Communication

Cleavage of Single Strand RNA Adjacent to RNA-DNA Duplex Regions by Escherichia coli RNase H1*

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RNase H1 from Escherichia coli cleaves single strand RNA extending 3′ from an RNA-DNA duplex. Substrates consisting of a 25-mer RNA annealed to complementary DNA ranging in length from 9–17 nucleotides were designed to create overhanging single strand RNA regions extending 5′ and 3′ from the RNA-DNA duplex. Digestion of single strand RNA was observed exclusively within the 3′ overhang region and not the 5′ overhang region. RNase H digestion of the 3′ overhang region resulted in digestion products with 5′-phosphate and 3′-hydroxyl termini. The number of single strand RNA residues cleaved by RNase H is influenced by the sequence of the single strand RNA immediately adjacent to the RNA-DNA duplex and appears to be a function of the stacking properties of the RNA residues adjacent to the RNA-DNA duplex. RNase H digestion of the 3′ overhang region was not observed for a substrate that contained a 2′-methoxy antisense strand. The introduction of 3′ deoxynucleotides at the 5′ terminus of the 2′-methoxy antisense oligonucleotide resulted in cleavage. These results offer additional insights into the binding directionality of RNase H with respect to the heteroduplex substrate.

RNase H hydrolyzes RNA in an RNA-DNA duplex (1). RNase H activity appears to be ubiquitous in eukaryotes and bacteria (2, 3). This family of enzymes functions as endonucleases exhibiting limited sequence specificity and requiring divalent cations to produce 5′-phosphate and 3′-hydroxyl termini (4).

RNase H1 from Escherichia coli is perhaps the best characterized isotype of this family of enzymes. The substrate requirements for RNase H1 have been studied extensively. The dissociation constants for various duplexes and E. coli RNase H1 indicate that this enzyme is a double strand RNA-binding protein that cleaves RNA only in an RNA-DNA duplex (5). Furthermore, the cleavage specificity of the enzyme appears to be due to the catalytic process and not the binding interaction between RNase H and the substrate duplex. For example, although RNase H exhibited a binding affinity for substrates containing 2′-methoxy modifications approximately equal to the unmodified RNA-DNA heteroduplex, RNase H activity was ablated by these modifications. Kinetic analyses of substrates containing RNA duplexed with chimeric oligonucleotides composed of a mixture of deoxy- and 2′-modified nucleotides demonstrated that the rate of cleavage was a function of the length of the deoxy portion of the chimeric oligonucleotide, i.e., the cleavage rate decreased with a diminishing number of contiguous deoxynucleotides, and complete loss in activity was observed for chimeric oligonucleotides containing less than 4 contiguous deoxynucleotides (6, 7). In all cases E. coli RNase H1 cleavage of the RNA was observed to occur exclusively within regions base-paired to DNA.

In this communication we report that E. coli RNase H1 can cleave single strand RNA extending 3′ from an RNA-DNA duplex. The fact that the products are 5′-phosphate and 3′-hydroxyl suggests that the enzyme cleaves single and double strand regions via the same mechanism. Furthermore, these data suggest that E. coli RNase H1 cleaves RNA in RNA-DNA duplexes through a DNase-like mechanism. A series of antisense oligodeoxynucleotides ranging in length from 9 to 17 were designed to target a 25-mer RNA producing a heteroduplex with overhanging single strand RNA regions of varying length and sequence composition at both the 3′ or 5′ termini (Fig. 1). The single strand RNA regions 3′ to the heteroduplex ranged in length from 5 to 16 residues and were positioned on the 25-mer RNA such that the sequence composition of the single strand RNA immediately 3′ of the heteroduplex consisted of either mixed purine and pyrimidine nucleotides, a string of three purines, or two pyrimidines. Heteroduplexes were preformed and then digested with RNase H, and the digestion products were analyzed by denaturing polyacrylamide gel electrophoresis. In addition, the secondary structures of the heteroduplex substrates were mapped using single strand-specific ribonucleases, as described previously (8).

Digestion outside of the heteroduplex site was observed for all of the oligodeoxynucleotide-RNA duplexes tested (substrates 1–6, Fig. 1). These cleavages were observed exclusively in the region 3′ of the heteroduplex. Under no circumstances was cleavage observed in the single strand RNA region 5′ to the heteroduplex and consistent with earlier reports. The 5′-most cleavage site observed on the RNA was 5 nucleotides downstream of the 3′ end of the oligodeoxynucleotide (7). The greatest number of single strand RNA cleavage sites was observed for the substrate containing the string of three purines within the single strand region immediately adjacent to the heteroduplex (substrate 6, Fig. 1). RNase H digestion of this substrate resulted in four cleavage sites within the single strand region of the RNA. The substrates containing mixed pyrimidine and purine sequences or two pyrimidines in the overhanging RNA resulted in fewer cleavage sites, with a maximum of two cleavage sites observed for these substrates (substrates 1–5, Fig. 1). Enzymatic structure mapping of the heteroduplexes with single strand-specific ribonucleases showed that the RNA regions flanking the heteroduplex were indeed single-stranded (Fig. 1), and thus alternative intramolecular or intermolecular structures were not formed.

The length of the single strand RNA sequence downstream of the heteroduplex (e.g. ranging from 5 to 16 residues for the substrates tested) appeared to have no effect on the number of cleavage sites within the single strand RNA region. For exam-

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ple, the substrate containing the longest single strand region, i.e. 16 nucleotides, exhibited only two cleavage sites outside of the heteroduplex (substrate 2, Fig. 1), whereas four single strand RNA residues of the substrate containing a 6-nucleotide single strand region were digested by RNase H (substrate 6, Fig. 1). These data suggest that the ability of E. coli RNase H1 to cleave single strand RNA regions is a function of the structure of these regions resulting from the sequence composition and not the length of the single strand RNA.

Analysis of the RNase H digestion products suggests that the overhanging single strand regions are cleaved by the same mechanism as the heteroduplex region (Fig. 2). Specifically, the cleavage products for both the heteroduplex and single strand overhanging regions were found to consist of a 5'-phosphorylated 5'-hydroxyl termini. These data are consistent with previous studies showing that E. coli RNase H1 cleaves RNA in an RNA-DNA duplex leaving a 5'-phosphorylated product (1). In addition these data suggest that the RNase H cleavage of the overhanging regions differs from many single strand-specific endoribonucleases which produce a 3'-phosphorylated product (9). These RNases follow a two-step mechanism involving the...

![Fig. 1](image1.png)

**Fig. 1.** A, denaturing polyacrylamide gel analysis of the RNase H digestion products of the oligonucleotide heteroduplex substrates, 1–15, shown in B. Antisense oligonucleotide (10 μM) was hybridized with 500 nM RNA and 30,000 cpm of 5'-end-labeled RNA for 15 h at 37 °C in 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, and 1 unit of Inhibit-ACE and then digested with 1.4 × 10⁻⁸ mg of E. coli RNase H for 30 min at 37 °C. Base hydrolysis ladder (lane 9) was prepared by incubation of 5'-end-labeled RNA at 90 °C for 5 min in 10 mM containing 100 mM sodium carbonate, pH 9.0. Enzymatic footprinting of DNA oligonucleotide (substrate 6) with RNases T1 and CL3 (lanes 7 and 8) was performed in 10 μL containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 5 μg of tRNA, and 30,000 cpm of 5'-end-labeled RNA and incubated for 5 min at 37 °C. Position of DNA oligonucleotide bound to the RNA is indicated by the boxed sequence. B, sites of RNase H digestion (1–6 and 10–13) and RNase T1 and CL3 (7 and 8) on the heteroduplex substrates. 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. Substrate numbers correspond to the lane numbers in A.

![Fig. 2](image2.png)

**Fig. 2.** Product analysis of substrate 2 (lanes 1–3) and substrate 4 (lanes 4–6). RNA was 3'-end-labeled using [³²P]cytidine bisphosphate (pCp) and RNA ligase as described previously (9). Hybridization reactions were prepared as described in Fig. 1 A, reactions were divided into equal aliquots with one aliquot subjected to dephosphorylation with alkaline phosphatase (from calf intestine) prior to digestion with RNase H. The digestion reactions were further divided into equal aliquots, again with one aliquot subjected to dephosphorylation. A, untreated hybridization reaction digested with RNase H are shown in lanes 3 and 6. Hybridization reactions treated with alkaline phosphatase prior to digestion with RNase H exhibited slower migration on the polyacrylamide gel due to the loss of the 3'-phosphate (lanes 2 and 5). RNase H digestion reactions treated with alkaline phosphatase exhibited a further reduction in migration due to the loss of the 5'-phosphate on the cleavage product (lanes 1 and 4).
use of the 2'-hydroxyl to perform a nucleophilic in-line attack on the phosphorus resulting in the formation of a 2',3'-cyclic phosphate intermediate. This trigonal bipyrimidal intermediate is then hydrolyzed to form the 3'-phosphorylated product (10). Although the catalytic pathway for RNase H remains unknown, the enzyme likely uses a catalytic mechanism similar to that observed for other nucleases which have been shown to produce a 5'-phosphorylated product (e.g. DNases, RNase P, and group I and II ribozymes) (11). These nucleases supply the nucleophile required to react directly on the phosphorus center, thus eliminating the requirement of the 2'-hydroxyl. RNase H has adopted this DNase-like mechanism presumably because in stacked and/or duplexed RNA the position of the 2'-hydroxyl is such that an in-line attack of the phosphorus is not possible via the 2'-hydroxyl (12).

Previous studies have shown that substrates containing RNA hybridized to 2'-methoxy oligonucleotides do not support RNase II activity (6, 13). To determine whether heteroduplex substrates containing 2'-methoxy antisense oligonucleotides would support RNase H digestion of single strand RNA overhangs, we designed a 2'-methoxy antisense analog (substrate 15, Fig. 1) of the antisense sequence that yielded the greatest number of cleavage sites outside of the heteroduplex region (substrate 6, Fig. 1). Consistent with previous studies, the heteroduplex substrate containing the 2'-methoxy antisense oligonucleotide did not support RNase H activity within the heteroduplex region. In addition, no cleavage of the single strand RNA region was observed with this substrate.

Clearly, digestion of the 3' overhanging RNA by RNase H requires that the antisense sequence consist of deoxynucleotides. To determine if oligonucleotides consisting of both deoxynucleotides and 2'-methoxynucleotides would support RNase H cleavage of the 3' overhang, we designed chimeric oligonucleotides with the deoxynucleotide portion on the 5' end of the antisense sequence adjacent to the 3' overhang region and varied the size of the deoxynucleotide portion of the chimeric oligonucleotide from 1 to 5 residues (substrates 10–14, Fig. 1). Again, the antisense sequence selected for this study corresponded to the oligodeoxynucleotide which yielded the greatest number of cleavages within the 3' overhang (substrate 6, Fig. 1). E. coli RNase H1 digestion of substrates 10–14 shows that three of the five substrates containing the chimeric oligonucleotides were cleaved by the enzyme. Furthermore, in all cases in which cleavage was observed with these substrates, RNase H cleavage occurred exclusively within the single strand RNA region. Interestingly, the substrates containing chimeric oligonucleotides exhibited fewer cleavage sites within the single strand RNA region as compared with the analogous unmoffified substrate (substrate 6, Fig. 1). These data also suggest that placement of a minimum of 3 deoxynucleotides at the 5' end of the antisense oligonucleotide was required to support RNase H cleavage. Kinetic analysis of the substrate containing the chimeric oligonucleotide with the 5-deoxynucleotide portion at the 5' end showed that this substrate was digested by E. coli RNase H1 at approximately the same rate as the unmodified RNA-DNA substrate (data not shown). Previous studies with chimeric oligonucleotides containing a string of 5 deoxynucleotides within other regions of the oligonucleotide showed that these substrates were digested 3- to 5-fold more slowly than the unmodified RNA-DNA substrate (5). Taken together these results suggest that placement of the deoxynucleotide portion at the 5' end of the chimeric oligonucleotide and adjacent to the 3' RNA overhang serves to effectively increase the size of the substrate portion of the chimeric oligonucleotide.

The cleavage patterns for the various substrates tested show that the 5'-most cleavage site on the RNA is 5 nucleotides from the 3' end of the oligodeoxynucleotide and that the 3'-most cleavage site extends 4 residues past the heteroduplex into the single strand 3' overhang region. These data are consistent with the observed structure and function of RNase H1. Structural studies suggest that the enzyme exhibits a selective "binding directionality" with respect to the RNA of the heteroduplex substrate such that the binding region on the enzyme is positioned several residues 5' to the catalytic region (14, 15) (Fig. 3). Furthermore, the binding interaction is sensitive to the structure of the substrate with the enzyme exhibiting a 40-fold greater affinity for RNA-DNA heteroduplexes than single strand RNA (9). Therefore, presented with an RNA-DNA substrate containing overhanging single strand RNA regions, the enzyme would preferentially bind to the heteroduplex region of the substrate. Binding of E. coli RNase H to the 5'-most end of the oligodeoxynucleotide duplexed with RNA would result in cleavage several nucleotides downstream on the RNA (e.g. yielding the observed cleavage 5–7 nucleotides from the 3' end). Binding of RNase H to the 5'-most end of the oligodeoxynucleotide duplexed with the RNA would result in cleavage within the downstream 3' overhang region of the RNA (e.g. yielding the observed cleavage 1–4 nucleotides from the 5' end). Said another way, RNase H-induced cleavages less than 5 nucleotides from the 3' end of the oligodeoxynucleotide or more than 4 residues within the 3'-overhanging RNA are not observed presumably because this would position the binding region of the enzyme within the overhanging single strand RNA, a region for which the enzyme exhibits significantly lower affinity (Fig. 3).

The greater number of cleavage sites observed for the substrate containing the string of purines may be due to the fact that single strand purines exhibit a greater propensity to continue stacking on the base pairs of a duplex (16, 17). Because the footprint of the enzyme appears to comprise a single helical turn on the duplex substrate and this binding/catalytic interaction is sensitive to the helical structure of the substrate, the extended helical structure created by the continued stacking of single strand RNA residues on the heteroduplex would serve to effectively increase the size of the substrate beyond the heteroduplex. Single strand sequences consisting of pyrimidines and mixed purines/pyrimidines also continue stacking but are in general limited to 1 or 2 residues immediately adjacent to the
duplex (16, 17). This lower propensity for stacking may explain why these substrates exhibited fewer cleavages within the 3'-overhang.

Given that cleavage of the 3'-overhanging RNA region by RNase H occurs with the enzyme bound to the heteroduplex region and that the 2'-hydroxyl does not appear to function as the nucleophile during catalysis, we designed RNA-DNA duplexes with 3'- and 5'-overhanging DNA regions to determine whether, provided with the heteroduplex binding site, RNase H would cleave single strand DNA adjacent to the RNA-DNA duplex. These substrates were patterned after substrates 4 and 6 (Fig. 1B), with the exception that 32P-end-labeled DNA was substituted for the RNA strand and RNA was substituted for the DNA strand. In both cases, no digestion of the DNA was observed for either the heteroduplex or single strand regions (data not shown). These data suggest that although the enzyme is capable of binding to the heteroduplex region, cleavage of the single strand overhanging region requires the presence of the 2'-hydroxyl. The role of the 2'-hydroxyl with respect to RNase H catalysis is unclear particularly in light of the fact that group I and II ribozymes, which also produce 5'-phosphorylated products, have been shown to cleave DNA (18, 19). These data support the proposed catalytic mechanism involving a single Mg2+ ion (14, 20, 21). This mechanism uses the 2'-hydroxyl to position the single water-bound Mg2+ ion and the amino acid residue (Asp-70) to activate the water nucleophile. Alternatively, the lack of cleavage observed within the overhanging single strand DNA region may be due to the difference in base-stacking properties between single strand RNA and DNA adjacent to the heteroduplex region. E. coli RNase H1 activity has been shown to be sensitive to the helical structure of the substrate, preferring A-form-like duplexes over B-form duplexes (5). Therefore, stacking of the single strand DNA on the heteroduplex region in a manner other than an A-form-like structure would likely result in the observed abrogation of RNase H activity.

In addition to the helical structure of the substrate, RNase H activity within the 3'-overhang appears to be sensitive to chemical modifications within the antisense oligonucleotide. For example, RNase H activity was abrogated by 2'-methoxy substitution within the antisense oligodeoxynucleotide sequence. Activity was restored with the introduction of a minimum of 3 deoxynucleotides at the 5'-end of the 2'-methoxy-substituted antisense oligonucleotide (substrates 10–12, Fig. 1). Previous studies have shown that the rate at which the RNA in a chimeric heteroduplex is digested by E. coli RNase H is a function of the length of the deoxynucleotide portion of the chimeric oligonucleotide (6, 7). Therefore, with longer RNAs, improved RNase H activity may be realized by placing the deoxynucleotide portion at the 5'-end of the chimeric antisense oligonucleotide and thereby effectively increasing the size of the substrate. Finally, these data suggest that for RNase H cleavage of single strand RNA to occur, at the minimum two criteria must be met. First, in order for the enzyme to bind to the substrate in a way that supports cleavage of the single strand RNA overhang, the duplex region must be positioned 5' to the cleavage site. Second the 5' end of the antisense sequence must consist of a minimum of 3 deoxynucleotides.

These data support previous observations that RNase H is both directional and processive (7, 14, 15, 22). Clearly the selective binding directionality exhibited by RNase H plays a crucial role in the biological processes involving the enzyme. For example, E. coli RNase H1 is believed to participate in DNA replication by removing the upstream RNA primers of Okazaki fragments (23). The binding specificity and polarity exhibited by the enzyme, i.e. binding region positioned several residues 5' to the catalytic region, is consistent with the orientation of the RNA primers which are positioned 5' with respect to the Okazaki fragments. Similar cleavage specificity with respect to the position of the RNA primers has been observed for calf thymus RNase H1 (24). Furthermore, RNase H activity is also often implicated in antisense oligodeoxynucleotide-mediated degradation of RNA. In a previous study examining E. coli RNase H1 activity on antisense oligonucleotide-induced RNA pseudo-halfknot structures we have shown that cleavage of the structured RNA is profoundly affected by the binding directionality of the enzyme (25). Taken together, these studies suggest that the binding polarity exhibited by RNase H1 has important implications both biologically and pharmacologically.

The demonstration that E. coli RNase H1 cleaves single strand RNA adjacent to heteroduplex regions via the same mechanism as it cleaves RNA within the heteroduplex, coupled with the demonstration that the enzyme is a double strand RNA-binding protein (5), suggests that the enzyme may have derived from genes coding for an RNA-binding protein and a DNase-like nuclease. The nuclease domain enables cleavage of stacked RNA whereas the double strand RNA binding domain serves either to provide greater substrate specificity or to modulate enzyme activity in a manner similar to that suggested for the double strand RNA binding domain of S. cerevisiae RNase H1 (26).

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