An ultraviolet crosslink in the hammerhead ribozyme dependent on 2-thiocytidine or 4-thiouridine substitution

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INTRODUCTION

A variety of approaches have been used to examine the hammerhead ribozyme. Substitution of all conserved residues with non-modified nucleotides confirmed the phylogenetically determined consensus sequence (1). This approach has been refined to include the use of modified nucleotides to evaluate the importance of individual functional groups on the bases, sugars and phosphates (for a review see 2). With the exception of the phosphate and 2'-hydroxyl at the cleavage site, these studies identify essential functional groups but fail to conclusively elucidate their involvement structurally or catalytically in the cleavage reaction.

Substitution of 4-thiouridine in the hammerhead has allowed the formation of UV-dependent crosslinks within the catalytic core (3,4). The replacement of several uridines, individually, with 4-thiouridine produced multiple crosslinks for each substituted uridine. Based on this data a 3-dimensional model has been proposed (5). As the locations of the crosslink were mapped on only one strand, the other end being assumed to be the substituted uridine, it is difficult to evaluate the validity of this model. As will be demonstrated in this work, a modified nucleotide can allow crosslinking without itself participating in the crosslink.

Recently the crystal structure of the hammerhead has been determined (6,7). Using different constructs and different crystallization conditions two different groups observed nearly identical structures (2). Subsequent demonstration that cleavage could occur within the crystal appears to provide conclusive evidence that the crystal structure is representative of the catalytically active solution structure. However, the structure fails to adequately explain the catalytic mechanism and conflicts with much of the mutagenesis data (for a review see 2). Further studies are clearly needed to fully understand the structure and mechanism of cleavage by the hammerhead domain.

Sulfur and oxygen have similar atomic structures, but sulfur-containing groups can perform many kinds of photoreactions and have altered metal ion binding specificity (4,8–12). Consequently, thio-substituted nucleosides have become valuable probes in structure–function studies of RNA. Among the sulfur-containing analogs, 4-thiouridine and the nucleotide phosphorothioates have been the most extensively studied. This is due in part to their interesting chemical and photochemical properties, but also because of their ease of introduction by transcription using bacteriophage RNA polymerases (13–15). For other thio-substituted ribonucleosides, such as thiocytidine, thioguanosine and thioinosine, other methods are needed, as they are not suitable substrates for RNA polymerase (unpublished observation). While chemical synthesis of RNA has become routine and some nucleotide modifications are compatible with synthetic methods, the sulfur groups on the base heterocycles of an oligoribonucleotide are incompatible with the normal conditions used in commercial automated oligonucleotide synthesizers. With great effort some thionucleotide-containing oligonucleotides have been synthesized.
using modified phosphoramidite procedures (16–18). For introduction of modified nucleotides, where transcription and chemical methods are not acceptable, the use of T4 RNA ligase is an attractive alternative (for a review see 19). Although Barrio had reported that some modified nucleoside 3′,5′-bisphosphates could be incorporated into oligoribonucleotides using T4 RNA ligase (20), application of the T4 RNA ligation reaction has largely been limited to construction of unmodified RNA molecules (21).

In this report we examine the use of 2-thiocytidine (s2C) and 4-thiouridine (s4U) to probe the structure and mechanism of action of the hammerhead ribozyme. We identify a UV crosslink between conserved residues of the catalytic core that requires s2 CDP or CDP, 0.1–0.15 U/µl T4 RNA ligase. The reaction mixtures were incubated at 16°C overnight. After the first ligation oligoribonucleotides were precipitated by addition of 0.15 vol 3.0 M NaOAc and 2.7 vol cold ethanol. The pellets were washed with 80% cold ethanol. The pellets were dissolved in the supplied buffer and dephosphorylated using shrimp alkaline phosphatase (US Biochemical) according to the manufacturer’s instructions. The reaction mixtures were phenol/chloroform extracted and precipitated with ethanol. Ligation of the precipitated oligoribonucleotides to the 3′-fragment was as described above.

All reactions were monitored by 20% denaturing polyacrylamide gel electrophoresis and visualized by autoradiography and/or silver staining. Preparative purification was performed after the second ligation reaction by electrophoresis into 20% denaturing polyacrylamide gels. The products were characterized by mass spectral analysis.

Materials and Methods

Synthesis and purification of RNA

T7 RNA polymerase was prepared as described (11). Oligoribonucleotides were synthesized by in vitro transcription with T7 RNA polymerase using synthetic DNA templates (22). Transcription reactions contained 40 mM Tris–HCl, pH 8.0, 20 mM MgCl2, 5 mM DTT, 1 mM spermidine, 0.01% Triton X-100, 40 mg/ml PEG 8000, 1.2 mM ATP, 0.2 mM acceptor oligoribonucleotide, 0.8 mM s4UTP. Transcription reactions were incubated at 37°C for 2.0–2.5 h. The products were separated by electrophoresis into 20% denaturing polyacrylamide gels. Transcripts were recovered using a crush and soak procedure and concentrated by ethanol precipitation.

Cytidine, 2-thiocytidine and pyrophosphoryl chloride were purchased from Sigma. Other chemicals were purchased from Aldrich or Sigma. Cytidine 3′,5′-bisphosphate (CDP) and thioctydine 3′,5′-bisphosphate (s2CDP) were synthesized using pyrophosphoryl chloride from corresponding unprotected nucleosides as described (20). The reactions were monitored using polyethyleneimine–cellulose TLC plates which were developed in 1.0 M LiCl: cytidine Rf 0.79; CDP Rf 0.43; 2-thiocytidine Rf 0.59; s2CDP Rf 0.24. The bisphosphates were purified by ion exchange chromatography on a DEAE–Sephadex A-25 column, eluted with 0–1.0 M triethylammonium bicarbonate (pH 7.4). The fractions containing nucleoside bisphosphates were collected and evaporated to dryness at room temperature in a vacuum centrifuge. Three additional evaporations from water were performed to remove volatile salts. The structure of the products were confirmed by mass spectrometry: s2CDP, m/e 418 (M-2H); CDP, m/e 402 (M-2). The 5′ (rbz-5′) and 3′ (rbz-3′) ribozyme fragments were prepared using standard methods on an Applied Biosystems DNA synthesizer. The deprotected and desalted synthetic oligonucleotides were purified in 20% denaturing polyacrylamide gels. T4 RNA ligase was obtained from New England Biolabs. The ligation reactions were performed as described with minor modifications for different oligoribonucleotides (19). Ligation was performed in a final volume of 200 µl composed of 50 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 10 mM DTT, 40 mg/ml PEG 8000, 1.2 mM ATP, 0.2 mM acceptor oligoribonucleotide, 0.8 mM s4UTP or CDP, 0.1–0.15 U/µl T4 RNA ligase. The reaction

Figure 1. (A) Hammerhead used for s2C substitution. Substrate (sub) and unmodified ribozyme (rbz-T) were prepared by transcription using T7 RNA polymerase (22). (B) Unmodified (rbz-L) and the s2C-containing (rbz-s2C) ribozyme were prepared by ligation (19). The rbz-5′ and rbz-3′ fragments were synthesized using commercially available phosphoramidites on a DNA synthesizer. In step one the 3′,5′-bisphosphate of cytidine or 2-thiocytidine was ligated to the 5′-fragment and the 3′-terminal phosphate was subsequently removed using shrimp alkaline phosphatase. The asterisk indicates the position of the cytidine or 2-thiocytidine added. In step two the product of step one was ligated to the 3′-fragment. The final product was separated on a denaturing polyacrylamide gel and isolated using a crush and soak procedure. The products were characterized by mass spectral analysis.

mRNA sequences, splicing, and antisense oligonucleotides.
Figure 2. Magnesium dependence of substrate cleavage by unmodified and s²C-modified ribozymes. Reactions were conducted in 8 μl 50 mM Tris–HCl, pH 8.0, and contained 1.8 μM substrate and 10 nM rbz-T, 10 nM rbz-L or 40 nM rbz-s²C. Reactions were initiated by addition of magnesium chloride at the concentrations indicated. The reactions were performed at 37°C for 10, 10 or 60 min for rbz-T, rbz-L or rbz-s²C respectively. The reactions were terminated by addition of 380 μl stop solution (74% ethanol, 13 mM EDTA). The terminated reactions were placed at –20°C for at least 1 h and centrifuged at 12,000 g and 4°C for 25 min. The pellet oligoribonucleotides were resuspended and analyzed by electrophoresis into 20% denaturing polyacrylamide gels.

Table 1. Cleavage kinetics for unmodified and s²C-modified ribozymes

<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>K_M (μM)</th>
<th>k_cat (1/min)</th>
<th>k_cat/k_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbz-L</td>
<td>0.92 ± 0.02</td>
<td>7.5 ± 0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>rbz-s²C</td>
<td>0.90 ± 0.15</td>
<td>0.16 ± 0.06</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Cleavage reactions were performed at 37°C in 50 mM Tris–HCl, pH 8.0, 10 mM MnCl₂. Ribozyme and substrate were combined with buffer and placed at 75°C for 3 min. The mixture was placed at 37°C and the reaction began by addition of manganese chloride. The reaction was stopped with addition of an equal volume of stop buffer containing 50 mM EDTA and fractionated in 20% denaturing polyacrylamide gels. The gels were quantitated using a Molecular Dynamics phosphorimager and kinetic constants determined from Eadie–Hofstee plots. Only time points having <15% cleavage were used.

Table 2. Single turnover cleavage kinetics (k_cat)

<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>Metal ion</th>
<th>t₁/₂ (min)</th>
<th>k_seq (per min)</th>
<th>k_Mn/k_Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbz-L</td>
<td>Mg²⁺</td>
<td>2.5 ± 0.3</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn²⁺</td>
<td>2.03 ± 0.3</td>
<td>0.34</td>
<td>1.26</td>
</tr>
<tr>
<td>rbz-s²C</td>
<td>Mg²⁺</td>
<td>149 ± 5</td>
<td>0.0046</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn²⁺</td>
<td>135 ± 13</td>
<td>0.0051</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Ribozyme and substrate were combined with buffer as indicated below. The mixture was incubated at 70°C for 3 min and cooled to 24°C. At 24°C the reaction began with addition of magnesium or manganese chloride. The final reaction conditions were 3.0 μM ribozyme and 1.5 μM substrate and either 50 mM Tris–HCl, pH 8.0, 50 mM MgCl₂ or 50 mM HEPES, pH 7.1, 10 mM MnCl₂.

RESULTS

The hammerhead motif used for 2-thiocytidine substitution is similar to one used by others (1,23,24; Fig. 1A). Thiocytidine was incorporated at position 3 of the ribozyme strand using T4 RNA ligase (Fig. 1B). For comparison, the unmodified ribozyme was prepared both using RNA ligase and by transcription using T7 RNA polymerase. The steady-state kinetic parameters and the metal ion dependence were determined for all modified and unmodified ribozymes using substrate prepared by in vitro transcription. Three ribozymes were examined: the unmodified ribozyme prepared by in vitro transcription, rbz-T; the unmodified ribozyme prepared by ligation, rbz-L; the 2-thiocytidine-containing ribozyme prepared by ligation, rbz-s²C. The unmodified ribozyme, synthesized with T4 RNA ligase, has similar cleavage activity and metal ion dependence to the unmodified transcript (Fig. 2). This indicates that the oligoribonucleotide synthesized using T4 RNA ligase is indistinguishable from that prepared by transcription. In contrast, the 2-thiocytidine-modified ribozyme was much less active than the corresponding unmodified ribozyme (Fig. 2). To determine the basis of the reduced cleavage rate for the modified ribozyme, the catalytic constants K_M and k_cat were determined for both rbz-L and rbz-s²C. For these determinations manganese chloride was used as the metal cofactor. The faster cleavage rates obtained with manganese were needed to obtain accurate data for both the modified and unmodified ribozymes. The data indicate that the single substitution of sulfur for oxygen reduces the k_cat of rbz-s²C by ~50-fold, while the K_M is unchanged (Table 1). To determine if the metal ion preference was altered by thiocytidine substitution, single turnover cleavage rates were determined in the presence of MgCl₂ or MnCl₂. For convenience, all reactions were performed at room temperature and at pH values of 8.0 or 7.1 for Mg²⁺ and Mn²⁺ catalyzed reactions respectively. The first order rate constant for the magnesium catalyzed reaction was reduced by almost 50-fold by the thio modification (Table 2). For the modified and unmodified ribozymes the cleavage activity in the presence of 10 mM MnCl₂ was much higher than in the presence of the same concentration of MgCl₂ (at least 20 times higher), consistent with other reports (25,26). The relative rates for magnesium versus manganese catalyzed cleavage for the modified ribozyme were not significantly different from those for the unmodified ribozyme. This lack of a significant ‘thio effect’ suggests that the role of the substituted oxygen atom is not direct coordination to an essential metal ion (26,27).

As other thionucleotides are known to be photoreactive (3,4,28), we examined whether the s²C-containing ribozyme could undergo UV crosslinking. Both 2-thiocytidine-modified and unmodified ribozymes were irradiated at 312 nm with and without unmodified substrate and analyzed using denaturing polyacrylamide gel electrophoresis. Irradiation was performed under four different metal ion conditions (no metal ion, 100 mM NaCl, 100 mM NaCl + 20 mM MgCl₂ and 20 mM MnCl₂) and at three different temperatures (0, 24 and 37°C). UV irradiation produced a new species of reduced mobility for the s²C-modified ribozyme. For the unmodified ribozyme a product of similar mobility was also produced, but the yield was significantly lower (Fig. 3). Crosslinking required the presence of both ribozyme and substrate. The UV crosslinking yield is not dependent on metal ions at low temperatures (Table 3). About 5% of the ribozyme–substrate complex was UV crosslinked under all four different metal ion conditions at 0°C. When the temperature was raised to 24 or 37°C the crosslinking...
Figure 3. UV crosslinking of rbz-L and rbz-s2C. Labeled substrate and non-labeled ribozyme (rbz-s2C, lanes 1–4, or rbz-L, lanes 5–8) were combined and irradiated at 312 nm at 24°C for 30 min. The concentrations of both ribozyme and substrate strand were 4.0 µM. Irradiation was performed in the presence of 20 mM MgCl₂ (lanes 1 and 5); 0.1 M NaCl, 20 mM MgCl₂ (lanes 2 and 6), 0.1 M NaCl (lanes 3 and 7) or no metal ion (lanes 4 and 8). The yield decreased sharply for the no metal ion sample, while the percent crosslinked was unchanged for the other three conditions (data not shown).

Table 3. Temperature and metal ion dependence of 2-thiocytidine-dependent UV crosslinking (%)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>No metal</th>
<th>0.1 M NaCl</th>
<th>0.1 M NaCl, 20 mM MgCl₂</th>
<th>20 mM MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.6</td>
<td>5.6</td>
<td>5.6</td>
<td>4.1</td>
</tr>
<tr>
<td>24</td>
<td>2.5</td>
<td>5.4</td>
<td>5.2</td>
<td>4.1</td>
</tr>
<tr>
<td>37</td>
<td>0.5</td>
<td>3.3</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Greater than 60% of the substrate underwent cleavage during crosslinking.

To identify the residues involved in the crosslink, 5′-end-labeled rbz-s2C or substrate was crosslinked to non-labeled substrate or rbz-s2C respectively. The crosslinked species was isolated from denaturing gels, subjected to limited alkaline digestion and fractionated on sequencing gels. Surprisingly, the crosslink on the ribozyme strand occurs not to the position substituted with s2C but an adjacent position, U4 (Fig. 4A). On the substrate strand the crosslink maps predominately to U16.1 (~70%) and, to a lesser extent, to U16.3 (~30%) (Fig. 4B).

The crosslinked ribozyme–substrate complex was purified by denaturing polyacrylamide gel electrophoresis and examined for its ability to undergo cleavage. No detectable cleavage was observed for the crosslinked species when incubated at various temperatures with either magnesium or manganese chloride (data not shown).

Since the crosslinked hammerhead is unable to undergo cleavage it is not clear that the crosslinked structure is representative of the correctly folded ribozyme. The nucleotide modification may stabilize an inactive conformation, one that is conducive to crosslink formation. Evidence to support that this may represent a relevant structure is provided by UV crosslinking of a 4-thiouridine-substituted hammerhead.

A previously well-characterized hammerhead (1,23,24; Fig. 5A) was examined for its ability to undergo UV crosslinking. The substrate (sub-2) and ribozyme (rbz-2) strands, alone and together, with and without MgCl₂, were irradiated at 312 nM for 45 min. No crosslinking was observed for this hammerhead when both the ribozyme and substrate were composed of only the four unmodified nucleotide bases (data not shown). In contrast, a crosslink was obtained when the substrate strand was fully substituted with s4U. This crosslink occurs only under conditions that are known to produce the ribozyme–substrate complex, i.e. when a matched substrate and ribozyme pair are irradiated in the presence of a divalent metal. The less active ATPαS-substituted ribozyme (29; Fig. 5B, lane 7) and inactive all-deoxynucleotide ribozyme (lane 9) also crosslink efficiently to the s4U-substituted substrate. The crosslink is an interstrand crosslink, since both strands are required for crosslinking and the crosslinked species migrates to the same position on a denaturing polyacrylamide gel whether the ribozyme or the substrate strand is labeled (data not shown). Crosslinking is not observed when the ribozyme is replaced by a non-complementary RNA (data not shown).
Figure 5. (A) The sequence of the hammerhead used for s^4U substitution. Ribozyme and substrate strands were prepared by in vitro transcription with T7 RNA polymerase (22). Unmodified substrate or ribozyme were prepared using only unmodified nucleotide triphosphates. s^4U modified ribozyme or substrate were prepared by replacing uridine triphosphate with 4-thiouridine triphosphate. (B) UV crosslinking of the s^4U-modified hammerhead. Crosslinking reactions contained 50 mM Tris–HCl, pH 8.0, 0.1 µM 5'-32P-end-labeled and s^4U-substituted substrate and either 0 or 0.4 µM non-labeled ribozyme. The reactions were incubated at 90°C for 30 s and then placed as a drop onto a sheet of parafilm on a bed of ice. For reactions containing magnesium, MgCl_2 was added to the drop to a final concentration of 20 mM. The drops were irradiated from above at a distance of no more than 2 cm using a 8 W 312 nM hand-held lamp producing 800 µW/cm² at 15 cm (FisherBiotech, catalog no. FB-UVM-80). Irradiation was performed for 30–45 min. The crosslink reactions were fractionated in denaturing polyacrylamide gels and visualized either by autoradiography or imaging on a Molecular Dynamics phosphorimager. Lanes 1 and 10 were not irradiated. wt indicates the wild-type, all-ribo, all-phosphoryl-containing ribozyme; deoxy indicates the all-deoxyribonucleotide-containing ribozyme; α-S-A indicates the ribozyme with phosphorothioate linkages 5' of all adenosine residues. Crosslinking was not observed when the fully s^4U-substituted ribozyme was irradiated in the presence of the unmodified substrate under the same conditions. The s^4U-dependent crosslink was mapped on the substrate and ribozyme strands. For these experiments ATPαS-substituted ribozyme was used because it produced a higher yield of the crosslinked ribozyme. The higher yields were needed to obtain sufficient material for the mapping experiments. The crosslink maps to U4 of the ribozyme and to U16.1 (and to a small extent U16.3) on the substrate (Fig. 6 A and B).

To determine the effect of s^4U substitution on cleavage activity, cleavage reactions were performed with both substituted and unsubstituted substrate. Under conditions similar to those used for crosslinking (i.e. single turnover) the substituted substrate is cleaved by the unsubstituted ribozyme, however, at a rate up to 32-fold slower than the unsubstituted substrate/ribozyme pair (Fig. 7, compare A–C with a–c). This decreased rate is due largely (if not entirely) to an increase in the apparent K_m for the reaction. This is evident from the fact that while both the substituted and non-substituted reactions are sub-saturating under conditions similar to those used for crosslinking (a and A), a 3-fold increase in ribozyme concentration saturates only the unsubstituted reaction (compare B and C with b and c).

Although substitution of s^4U in the substrate does not block cleavage, formation of the crosslink does. Under various conditions, including elevated temperature and replacement of MnCl_2 by MgCl_2, no cleavage of the crosslinked species was observed (data not shown).
DISCUSSION

The crosslink described here provides a structural constraint that offers insight into the folded structure of the hammerhead. However, in the light of other reports that suggest that the hammerhead has the potential to exist in multiple conformations (4) one must be careful in interpreting these results. It is possible that the structures of our crosslinked hammerheads are representative of an alternative misfolded conformation. We believe this to be unlikely. Using hammerheads of different sequence, substituted with different modifications at different positions, we induced the same residues to crosslink, i.e. U4 to U16.1 and to a lesser extent U4 to U16.3. This would at least suggest that the two crosslinked ribozymes are both in the same unique conformation. As the crosslinked ribozymes are unable to undergo cleavage, it is possible that the crosslinked structure represents a conformation unrelated to the active conformation. This seems unlikely. If each modification is perturbing the active conformation to stabilize an alternative and crosslinkable conformation, it is unlikely that these two different modifications in hammerheads of different sequence would stabilize the same alternative conformation. In the light of this we believe that the crosslinks are relevant to the active solution structure of the hammerhead ribozyme.

Our results for the s4U-dependent crosslink differ from those reported by others (4). In those studies multiple crosslinks were found to occur to the ribozyme strand, in contrast to our single crosslink to U4. It is difficult to reconcile the difference for several reasons. First, the location of the crosslink on the ribozyme strand was not determined (4). It was assumed to be the position substituted with s4U. In the light of our finding that s4C can induce a crosslink without participating in the crosslink, this assumption is somewhat tenuous. Second, our crosslinks were generated with irradiation at 312 nm, in contrast to the 365 nm used by Woisard et al. This could favor generation of the classical cyclobutane dimer in our studies, in contrast to the 4–5 addition typical of s4U. Finally, the hammerhead used by Woisard et al. was of the I/III configuration (4), in contrast to the I/II configuration used in these studies (30). Since our stem III is only 3 bp in length, closed by a 3 nt loop, it is likely to exist in a more open conformation due to the reduced stability conferred by substitution of 2 of the 3 bp with s4U.

The crosslink for the thiocytidine-modified hammerhead is likely to be a cyclobutane dimer, as this is the only type of crosslink known to form efficiently between unmodified uridines (for a review see 31). For the s4U-modified hammerhead two possibilities exist. The crosslink may be of the 4–5 type, as observed between U8 (s4U) and C13 in Escherichia coli tRNAVal (32–34). However, in the light of the thiocytidine-dependent crosslink it seems likely that the s4U-induced crosslink is also of the cyclobutane type. The likelihood of these different mutations producing the same pattern of crosslinks but differing in the nature of the crosslink seems highly improbable. Nevertheless, either type could be accommodated with only a small change in position of one of the two nucleotide bases involved.

On examination of the hammerhead crystal structure (6–7,35) it is not clear how these two different modifications can allow formation of the same crosslink. Residues U4 and U16.1 are 12.7 Å apart, with A6 in between them. A major change in conformation would be required to allow crosslinking to occur. In fact, from the structure it is more reasonable to believe that U16.1 would crosslink to U7. U7 and U16.1 are only 3.3 Å apart, with no intervening residues. A minor change in conformation would appear to be sufficient for this to occur. Thiouridine substitution of U16.1 should aid in this, as O4 of U16.1 participates in the only hydrogen bond to A15.1 (6). The loss of this hydrogen bond, as a result of 4-thiouridine substitution, could allow the needed conformational flexibility, to allow better overlap with and, consequently, crosslinking to U7.

The incompatibility of the crosslink with the crystal structure could suggest that the crosslink is probing a misfolded structure. We believe this to be unlikely. These two modifications are each expected to eliminate only a single hydrogen bond acceptor. It is...
difficult to imagine that the loss of a single hydrogen bond would be sufficient to cause gross misfolding and this is supported by the only modest effect on cleavage that these two modifications cause. Even more difficult to imagine is the possibility that these two different modifications in different parts of the molecule can allow misfolding into the same alternative conformation. Nevertheless, significant concerns have been raised regarding inconsistencies between the alternative misfolded conformation. We believe it is more likely that residues U4 and U16.1 are in close proximity in the active solution structure but are unable to allow misfolding into the same alternative conformation. We are grateful to Darrell Davis, James McCloskey and Michael Pierce for their critical reading of this manuscript. We also thank Robert Schackman of the University DNA/Peptide Core facility, which received support through a NIH Cancer Center Support Grant, CA42014, to the Huntsman Cancer Institute. This work was supported by a grant to D.E.R. from the NIH, 5R29AI34278-03.

ACKNOWLEDGEMENTS

We are grateful to Darrell Davis, James McCloskey and Michael Pierce for their critical reading of this manuscript. We also thank Robert Schackman of the University DNA/Peptide Core facility for synthesis of DNA and RNA oligonucleotides. Mass spectral analysis was performed by the Electrospray Mass Spectrometry Core facility, which received support through a NIH Cancer Center Support Grant, CA42014, to the Huntsman Cancer Institute. This work was supported by a grant to D.E.R. from the NIH, 5R29AI34278-03.

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