shown to be homologous to E. coli RNase H2 (28). It is referred to as human RNase H2.

In this communication we provide the first detailed characterization of the enzymological properties of human RNase H1 and compare its properties to those of the homologous protein E. coli RNase H1. These studies provide a basis to begin to develop a better understanding of the biological and pharmacological roles of the human RNase H family and to design antisense drugs that interact more effectively with the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—T4 polynucleotide kinase was purchased from Promega (Madison, WI). $[^{32}P]$ATP and $[^{32}P]$cytidine bisphosphate were purchased from ICN (Irvine, CA). RNase inhibitor was from 5 Prime → 3 Prime, Inc. (Boulder, CO). Calf intestine alkaline phosphatase (CIP) and T4 RNA ligase were purchased from Roche Molecular Biochemicals. Some oligodeoxynucleotides were purchased from Retrogen Inc. (San Diego, CA). The oligodeoxynucleotides were greater than 90% full-length material as determined by capillary gel electrophoresis analysis.

1 H. Wu, unpublished data.

2 The abbreviation used is: CIP, calf intestine alkaline phosphatase.
ysis. Human RNase H1 with a His-tag was expressed and purified from a bacterial expression system as described previously (16).

Oligonucleotide Synthesis—Synthesis of 2'-methoxy, 2'-fluoro, 2'-propoxy, and deoxy chimeric oligonucleotides was performed using an Applied Biosystems 380B automated DNA synthesizer as described previously (29, 30). Purification of oligonucleotides was also as described previously (29, 30). Purified oligonucleotides were greater than 90% full-length material as determined by capillary gel electrophoretic analysis.

Properties of Purified Human RNase H1—The effects of various reaction conditions on the activity of human RNase H1 were evaluated (Fig. 1). The optimal pH for the enzyme in both Tris-HCl and phosphate buffers was 7.0–8.0. At pH values above pH 8.0, enzyme activity was reduced. However, this could be due to instability of the substrate or effects on the enzyme, or both. To evaluate the potential contribution of changes in ionic strength to the activities observed at different pH values, two buffers, NaH2PO4 and Tris-HCl, were studied at pH 7.0 and gave the same enzyme activity even though the ionic strengths differed. Enzyme activity was inhibited by increasing ionic strength (Fig. 1B) and N-ethylmaleimide (Fig. 1C). Activity increased as the temperature was raised from 25 to 42 °C (Fig. 1D). Mg2+ stimulated enzyme activity with an optimal concentration of 1 mM. At higher concentrations, Mg2+ was inhibitory (Fig. 1E). In the presence of 1 mM Mg2+, Mn2+ was inhibitory at all concentrations tested (Fig. 1F). The purified enzyme was quite stable and easily handled. In fact, the enzyme could be boiled and rapidly or slowly cooled without significant loss of activity (Fig. 1D). The initial rates of cleavage were determined for four duplex substrates studied simultaneously. The initial rate of cleavage for a phosphodiester DNA-RNA duplex was 1050 ± 203 pmol liter−1 min−1 (Table I). The initial rate of cleavage of a phosphorothioate oligodeoxynucleotide duplex was approximately 4-fold faster than that of the same duplex comprised of a phosphodiester antisense oligodeoxynucleotide (Table I). The initial rates for 17-mer and 20-mer substrates of different sequences were equal (Table IB). However, when a 25-mer heteroduplex containing the 17-mer sequence in the center of the duplex was digested (RNA No. 3), the rate was 50% faster. Interestingly, the K0.5 of the enzyme for the 25-mer duplex was 40% lower than that for the 17-mer, whereas the Vmax values for both duplexes were the same (see Table III), suggesting that with the increase in length, a larger number of cleavage sites are available, resulting in an increase in the number of productive binding interactions between the enzyme and substrate. As a result, a lower substrate concentration is required for the longer duplex to achieve a cleavage rate equal to that of the shorter duplex.

To better characterize the substrate specificity of human RNase H1, duplexes in which the antisense oligodeoxynucleotide was modified in the 2′ position were studied. As previously reported for E. coli RNase H1 (18–21), human RNase H1 was unable to cleave substrates with 2′ modifications at the cleavage site of the antisense DNA strand or the sense RNA strand (Table II). For example, the initial rate of cleavage of a duplex containing a phosphorothioate oligodeoxynucleotide and its complement was 3400 pmol liter−1 min−1, whereas that of its 2′-propoxy-modified analog was undetectable (Table II). A duplex comprised of a fully modified 2′-methoxy antisense strand also failed to support any cleavage (Table II). The placement of 2′-methoxy modifications around a central region of oligodeoxynucleotides reduced the initial rate (Table II). The smaller the central oligodeoxynucleotide “gap,” the lower the initial rate. The smallest “gap-mer” for which cleavage could be measured was a 5 deoxynucleotide gap. These data are highly consistent with observations we have previously reported for E. coli RNase H1, except that for the bacterial enzyme, the minimum gap size was 4 deoxynucleotides (18, 20, 21).

The K0.5 and Vmax of human RNase H1 for three substrates are shown in Table III. The K0.5 values for all three substrates were substantially lower than those of E. coli RNase H1 (Table III) (18, 19). As previously reported for E. coli RNase H1, the Km for a phosphorothioate-containing duplex was lower than...
that of a phosphodiester duplex. The V\textsubscript{max} of the human enzyme was 30-fold lower than that of the E. coli enzyme. The V\textsubscript{max} for the phosphorothioate-containing substrate was less than the phosphodiester duplex. This is probably due to inhibition of the enzyme at higher concentrations by excess phosphorothioate single strand oligonucleotide (see below), as the
Effects of phosphorothioate substitution and substrate length on digestion by human RNase H1

Oligoribonucleotides were preannealed with the complementary antisense oligodeoxynucleotide at 10 and 20 °C and subjected to digestion by human RNase H1. The 17-mer (RNA No. 1) and 25-mer (RNA No. 3) RNA sequences are derived from Ha-Ras oncogen (51), and the 25-mer RNA contains the 17-mer sequence. The 20-mer (RNA No. 2) sequence is derived from human hepatitis C virus core protein coding sequence (52). The initial rates were determined as described under "Experimental Procedures." A, comparison of the initial rates of cleavage of an RNA-phosphodiester (P=O) and an RNA-phosphorothioate (P=S) duplexes. B, comparison among duplexes of different sequences and lengths.

**Table I**

| RNA No. | RNA | Antisense DNA | Initial rate |
|---------|-----|--------------|-------------|
|         |     | 17-mer P=O   | 17-mer P=S  |
| A       | 1   | 6650 ± 266   | 4034 ± 168  |
| B       | 1   | 3056 ± 56    | 1081 ± 33   |
|         | 2   | 3030 ± 81    | 605 ± 81    |
|         | 3   | 300 ± 56     | 17-mer P=O  |
|         |     | 17-mer P=O   | 17-mer P=S  |
|         | 4*  | 3400 ± 384   | 20-mer P=O  |
|         |     | 20-mer P=O   | 20-mer P=S  |

**Table II**

Effects of 2'-substitution and deoxy-gap size on digestion rates by human RNase H1

Substrate duplexes were hybridized, and initial rates were determined as described in Table 1 and described under "Experimental Procedures." The 17-mer RNA is the same used in Table 1, and the 20-mer RNA (UGGUGGCGGCUUGGGCGGCUU, RNA No. 4) was derived from the protein kinase C C (53) sequence. The 17-mer and 20-mer P=S oligonucleotides were fully deoxyporphosphorothioate-containing No. 2'-modifications. The 9, 7, 5, 4, and 3 deoxy gap oligonucleotides were 17-mer oligonucleotide with a central portion consisting of nine, seven, and five, and four deoxynucleotides flanked on both sides by 2'-methoxynucleotides (also see Fig. 2). Boldface sequences indicate the position of the 2'-methoxyl-modified residues. The italic sequences indicate the position of the 2'-propoxy-modified residues.

**Table III**

Kinetic constants for RNase H1 cleavage of RNA-DNA duplexes

The RNA-DNA duplexes in Table 1 were used to determine $K_m$ and $V_{max}$ of human and E. coli RNase H1 as described under "Experimental Procedures." The $K_m$ (nm) and $V_{max}$ (pmol liter$^{-1}$ min$^{-1}$) values for human RNase H1 and E. coli RNase H1 are shown in Table III.

**Table IV**

Binding constants and specificity of RNase H1

$K_d$ values were determined as described under "Experimental Procedures." The $K_d$ values for E. coli RNase H1 were derived from previously reported data (21). The competing substrates (competitive inhibitors) used in the binding study are divided into two categories: single strand (ss) oligonucleotides and oligonucleotide duplexes all with the 17-mer sequence as in Table 1 (RNA No. 1). The single strand oligonucleotides included ssRNA, ssDNA, as fully modified 2'-methoxy phosphodiester oligonucleotide (ssDNA, P=S), and ss full phosphorothioate deoxynucleotide (ssDNA, P=S). The duplex substrates include DNA-DNA duplex, RNA-RNA duplex, DNA-RNA duplex, RNA-DNA duplex, and RNA-DNA duplex. Dissociation constants are derived from $3$ slopes of Lineweaver-Burk and/or Augustisson analysis. Estimated errors for the dissociation constants are $2$-fold. Specificity is defined by dividing the $K_d$ for duplex by the $K_d$ for an RNA-RNA duplex.

**Human RNase H1**

methoxy oligonucleotide as the noncleavable substrate is considered the most like DNA-RNA. Table IV shows the results of these studies and compares them to previously reported results for the E. coli enzyme performed under similar conditions (20, 21). Clearly, the affinity of the human enzyme for its DNA-RNA like substrate (DNA-2'-methoxy) was substantially greater than that of the E. coli enzyme, consistent with the differences observed in $K_m$ (Table III).

$E. coli$ RNase H1 displays approximately equal affinity for RNA-RNA, RNA-2'-methoxy, and DNA-2'-methoxy duplexes (Table IV). The human enzyme displays similar binding properties but is more able to discriminate between various duplexes. For example, the $K_d$ for RNA-RNA was approximately 5-fold lower than the $K_d$ for DNA-2'-methoxy. This is further demonstrated by the $K_d$ for the RNA-2'-fluoro duplex. The $K_d$ for the DNA-2'-fluoro duplex was slightly greater than for the RNA-2'-fluoro duplex and the RNA-RNA duplex but clearly lower than for other duplexes. Thus, both enzymes can be considered double strand RNA-binding proteins. However, human RNase H1 is somewhat less specific for duplexes as compared with single strand oligonucleotides than the $E. coli$ enzyme. The enzyme bound to single strand RNA and DNA only
20-fold less well than an RNA-RNA duplex, whereas the *E. coli* enzyme bound to single strand DNA nearly 600-fold less than to an RNA-RNA duplex (Table IV). The affinity of a single strand phosphorothioate oligodeoxynucleotide for both enzymes was significant relative to the affinity for the natural substrate and accounts for the inhibition of the enzymes by members of this class oligonucleotides. Remarkably, human RNase H1 displayed the highest affinity for a single strand phosphorothioate oligodeoxynucleotide. Thus, this noncleavable substrate is a very effective inhibitor of the enzyme, and excess phosphorothioate antisense drug in cells might be highly inhibitory.

**Site and Sequence Preferences for Cleavage**—Fig. 2 shows the cleavage pattern for RNA duplexed with its phosphorothioate oligodeoxynucleotide for both enzymes was significant relative to the affinity for the natural substrate and accounts for the inhibition of the enzymes by members of this class oligonucleotides. Remarkably, human RNase H1 displayed the highest affinity for a single strand phosphorothioate oligodeoxynucleotide. Thus, this noncleavable substrate is a very effective inhibitor of the enzyme, and excess phosphorothioate antisense drug in cells might be highly inhibitory.

**Site and Sequence Preferences for Cleavage**—Fig. 2 shows the cleavage pattern for RNA duplexed with its phosphorothioate oligodeoxynucleotide and the pattern for several gap-mers. In the parent duplex, RNA cleavage occurred at a single major site with minor cleavage noted at several sites 3' to this major cleavage site that was 8 nucleotides from 5'-end of the RNA. Note that the preferred site occurred at a GU dinucleotide. Cleavage of several gap-mers occurred more slowly, and the major cleavage site was at a different position from that of the parent duplex. Furthermore, in contrast to the observations we have made for *E. coli* RNase H1 (18), the major cleavage site in gap-mers treated with human RNase H1 did not occur at the nucleotide apposed to the nucleotide adjacent to the first 2'-methoxy nucleotide in the wing hybridized to the 3' portion of the RNA.

To further evaluate the site and sequence specificities of human RNase H1, cleavage of substrates shown in Figs. 3 and 4 was studied. In Fig. 3, the sequence of the RNA is displayed below the sequencing gels, and the length and position of the complementary phosphodiester oligodeoxynucleotide is indicated by the solid line below the RNA sequence. The arrows indicate the sites of the enzyme digestion, and the size of the arrows reflect the relative cleavage intensities.

**Fig. 2.** Denaturing polyacrylamide gel analysis of human RNase H1 cleavage of 17-mer RNA-DNA gap-mer duplex. Antisense oligonucleotides were hybridized with 5'-end-labeled sense RNA as described under “Experimental Procedures,” then digested with RNase H1 for 30 and 60 min at 37 °C. A base hydrolysis RNA ladder was prepared as described under “Experimental Procedures.” The RNA ladder was sequenced with RNases T1, CL3, and A1 (data not shown). For each substrate, the RNA sequences (5'-3') are shown above the DNA sequence. Boxed sequences indicate the position of the 2'-methoxy-modified residues. The arrows indicate the sites of the enzyme digestion, and the size of the arrows reflect the relative cleavage intensities.

**Fig. 3.** Analysis of human RNase H1 cleavage of a 25-mer Ras RNA hybridized with phosphodiester oligodeoxynucleotides of different lengths. Antisense oligonucleotides with different lengths from 6- to 17-mer were hybridized with 5'-end-labeled 25-mer sense Ha-Ras RNA as described under “Experimental Procedures,” then digested with RNase H1 at 37 °C for a time course of 0, 2, 5, and 10 min shown on the gel (left to right) for each substrate (A to F). A 25-mer RNA ladder was prepared and sequenced as described the legend for Fig. 2. For each substrate, the RNA sequences (5'-3') are shown in the figure, and antisense DNA sequences were indicated by the solid line below the RNA sequence. The arrows indicate the sites of the enzyme digestion, and the size of the arrows reflect the relative cleavage intensities.
that the 6-nucleotide duplex was fully hybridized, the reactions were carried out at a 50:1 DNA-RNA ratio (data not shown). Fourth, the figure shows that for duplexes smaller than the nine base pairs, the slower the cleavage.

The site and sequence specificities are further explored in Fig. 4. That the enzyme selects a position from 8 to base pair 12. Interestingly, the sequence at both sites was 8–12 nucleotides from the 5'-RNA-DNA terminus. Finally, optimal cleavage seemed to occur when a GU dinucleotide was located 8–12 nucleotides from the 5'-RNA-3'-DNA terminus of the duplex.

To address both the mechanism of cleavage and processivity, the cleavage of 5'-labeled and 3'-labeled substrates was compared (Fig. 5). Lane C shows that CIP treatment before and after digestion with human RNase H1 resulted in a shift in the mobility of the digested fragments, suggesting that human RNase H1 generates cleavage products with 5'-phosphates. Thus, it is similar to E. coli RNase H1 in this regard (20). A second intriguing observation is that the addition of [32P]cytidine to the 3'-end of the RNA caused a shift in the position of the preferred cleavage site (A versus B or C). The four cleavage sites in the center of the duplex observed with a 5'-phosphate-labeled RNA were observed in 3'-[32P]cytidine-labeled substrates. However, the main cleavage site shifted from base pair 8 to base pair 12. Interestingly, the sequence at both sites was GU. Thus, it is conceivable that the enzyme selects a position 8–12 nucleotide from the 5'-RNA-3'-DNA terminus then
cleaves at a preferred dinucleotide such as GU. Third, this figure considered along with the cleavage patterns shown in Figs. 3 and 4 demonstrates that this enzyme displays minimal processivity in either the 5’ or 3’ direction. In no time-course experiment using any substrate have we observed a pattern that would be consistent with processivity. The possibility that the failure to observe processivity in Figs. 3 and 4 was due to processivity in the 3’ to 5’ direction is excluded by the results in Fig. 5. Again, this is significantly different from observations we have previously reported for E. coli RNase H1 (18).

**DISCUSSION**

**General Properties of Human RNase H1 Activity**—In this study, we have characterized the properties of human RNase H1. As the protein studied was a His-tag fusion and was denatured and refolded, it is possible that the activity of the enzyme in its native state might be greater than we have observed. However, basic properties reported in this paper are certainly likely to reflect the basic properties of the native enzyme. Numerous studies have shown that a His-tag does not interfere with protein folding and crystallization (35, 36), kinetic and catalytic properties (37, 38), or nucleic acid binding properties (39, 40), since it is very small (few amino acids), and its pK is near neutral. As shown in this and our previous (16) studies, this Histag fusion protein did behave like other RNase H proteins (6, 7). It cleaved specifically the RNA strand in RNA-DNA duplexes, resulting in cleavage products with 5’-phosphate termini (Fig. 5), and was affected by divalent cations (Fig. 1). Optimal conditions for human RNase H1 were similar phate termini (Fig. 5), and was affected by divalent cations proteins (6, 7). It cleaved specifically the RNA strand in RNA-

Numerous studies have shown that a His-tag does not interfere in its native state might be greater than we have observed. As shown in this and our previous (16) studies, the His-tag fusion protein did behave like other RNase H proteins (6, 7). It cleaved specifically the RNA strand in RNA-DNA duplexes, resulting in cleavage products with 5’-phosphate termini (Fig. 5), and was affected by divalent cations (Fig. 1). Optimal conditions for human RNase H1 were similar but not identical to E. coli RNase H1. For the human enzyme, the Mg2+ optimum was 1 mM, and 5 mM Mg2+ was inhibitory. In the presence of Mg2+, both enzymes were inhibited by Mn2+. The human enzyme was inhibited by N-ethylmaleimide and was quite stable, easily handled, and did not form multimeric structures (Fig. 1). The ease of handling, denaturation, refolding, and stability in various conditions suggest that the human RNase H1 was active as a monomer and has a relatively stable preferred conformation.

Studies on the structure and enzymatic activities of a number of mutants of E. coli RNase H1 have recently led to a hypothesis to explain the effects of divalent cations termed an activation/attenuation model (41). The effects of divalent cations on human RNase H1 are complex and are consistent with the suggested activation/attenuation model. The amino acids proposed to be involved in both cation binding sites are conserved in human RNase H1 (16).

**Positional and Sequence Preferences and Processivity**—The site and sequence specificity of human RNase H1 differ substantially from E. coli RNase H1. Although neither enzyme displays significant sequence specificity (Ref. 18 and Figs. 2–5), the human enzyme displays remarkable site specificity. Figs. 2–4 show that human RNase H1 preferentially cleaved 8–12 nucleotides 3’ from the 5’-RNA-3’-DNA terminus of a DNA-RNA duplex irrespective of whether there were 5’ or 3’ RNA or DNA overhangs. The process by which a position is selected and then within that position on the duplex a particular dinucleotide is cleaved preferentially must be relatively complex and influenced by sequence. Clearly, the dinucleotide, GU, is a preferred sequence. In Fig. 3, for example, all the duplexes contained a GU sequence near the optimal position for the enzyme, and in all cases, the preferential cleavage site was GU. Additionally, in duplexes A and B a second GU was also cleaved, albeit at a very slow rate. The third site in duplexes A and B cleaved was a GG dinucleotide 7 base pairs from the 3’-RNA-5’-DNA terminus. Thus, the data suggest that the enzyme displays strong positional preference and, within the appropriate site, slight preference for GU dinucleotides.

The strong positional preference exhibited by human RNase H1 suggests that the enzyme fixes its position on the duplex via the 5’-RNA-3’-DNA terminus. Interestingly, the in vitro cleavage pattern observed for the enzyme is compatible with its proposed in vivo role, namely, the removal of RNA primers during DNA replication of the lagging strand. The average length of the RNA primer ranges from 7 to 14 nucleotides (42). Consequently, synthesis of the lagging strand results in chimeric sequences consisting of 7–14 ribonucleotides at the 5’ terminus with contiguous stretches of DNA extending in the 3’ direction. The positional preference observed for human RNase H1 (i.e. 8–12 residues from the 5’ terminus of the RNA) would suggest that cleavage of the chimeric lagging strand by RNase H1 would occur at or near the RNA-DNA junction. The removal of residual ribonucleotides following RNase H digestion has been shown to be performed by the endonuclease FEN1 (43).

Fig. 4 provides additional insight into the positional and sequence preferences of the enzyme. When there was a GU dinucleotide present in the correct position in the duplex, it was cleaved preferentially. When a GU dinucleotide was absent, AU was cleaved as well as other dinucleotides. For duplex G, both a GU and a GG dinucleotide were present within the preferred site, and in this case the GG dinucleotide was cleaved slightly more extensively than the GU dinucleotide. Clearly, additional duplexes of different sequences must be studied before definitive conclusions concerning the roles of various sequences within the preferred cleavage sites can be drawn.

In Fig. 5, the 3’ terminus of the RNA was labeled with [32P]Pcylidine. In this case the same four nucleotides were cleaved as when the RNA was 5’-labeled (Fig. 5, panels B and C). However, the GU closer to the 3’ terminus of the RNA was cleaved at least as rapidly as the 5’-GU. Interestingly in studies on the partially purified enzyme, differences in the cleavage pattern were also observed when 5’-labeled substrates were compared with 3’-labeled substrates (6). At present, we have no explanation for this observation, but one possibility is that the presence of a 3’-phosphate on an oligonucleotide substrate affects the scanning mechanism the enzyme uses to select preferred positions for cleavage.

In a duplex comprised of RNA annealed to a chimeric oligonucleotide with an oligodeoxynucleotide center flanked by 2’-modified nucleotide wings, the cleavage by human RNase H1 was directed to the DNA-RNA portion of the duplex, as was observed for E. coli RNase H1 (18, 20). However, within this region, the preferred sites of cleavage for the human enzyme differed from E. coli RNase H1. E. coli RNase H1 preferentially cleaved at the ribonucleotide apposed to first 2’-modified nucleotide in the wing of antisense oligonucleotide at the 3’-end of the RNA (18). In contrast, the human enzyme preferentially cleaved at sites more centered within the gap until the gap was reduced to 5 nucleotides. Furthermore, the minimum gap size for the human enzyme was 5 nucleotides, whereas that of E. coli RNase H1 was 4 nucleotides (18). These differences in behavior suggest differences in the structures of the enzymes and their interactions with substrate that will require additional study.

We have reported that although E. coli RNase H1 degrades the heteroduplex substrate in a predominantly distributive manner, the enzyme displays modest 5’-3’ processivity. In contrast, human RNase H1 evidences no 5’-3’ or 3’-5’ processivity, suggesting that the human enzyme hydrolyzes the substrate in an exclusively distributive manner. The lack of processivity observed with the human RNase H1 may be a function of the significantly tighter binding affinity (Table IV), thereby reduc-

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3 L. B. Blyn, personal communication.
ing the ability of the enzyme to move on the substrate. Alternatively, human RNase H1 appears to fix its position on the substrate with respect to the 5′-RNA-3′-DNA terminus, and this strong positional preference may preclude cleavage of the substrate in a processive manner (Fig. 5). Thus, despite the facts that the enzymes are both metal-dependent endonucleases that result in cleavage products with 5′-phosphates (Fig. 5) and both can cleave single strand 3′-RNA overhangs (Fig. 5 and Ref. 20), these enzymes display substantial differences.

E. coli RNase H1 has been suggested to exhibit “binding directionality” with respect to the RNA of the substrate such that the primary binding region of the enzyme is positioned several nucleotides 5′ to the catalytic center (13). This results in cleavage sites being restricted from the 5′-RNA-3′-DNA end of a duplex and cleavage sites occurring at the 3′-RNA-5′-DNA end of the duplex and in 3′ single strand overhangs. The human enzyme behaves entirely analogously. Thus, we conclude that human RNase H1 likely has the same binding directionality as the E. coli enzyme.

Substrate Binding—RNA-RNA duplexes have been shown to adopt an A-form conformation (44, 45). Many 2′ modifications shift the sugar conformation into a 3′-endo pucker characteristic of RNA (9, 46–48). Consequently, when hybridized to RNA, the resulting duplex is A form, and this is manifested in a more stable duplex. 2′-Fluoro oligonucleotides display duplex-forming properties most like RNA, whereas 2′-methoxy oligonucleotides result in duplex intermediate information between DNA-RNA and RNA-RNA duplexes (20).

The results shown in Table IV demonstrate that like the E. coli enzyme, human RNase H1 is a double strand RNA-binding protein. Moreover, it displays some ability to discriminate between various A-form duplexes (Table IV). The observation that the $K_a$ for an RNA-2′-F duplex is equal to that for an RNA-RNA duplex suggests that 2′-hydroxy group is not required for binding to the enzyme. Nevertheless, we cannot exclude the possibility that bulkier 2′ modifications, e.g., 2′-methoxy or 2′-propyl, might sterically inhibit the binding of the enzyme as well as alter the A-form quality of the duplex. The human enzyme displays substantially greater affinity for all oligonucleotides than the E. coli enzyme, and this is reflected in a lower $K_a$ for cleavable substrates (Tables III and IV). In addition, the tighter binding affinity observed for human RNase H1 may be responsible for the 20-fold lower $V_{\text{max}}$ when compared with the E. coli enzyme. In this case, assuming that the E. coli and human enzymes exhibit similar catalytic rates ($K_{\text{cat}}$), then an increase in the binding affinity would result in a lower turnover rate and ultimately a lower $V_{\text{max}}$.

The principal substrate binding site in E. coli RNase H1 is thought to be a cluster of lysines that are believed to bind to the phosphates of the substrates (13). The interaction of the binding surface of the enzyme and substrate is believed to occur within the minor groove. This region is highly conserved in the human enzyme (16). In addition, eukaryotic enzymes contain an extra N-terminal region of variable length containing an abundance of basic amino acids (16, 17). This region is homologous with a double strand RNA binding motif and indeed in the Saccharomyces cerevisiae RNase H has been shown to bind to double strand RNA (17, 49). The N-terminal extension in human RNase H1 is longer than that in the S. cerevisiae enzyme and appears to correspond to a more complete double strand RNA binding motif. Consequently, the enhanced binding of human RNase H1 to various nucleic acids may be due to the presence of this additional binding site.

Biological Roles and Implications for Antisense Drug Design—As discussed previously, the positional preferences of human RNase H1 argue that the proposal that it may be involved in DNA replication may be correct (42). However, the lack of processivity would suggest that the enzyme is suboptimally designed for this task, but considering the involvement of FEN1 in DNA replication, processive cleavage of the RNA by RNase H may be unnecessary. Clearly, more work is required before any conclusions can be drawn.

Although RNase H enzymes have been suggested to be involved in the effects of DNA-like antisense drug, to date no studies have directly demonstrated this nor determined which isotypes may be involved. We now have the tools to begin to answer these questions. If human RNase H1 is involved, our studies suggest that excess single strand phosphorothioate oligonucleotides in cells would be highly inhibitory, resulting in loss of effectiveness at higher concentrations. Furthermore, the binding preference human RNase H1 displays for A-form duplexes suggests that binding of the enzyme would be enhanced by appropriate 2′ modifications. However, cleavage rates are lower in chimeric duplexes, so the design of optimal 2′-modified gap-mers may be challenging.

Clearly, if the positional and sequence preferences observed for oligonucleotide substrates were for RNA species bound to DNA-like antisense drug, the implications would be substantial. For example, the placement of DNA gaps centered around a GU dinucleotide would be of value. Furthermore, since the positional preference of the enzyme was evident even when there were 5′- and 3′-RNA overhangs, positioning DNA gaps 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex and creating a GU within that area could be beneficial. Also, locating antisense drugs at the 5′-end of an RNA should be of value. However, it is clear that many DNA-like antisense drugs bind to RNA species at sites distal from the 5′ terminus of the RNA and still result in loss of RNA, presumably via RNase H-mediated cleavage (50). Thus, much more work is required before conclusions can be drawn and the information can be used to design better antisense drugs.

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