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Modulation of RNase H Activity by Modified DNA Probes: Major Groove vs Minor Groove Effects

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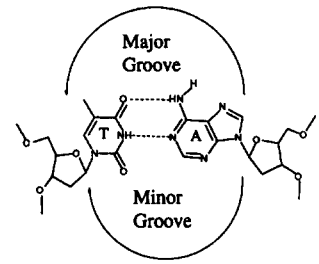
Abstract—We have previously prepared ribozyme mimics and chemical nucleases from modified DNA containing pendant bipyridine and terpyridine groups. The ability of these modified DNA probes to support RNase H cleavage of complementary RNA is described. DNA/RNA duplexes were formed using DNA probes designed to deliver metal complexes *via* either the major groove or the minor groove of the duplex. The duplexes were treated with *Escherichia coli* RNase H. Modifications in the major groove produced the same RNA cleavage pattern as unmodified DNA probes. However, minor groove substituents inhibited RNA cleavage over a four-base region. Comparison was made with a DNA probe containing a 2′-OMe modification. Our results support enzyme binding in the minor groove of a DNA/RNA duplex. We do not observe cleavage directly across from the modified nucleoside. The RNA cleavage efficiency effected by RNase H and a DNA probe decreases as follows: unmodified DNA ≥ C-5 modified DNA ≫ C2′-modified DNA > C1′-modified DNA. Results with 28-mer RNA substrates roughly parallel those obtained with a 159-mer RNA target. The differences observed between low and high MW RNA substrates can be explained by a much higher enzyme–substrate binding constant for the high MW target. © 1997 Elsevier Science Ltd.

Introduction

RNase H forms a family of enzymes with both endonuclease and 3′–5′ exonuclease activity. RNase H cleaves the RNA strand of RNA/DNA duplexes to yield a 5′-phosphate and a 3′-hydroxyl. It plays an important role in the life cycle of HIV, DNA replication, and possibly in the destruction of mRNA during antisense gene regulation.[1–3] Much work has been done to characterize this enzyme and its substrate specificity.[4–7] The enzyme is thought to bind in the minor groove of a DNA/RNA double helix by recognizing the sugar conformation of the DNA nucleotide complementary to the site of RNA cleavage.[8,9] The active-site residues essential for catalysis, as found by site-directed mutagenesis of *Escherichia coli* RNase H, are Asp^{10}, Glu^{48}, and Asp^{70}. Residues Cys^{13}, Asn^{14}, Asn^{44}, and Gln^{72} are involved in substrate recognition.[8]

We have designed ribozyme mimics to act as catalytic antisense agents by catalyzing transesterification and hydrolysis of the target RNA.[10–17] Other groups have also pursued this strategy.[18–23] Our reagents contain hydrolytically active metal complexes which are covalently linked to DNA at strategic positions. The reagents are designed to attack complementary RNA across either the major or minor groove of an RNA/DNA duplex. A variety of important DNA analogues, including many 2′-modified nucleotides, do not support RNase H-mediated hydrolysis of complementary RNA.[5,7,24] Due to the possible importance of a catalytic antisense process mediated by RNase H,[1,3,7] we wanted to determine the viability of our ribozyme mimics as templates for RNase H cleavage of RNA targets. Structures of the ribozyme mimics employed in the current study are shown in Scheme 1. Given the structural requirements of RNase H, we hoped to learn about the conformation of heteroduplexes formed between these modified DNA probes and complementary RNA. We also wished to determine if our DNA probes with pendant polypyridine ligands had an inhibitory effect on the metalloenzyme active site of



Major Groove Modified Probes

3′ GAA ATC TCT GAX ACA TC 5′

X =

probe 2

3′ GAA ATC TCT GAX ACA TC 5′

X =

probe 3

Minor Groove Modified Probes

3′ GAA ATC TCT GAX ACA TC 5′

X =

probe 4

3′ GAA ATC TCT GXT ACA TC 5′

X =

probe 5

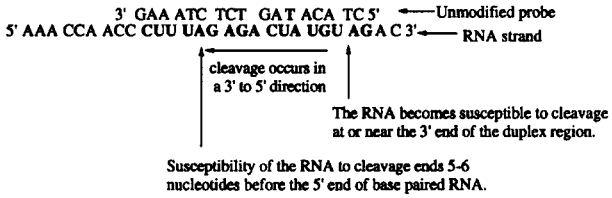
Scheme 1.

RNase H. Finally, by using 28-mer and 159-mer RNA substrates, we were able to investigate how substrate MW affects the relative importance of endonucleolytic and exonucleolytic pathways for RNase H.

We report that our modified DNA probes are indeed templates for RNase H-mediated cleavage of RNA. Observed cleavage patterns support a mechanism in which the enzyme binds in the minor groove of the RNA/DNA duplex. We report examples of 2′-*O*-alkyl-substituted nucleotides that do support RNase H cleavage of an RNA target, directly across the duplex, at their complementary ribonucleotides. We also report RNase H digestion of target RNA outside the duplex region. Our results support both endonuclease and processive 3′–5′ exonuclease activity for *E. coli* RNase H acting on a high MW substrate, but only endonuclease activity was observed with a low MW substrate.

Results

Based on literature studies,[25] the expected cleavage of the 5′-end labeled 28-mer substrate by RNase H and probe **1**, the unmodified DNA control, are shown below.



Cleavage of the 28-mer RNA was examined for both 5′- and 3′-end labeled substrates and analyzed by gel electrophoresis. The results are summarized in Figure 1, and data are shown in Figures 2–4. Numbering of the target nucleotides (NTs) proceeds from the 5′-end of the RNA, as shown below (note that 3′-end labeling adds pCp to the 3′ end of this sequence, generating a 29-mer):

5′-$A_1A_2A_3$ $C_4C_5A_6$ $A_7C_8C_9$ **$C_{10}U_{11}U_{12}$** **$C_{13}A_{14}G_{15}$** **$A_{16}G_{17}A_{18}$** **$C_{19}U_{20}A_{21}$** **$U_{22}G_{23}U_{24}$** **$A_{25}G_{26}A_{27}$** C_{28}-3′

The duplex is formed by pairing of NTs 10–26, shown in **bold** above, with the 17-mer DNA probes. For all of the assayed duplexes, we found that RNA cleavage starts at or near NT 23 on the RNA strand. As shown in Figure 1, cleavage does not continue to C^{10}, but stops short of the end of the duplex, at positions 12 or 14.

Reaction products from the 5′-end labeled RNA are shown in Figure 2. A T^1 nuclease lane shows cleavage at G residues and allows identification of RNase H hydrolysis products. The true cleavage products were synthesized and run on a gel next to the RNase H cleavage products for exact assignments (data not shown). In control experiments with all-DNA probe **1** (lanes 2–4), RNase H cleavage spans the 12 NTs from positions 12–23. Under these conditions, preferred cleavage occurs at NTs 14 and 19.

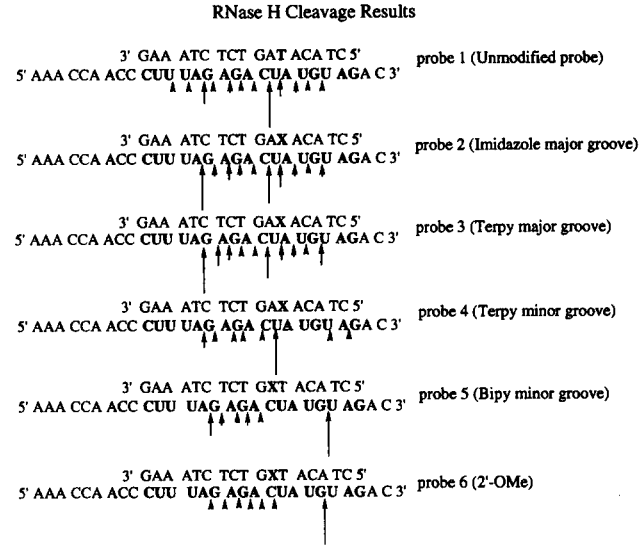


Figure 1. Cleavage histograms for the 5′-end labeled 28-mer RNA by RNase H and probes **1–6**. X indicates the position of the modified nucleotide. See Scheme 1 for structures of the modified nucleotides.

Probes **2** and **3** deliver their pendant reagents in the major groove of the DNA/RNA duplex (Scheme 1). Hydrolysis of the RNA target with RNase H, supported by probes **2** and **3**, is illustrated in Figure 2. The cleavage patterns shown in lanes 5–8 (probe **2**) and lanes 9–12 (probe **3**) are very similar to those obtained

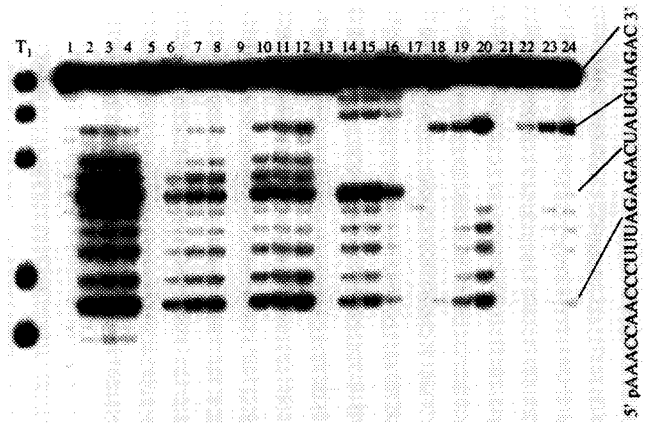


Figure 2. Cleavage of 5′-end labeled RNA with DNA probes and *E. coli* RNase H. Lane T_1 is digestion of RNA with RNase T_1. Lanes 1–4 cleavage products from duplex formed with probe **1**. Lanes 5–8, 9–12, 13–16, 17–20, and 21–24 correspond to cleavage with probes **2**, **3**, **4**, **5**, and **6** respectively. Lanes 1, 5, 9, 13, 17, and 21 serve as controls with no enzyme added and a reaction time of 15 min. Lanes 2, 6, 10, 18, and 22 show cleavage 1 min after the addition of enzyme. Lanes 3, 7, 11, 15, 19, and 23 show cleavage 5 min after the addition of enzyme. Lanes 4, 8, 12, 16, 20, and 24 show cleavage 15 min after the addition of enzyme. All reactions where carried out in a total volume of 10 μL. All duplexes were pre-annealed by heating to 80 °C and cooled slowly to room temperature. The enzyme was added and the reaction proceeded at 37 °C. Aliquots of 3 μL were taken at the above time points, added to loading dyes and placed on dry ice. Amounts of 0.5 units of RNase H were used for each reaction, the concentration of each probe was 5.0 μM, and the concentration of labeled RNA was ca 10^{-8} M. Samples were run on a 20%, 8 M urea, PAGE gel and exposed on a Molecular Dynamics Phosphorimager.

with the unmodified DNA probe: hydrolysis begins at nucleotide 23 and spans 10 NTs to position 14. Preferred sites of cleavage are again seen at positions 14 and 19, although less overall cleavage is seen with these probes than with probe **1**. Thus, our pendant groups located in the major groove have no significant effect on the enzyme's specificity. This is consistent with enzyme–substrate binding in the minor groove, and agrees with the effects of major-groove modifications reported recently by Crooke et al.[25]

In contrast, probes **4** and **5**, which were designed to deliver ligands in the minor groove, do alter the enzymatic cleavage pattern. In Figure 2, lanes 14–16 show that probe **4** initiates cleavage at positions 24 and 25; however, inhibited cleavage is observed over a 4-NT region, between positions 19 and 24. Cleavage resumes at nucleotide 19, and continues for 6 NTs until it reaches position 14. As found with **1–3**, probe **4** promotes enhanced cleavage at NTs 14 and 19. As illustrated in Figure 1, the modified bases in probes **2–4** occur immediately opposite NT 19.

The cleavage pattern for probe **5** (Fig. 2, lanes 17–20) roughly parallels that of probe **4**. Thus, hydrolysis spans NTs 14–23, but is inhibited over a 4-NT region between positions 18 and 23. As seen in Figures 1 and 2, this inhibition is shifted by 1 NT relative to results with probe **4**. Consistent with this shift, probe **5** contains a modified adenosine complementary to RNA position 20, while probe **4** contains a modified thymidine complementary to NT 21.

Lanes 21–24 (Fig. 2) show results from RNA hydrolysis supported by the 2′-OMe-substituted probe **6**, which is closely analogous to probe **5**. Both probes **5** and **6** show enhanced cleavage at NT 23 (compare lanes 19 and 24 of Fig. 2). In contrast to probe **5**, however, probe **6** inhibits cleavage over only 3 NTs (U20–U22), and supports relatively weak cleavage to the 5′-side of this inhibited region.

We also examined reactions of the 3′-end labeled RNA substrate. We wished to determine if the enzyme bypassed the modified NTs in a processive manner. If the enzyme bound only to the 3′ end of the base-paired RNA, and cleaved in a processive manner until the termination site, a single radiolabeled product would be observed in these experiments.[26] Analysis of these reactions by PAGE, shown in Figure 3, shows cleavage sites analogous to those observed in 5′-end labeled experiments. Similar results were expected, but *not* observed, in the study reported by Schatz.[26] Preferred hydrolysis sites and regions of inhibition seen in Figure 3 also parallel those found in Figure 2. We can therefore conclude that, under our conditions, endonuclease activity was observed and no evidence was found for exonuclease digestion. Hydrolysis initiates throughout the heteroduplex region, except in the inhibited areas that result from substituents in the minor groove.

The relevance of these studies to high-MW RNA targets was assessed with a 159-mer substrate that is a fragment of mRNA from the HIV *gag* gene.[16] The 159-mer contains the same recognition sequence as our 28-mer RNA target. Both 5′- and 3′-end labeled substrates

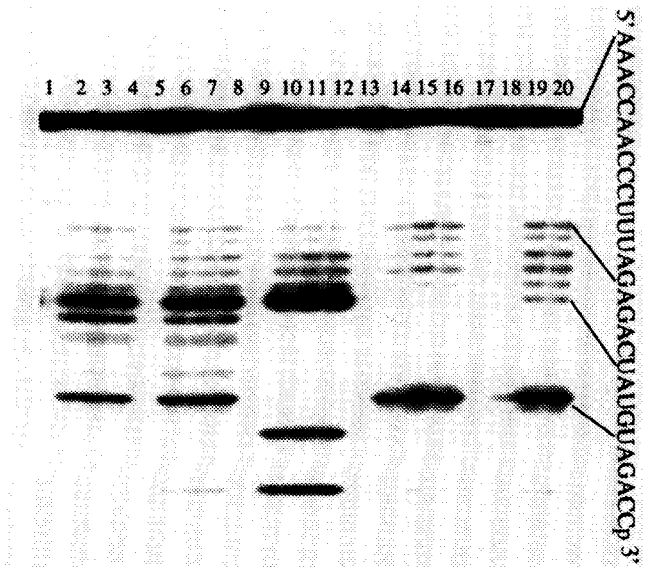


Figure 3. Cleavage of 3′-end labeled RNA with DNA probes and *E. coli* RNase H. Lanes 1–4, 5–8, 9–12, 13–16, and 17–20 correspond to cleavage with probes **2**, **3**, **4**, **5**, and **6** respectively. Cleavage reactions were carried out under the same conditions given for Figure 2.

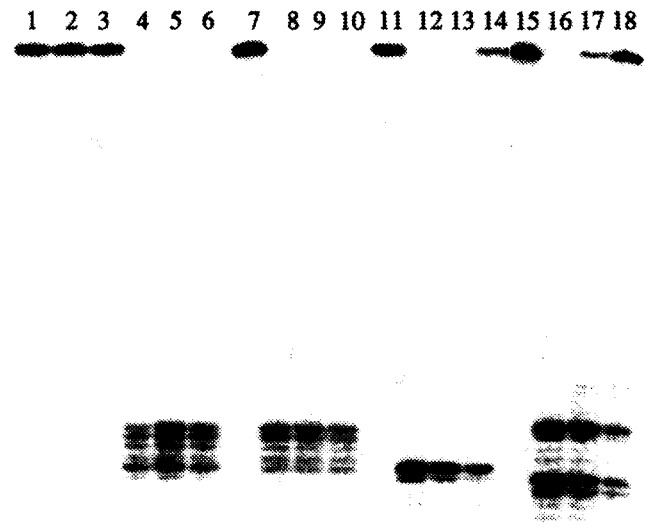


Figure 4. Cleavage of the 3′-end labeled 159-mer RNA, a fragment of the mRNA from the HIV *gag* gene, with hybridized probes and *E. coli* RNase H. Lane 1 shows the RNA starting material; lane 2 shows RNA treated with enzyme but no DNA probe. Lanes 3–6, 7–10, 11–14, and 15–18 correspond to cleavage with **1**, **3**, **5**, and **4** respectively. Lanes 3, 7, 11, and 15: no enzyme added. Lanes 4, 8, 12, 16: 0.05 units of enzyme. Lanes 5, 9, 13, and 17: 0.005 units of enzyme. Lanes 6, 10, 14, and 18: 0.0005 units of enzyme. All reactions were carried out in 5 μL total volume. The RNA was incubated with the probes and RNase inhibitor in the RNase H buffer for 20 min at 37 °C. The appropriate concentration of enzyme was then added and the reaction was maintained at 37 °C for 15 min. The reactions were stopped by adding 3 μL of loading dye. The concentration of each probe was 10 μM, and the labeled RNA was ca 10^{-8} M. Samples were run on a 20%, 8 M urea PAGE gel and exposed to the phosphorimager.

were examined. Cleavage of the 3′-end labeled 159-mer (Fig. 4) resembles hydrolysis of the 28-mer target, but some differences are apparent. Hydrolysis occurred on both sides of a zone of inhibition with probe **4** (lanes 16–18), which also supported cleavage outside the duplex region. With probe **5**, RNase H digestion of the 159-mer, seen in lanes 12–14, is shifted one NT from the products generated with probe **4**. Also with **5**, inhibition occurs in the expected region, but cleavage of the 159-mer further down the RNA, toward the 5′ end, is barely detectable with the phosphorimager. We also examined RNase digestion of the 5′-end labeled 159-mer with probes **1**, **3**, **4**, and **5** (data not shown). These reactions were closely analogous to results reported by Schatz:[26] one major band was seen in each case, originating from digestion of the RNA by the 3′–5′ exonuclease activity of RNase H. As Figure 4 shows, cleavage of the 159-mer starting material proceeded to completion with all amounts of enzyme examined, 0.05–0.0005 units, for probes **1** and **3**. However, in the reaction of the 159-mer with probe **5** and 0.0005 units of RNase H, some starting material remained after 20 min. Furthermore, even 0.005 units of enzyme failed to cleave 100% of the substrate when probe **4** was added.

Discussion

For similar concentrations of DNA and RNA, the enzyme is much more active towards the 159-mer than towards the 28-mer substrate: while 0.5 units of RNase H gave only partial cleavage of the 28-mer in 15 min, 10- to 1000-fold smaller amounts of enzyme completely cleaved the 159-mer in 20 min (Fig. 4). Electrostatic attraction between enzyme and substrate must be greatly enhanced for polymeric RNA. This greater binding must allow processive exonuclease digestion of the 159-mer substrate under conditions where the 28-mer RNA is only subject to *endonucleolytic* cleavage.

The efficiency of RNase H degradation is, in some cases, higher with unmodified DNA than with the modified probes. Thus, as shown in Figure 2, cleavage of the 28-mer substrate with enzyme and (unmodified) probe **1** is more efficient than cleavage with any of the modified probes (**2–6**). In slight contrast, cleavage of the 159-mer substrate with probes **1** and **3** appears equivalent in Figure 4, and both probes gave 100% cleavage under the reaction conditions. However, in this same experiment, cleavage of the 159-mer with probes **4** and **5** did not proceed to completion when <0.005 units of RNase H were added. Thus, minor groove modifications (probes **4** and **5**) diminished the efficiency of RNA cleavage. Overall, the RNA cleavage efficiency effected by RNase H and a DNA probe decreases as follows: unmodified DNA $\geq$ C-5 modified DNA $\gg$ C2′-modified DNA > C1′-modified DNA.

Cleavage of the 5′-end labeled substrate is summarized in Figure 1 for each of the probes. These results compare favorably to recent work of Crooke et al.,[25] although some differences are apparent. Crooke and coworkers constructed DNA probes with a modified adenosine building block, N2-imidazolylpropyladenosine. On binding to complementary RNA, this modification should reside in the minor groove of the resulting duplex. Crooke et al. found no RNase H cleavage of the RNA directly opposite the modified base. In their study, RNA cleavage was blocked at only two RNA positions, while we observed inhibition over a four NT region. In both cases, RNA cleavage was blocked at nucleotides located to the 3′ side of the minor groove modification. Further direct comparison of both cases is difficult because the probe used by Crooke had five deoxy residues flanked by 2′-*O*-methylnucleotides (which are not substrates for RNase H).

To explain our observed inhibition, it is helpful to examine the crystal structure of *E. coli* RNase H.[27,28] The 'handle' region of the enzyme has a high percentage of cationic amino acids which present a highly positive charge in a confined space. This handle region is probably responsible for the initial, nonspecific, binding of the enzyme to the duplex. Kanaya[29] found that if one of these residues is replaced with a neutral amino acid, the K^m of the enzyme increases. Interestingly, crystallography shows that the RNase H domain of HIV reverse transcriptase[30] lacks this handle region, and therefore cannot bind to a duplex when separated from the rest of the reverse transcriptase.

After RNase H binds to the duplex, the enzyme needs a way to identify true substrates (i.e., DNA/RNA duplexes vs nonsubstrates such as RNA/RNA duplexes). A recognition area near the active site and consisting of residues Cys^{13}, Asn^{16}, Asn^{44} and Gln^{72},[8] apparently makes the key sugar contacts that identify a suitable substrate.[8,9]

Yang et al.[28] found that when they docked their crystal structure of *E. coli* RNase H to the minor groove of an A-type duplex, near the catalytic core of the enzyme, the interaction spanned about four nucleotides on each strand. In the enzyme–substrate complex, the active site lies to the 3′ side of the recognition site, along the RNA strand. Thus, cleavage lags behind recognition, as the enzyme traverses the RNA in a 3′–5′ direction. We observed 3- and 4-NT regions of inhibition with probes **4–6**. These probes introduce steric bulk in the minor groove, where recognition and binding take place. Sterically large 2′- and 1′-substituents gave inhibition over 4 NTs, while the smaller 2′-OMe group inhibited cleavage over only a 3 NT region. These inhibited regions may be a footprint for the minimum number of base pairs needed for recognition and cleavage by RNase H.

Reid[9] found that the minor groove of a DNA/RNA duplex, which is narrower than the corresponding groove in an RNA/RNA duplex, evidently allows RNase H to contact both nucleic acid strands in a manner that triggers cleavage. Based on solution NMR studies, Reid reported that more than four successive DNA residues were required to generate this active conformation in an

RNA/DNA duplex. This agrees with our inhibition length of four bases.

We plan to investigate the structure of the DNA/RNA duplexes formed with these probes. We have performed both hydrolytic and oxidative cleavage of RNA targets and oxidative cleavage of DNA targets with ribozyme mimics **3–5**. Product distributions, kinetic analyses and metal dependence will be described elsewhere, and cleavage patterns will be compared with RNase H digestion footprints.[31,32]

Conclusion

In summary, we examined RNase H digestion of 28- and 159-mer RNA targets supported by modified and unmodified 17-mer DNA probes. We found that our modifications targeted to the minor groove of a DNA/RNA duplex partially inhibit RNase H activity, whereas our modifications targeted to the major groove allow complete RNA digestion. This is consistent with a model where the enzyme recognizes the duplex through contacts in the minor groove, and where the active site lags behind the recognition site, on the RNA strand. Cleavage initiates at 10–12 of the 17 base-paired ribonucleotides, nearest the 3′-end of the RNA. The RNA cleavage efficiency effected by RNase H and a DNA probe decreases as follows: unmodified DNA $\geq$ C-5 modified DNA $\gg$ C2′-modified DNA > C1′-modified DNA. Both endo- and exonuclease activity were observed with the 159-mer RNA substrate, but exonuclease activity was suppressed with the lower MW target. It is important to consider effects of substrate MW on binding and dissociation steps when relating studies on short modified oligonucleotides to mechanisms derived from polymeric substrates.

Experimental

On an Applied Biosystems 380B DNA Synthesizer, we synthesized modified probes **2** and **3**, which contain pendant groups that point into the major groove of a DNA/RNA duplex, and probes **4** and **5**, with modifications that point into the minor groove (Scheme 1).[17,33] We purchased probe **6** from Oligos Etc.; it is analogous to probe **5**, but contains a 2′-*O*-methyladenosine in place of the 2′-*O*-alkylbipyridine residue (Scheme 1). We also used an unmodified DNA probe (**1**) to serve as a control. The 159-mer substrate was synthesized by runoff transcription as previously described.[16] All probes were purified by polyacrylamide gel electrophoresis, visualized by UV-shadowing, excised from the gels and desalted on Sephadex G-25 columns. The probes were characterized by UV spectrophotometry, gel electrophoresis, and MALDI-TOF mass spectrometry.

The purified probes were annealed to a 28-mer RNA target and reacted with 0.5 units of *E. coli* RNase H (U.S.B.). The RNA was chemically synthesized by Oligos Etc. and radiolabeled at the 5′-end, using T4 Polynucleotide Kinase and [γ-^{32}P] ATP, or at the 3′-end, using T4 RNA ligase (New England Biolabs) and [5′-^{32}P] pCp (Amersham). Other experimental details are provided in the captions for Figures 2–4.

Acknowledgements

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