Differential effects of the protein cofactor on the interactions between an RNase P ribozyme and its target mRNA substrate

Amy W. Hsu¹, Ahmed F. Kilani¹, Kwa Liou¹, Jarone Lee¹ and Fenyong Liu^{1,2,*}

¹Program in Infectious Diseases and Immunity and ²Program in Comparative Biochemistry, School of Public Health, University of California, Berkeley, CA 94720, USA

Received April 27, 2000; Revised and Accepted June 23, 2000

ABSTRACT

RNase P from *Escherichia coli* is a tRNA-processing enzyme and consists of a catalytic RNA subunit (M1 RNA) and a protein component (C5 protein). M1GS, a gene-targeting ribozyme derived from M1, can cleave a herpes simplex virus 1 mRNA efficiently *in vitro* and inhibit its expression effectively in viral-infected cells. In this study, the effects of C5 on the interactions between a M1GS ribozyme and a model mRNA substrate were investigated by site-specific UV crosslink mapping. In the presence of the protein cofactor, the ribozyme regions crosslinked to the substrate sequence 3' immediately to the cleavage site were similar to those found in the absence of C5. Meanwhile, some of the ribozyme regions (e.g. P12 and J11/12) that were crosslinked to the leader sequence 5' immediately to the cleavage site in the presence of C5 were different from those regions (e.g. P3 and P4) found in the absence of the protein cofactor and were not among those that are believed to interact with a tRNA. Understanding how C5 affects the specific interactions between the ribozyme and its target mRNA may facilitate the development of gene-targeting ribozymes that function effectively *in vivo*, in the presence of cellular proteins.

INTRODUCTION

Ribonuclease P (RNase P) is a ribonucleoprotein complex responsible for the maturation of the 5' termini of tRNAs (1,2). In bacteria, the RNase P holoenzyme contains a catalytic RNA subunit of 350–500 nt and a small highly basic protein subunit of 100–150 amino acids (e.g. M1 RNA and C5 protein in *Escherichia coli*), both of which are required for activity *in vivo*. Under certain conditions *in vitro*, M1 RNA acts as a catalyst and cleaves ptRNAs (precursor to tRNAs) in the absence of C5 protein (3). Phylogenetic analyses, combined with biochemical studies and computer modeling, have established the models for the secondary and three-dimensional structures of M1 RNA (4–7). Recently, the tertiary structure of the protein component of *Bacillus subtilis* RNase P has been

determined to 2.6 Å resolution (8). These studies have provided a foundation for understanding the mechanism of RNase P catalysis and the interactions among the ribozymes, the protein cofactors and the substrates.

One of the unique features of RNase P holoenzyme and its catalytic RNA is their ability to recognize the structures, rather than the sequences of their substrates, which gives them the ability to hydrolyze different natural substrates *in vivo* or *in vitro*. The interactions between M1 RNA and a ptRNA substrate have been extensively studied by site-directed mutagenesis, kinetic analyses, UV crosslinking, chemical footprinting and interference experiments (6,9–19). These studies have revealed the regions of M1 RNA that are in close proximity to a ptRNA substrate. Moreover, these studies have led to the identification of the regions of M1 RNA that bind to the 3' CCA sequence and interact with a part of the T-stem and T-loop of a ptRNA (9,12,20,21). More recently, UV crosslinking experiments have suggested that the P4, J18/2, J15/16 and P3 regions are in close contact with the 5' leader sequence of a ptRNA, with nucleotides G₃₃₂ and A₃₃₃ of M1 RNA in close proximity to the nucleotide 5' adjacent to the cleavage site (16,22–24).

Studies on the function of C5 protein have shown that the holoenzyme has broader substrate specificity and higher catalytic efficiency than the catalytic RNA component alone (25,26). C5 protein appears to increase the affinity of the enzyme to the substrate but not to the product (19,27,28). The effect of the *E.coli* and *B.subtilis* RNase P protein subunits in enhancing the cleavage of a ptRNA is correlated with the length of the 5' leader sequence of the substrate (28,29). It was recently reported that the *B.subtilis* RNase P protein component, modified at single cysteine residues with a photochemical crosslinking agent, can crosslink to nucleotides in the 5' leader sequence of a ptRNA (27). Meanwhile, UV crosslinking studies have suggested that C5 enhances the yields of certain but not all M1 RNA–ptRNA complexes found in the absence of the protein cofactor (16). These studies have highlighted the importance of the protein cofactor in enhancing specific interactions between the ribozyme and its ptRNA substrates.

Studies on substrate recognition by RNase P have also revealed that a small model substrate can be cleaved efficiently by M1 ribozyme (Fig. 1A). This model substrate contains a structure equivalent to the acceptor stem, the T-stem, the 3'

*To whom correspondence should be addressed at: School of Public Health, 140 Warren Hall, University of California, Berkeley, CA 94720, USA.
Tel: +1 510 643 2436; Fax: +1 510 642 6350; Email: liu_fy@uclink4.berkeley.edu

Figure 1. (A) Schematic representation of natural substrates (ptRNA and p4.5S RNA), a small model substrate (EGS:mRNA) for ribonuclease P and M1 RNA from *E.coli*, and a complex formed between a M1GS RNA and its mRNA substrate (S). The structural components common to both ptRNA and p4.5S RNA substrates are highlighted. The site of cleavage by RNase P or M1 RNA is marked with a filled arrow. (B) Schematic representation of the substrates used in the study. The uridine positions that were incorporated with the photoactive 4-thio-uridine nucleotides are highlighted. The targeting sequences that bind to the guide sequences of the ribozymes are boxed. The regions upstream and downstream from the targeting sequence represent the 5' leader sequence and the 3' tail sequence, respectively. (C) Nuclease (RNase T1 and V) cleavage mapping of the secondary structure of the M1 region of M1-S3 in the presence of MgCl₂ [buffer F (50 mM Tris-HCl pH 7.5, 100 mM NH₄Cl, 20 mM MgCl₂)] or CaCl₂ [buffer G (50 mM Tris-HCl pH 7.5, 100 mM NH₄Cl, 20 mM CaCl₂)]. The predicted secondary structure of M1 RNA is presented as described previously (4,54). This is a summary of several independent experiments. Only the results that were reproducible are included.

CCA sequence and the 5' leader sequence of a tRNA molecule (30). Accordingly, M1 catalytic RNA can cleave a mRNA sequence if the mRNA substrate forms a hybrid complex with its complementary sequence (external guide sequence or EGS) (Fig. 1A) (31). Moreover, a sequence-specific mRNA-cleaving ribozyme, M1GS RNA, can be constructed by linking a guide sequence covalently to M1 RNA (Fig. 1A) (32,33). We have previously shown that M1GS ribozymes cleave the mRNA sequence encoding thymidine kinase (TK) of herpes simplex virus 1 (HSV-1) (33–36). When the ribozymes were expressed in mammalian cells infected with HSV-1, both the viral TK mRNA and protein levels were reduced by ~75% (33).

RNA enzymes are being developed as promising gene-targeting reagents to specifically cleave RNA sequences of choice (37–39). Targeted cleavage of mRNA by RNase P ribozyme provides a unique approach to inactivate any RNA of known sequence expressed *in vivo*. Further studies to investigate the mechanism of how a M1GS RNA interacts with a mRNA substrate should provide insight into how to improve the catalytic efficiency and sequence specificity of the ribozyme. Using a nuclease footprint analysis and a site-specific UV crosslinking approach, we have recently mapped the regions of the ribozymes that are in close contact with a mRNA model substrate (35,36). In this study, we have determined the regions of M1GS RNA that are in close proximity to a TK mRNA substrate in the presence of C5 by site-specific UV crosslink mapping. Understanding the effects of C5 protein on the interactions between a M1GS ribozyme and a mRNA model substrate may provide insight into developing effective RNase P ribozymes that recognize their mRNA substrates efficiently *in vivo* in the presence of cellular proteins.

MATERIALS AND METHODS

Synthesis of RNase P holoenzyme, ribozymes and substrates

C5 protein was kindly provided by Dr Venkat Gopalan of Ohio State University. The oligonucleotides used in this study were synthesized with a DNA oligonucleotide synthesizer. Uniformly radiolabeled RNA molecules were synthesized *in vitro* by T7 RNA polymerase in the presence of [α - 32 P]GTP. Plasmids pFL117 and pFL120 contain the DNA sequences that encode M1 RNA and substrate tk46 driven by the T7 RNA polymerase promoter, respectively (33). The DNA sequences that encode M1GS ribozymes were constructed by PCR using *Pvu*II-digested pFL117 as the template and oligonucleotide AF25 (5'-GGAATTCTAATACGACTCACTATAG-3') as the 5' primer. The 3' PCR primers for the DNA sequences that encode ribozymes M1-S2 and M1-S3 are oligonucleotides S5-GS (5'-GTGGTGTCCCGCGCCCGACTATGACCATG-3') and S3-GS (5'-GTGGTGTTCGCGCCCGACTATGACCATG-3'), respectively.

To construct plasmid pS5 that contained the DNA sequence coding for substrate tk46S5, the DNA sequence was generated by PCR using pFL120 as the template and oligonucleotides AF25 and S5 (5'-CGCGGATCCGCGGAAAGGTCGGGC-GCGGGCGCGTGTGGTGGCGGGGTC-3') as the 5' and 3' primers, respectively, and was subsequently cloned into pUC19 vector. The DNA sequences in plasmids pS2 and pS3 that encode substrates tk46S2 and tk46S3 were generated by

PCR using pS5 as the template and the 5' primer AF25. The oligonucleotides used as the 3' primers for construction of pS2 and pS3 were S2 (5'-CGCGGATCCGCGGCCTGGTCGGG-CGCGGGCAAATGTTGGTGGC-3') and S3 (5'-CGCGG-ATCCGCGGCCTGGTCGGGCGCAAACGCGTGTGG-3'), respectively. The ribozymes and RNA substrates were synthesized *in vitro* from these DNA templates by T7 RNA polymerase as described previously (35,36,40). The substrates that contained photoactive 4-thio-uridines were synthesized by carrying out transcription reactions in 40 mM Tris-HCl pH 7.9, 6 mM MgCl₂, 10 mM DTT, 2 mM spermidine, 2 mM ATP, 2 mM CTP, 2 mM GTP, 4 mM 4-thio-UTP (Amersham, Arlington Heights, IL), and 20 μ Ci of [α - 32 P]GTP, with T7 RNA polymerase for 16 h at 37°C. Substrates and ribozymes prepared by transcription *in vitro* were further purified in denaturing 8% polyacrylamide gels prior to use. Prior to crosslinking and kinetic analyses, the ribozymes and substrates were incubated separately in buffer H (50 mM Tris pH 7.5, 100 mM NH₄Cl) at 80°C for 2 min and then cooled slowly to room temperature to allow renaturation of ribozyme and substrate structures. The assembly of the ribozyme-C5 complexes was carried out by mixing the ribozyme and C5 protein in a ratio of 1:20 as described previously (25,40).

Kinetic analyses of the reactions catalyzed by the ribozymes

The cleavage reactions of the substrates by different ribozymes were carried out either in the absence or the presence of C5 protein in buffer F (50 mM Tris pH 7.5, 100 mM NH₄Cl, 20 mM MgCl₂) as described previously (33,40). The assembly of the ribozyme-C5 complexes was carried out by mixing the ribozyme and C5 protein in a ratio of 1:20. The cleavage products were separated in 15% polyacrylamide gels and analyzed with a STORM840 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Assays to determine the observed reaction rate (k_{obs}) and the values of $k_{\text{cat}}/K_{\text{m}}$ were performed in buffer H (50 mM Tris pH 6.5, 100 mM NH₄Cl, 20 mM MgCl₂) as described previously (40,41). Briefly, analyses were performed with a trace amount of radioactive substrate and an excess of ribozyme. The concentration of radioactive substrate was <0.1 nM and the concentrations of ribozyme tested ranged from 0.5 to 100 nM. The ribozymes and ribozyme-C5 complexes were incubated with the radiolabeled substrates at 37°C. Aliquots were withdrawn from reaction mixtures at regular intervals (from 0 to 720 min). The cleavage products were separated on 15% denaturing gels, autoradiographed and quantitated with a STORM840 phosphorimager. Variants of the amount of substrate did not affect the observed cleavage rate (k_{obs}) at a fixed excess ribozyme concentration and the reaction followed pseudo-first-order kinetics. Pseudo-first-order rate constants of cleavage (k_{obs}) were assayed at each ribozyme concentration by the slope of a plot $-\ln [(Ft - Fe) / (1 - Fe)]$ versus time using Kaleidagraph program (Synergy Software, Reading, PA). Ft and Fe represent the fraction of the substrate at time t and the end point (>10 h) of the experiments, respectively. The values of the overall cleavage rate ($k_{\text{cat}}/k_{\text{m}}$) were calculated by the slope of a least-squares linear regression (Kaleidagraph) of a plot of the values of k_{obs} versus the concentrations of the ribozymes. These values were the average of three experiments.

To assay the catalytic activity of the crosslinks, the crosslinked species were purified from denaturing gels, either

diluted 100-fold first or directly incubated at 37°C in buffer F in the absence or presence of 100 nM of C5 protein (>20-fold excess to the ribozyme). The reactions were stopped after 1–2 h incubation by adding an equal volume of 8 M urea. The cleavage products were separated on 4% polyacrylamide denaturing gels and quantitated with a Molecular Dynamics STORM840 phosphorimager.

UV crosslinking, primer extension and RNase mapping experiments

UV crosslinking was carried out essentially as described previously (35,42). Radiolabeled RNA substrates (5–100 nM) were incubated with ribozymes (5–100 nM) in buffer G (50 mM Tris pH 7.5, 100 mM NH₄Cl, 20 mM CaCl₂) for 5 min at 25°C either in the absence or presence of C5 protein (100–1000 nM). Then, the reaction mixture was exposed to UV light (365 nm) on ice for 5–30 min. The crosslinked species were separated in denaturing gels and visualized by autoradiography. The conjugates were then excised from the gels and further purified. RNA samples were recovered by phenol/chloroform extraction and subsequent ethanol precipitation.

The oligonucleotide primers used for primer extension experiments were radiolabeled at their 5' termini by T4 polynucleotide kinase in the presence of [γ -³²P]ATP. The oligonucleotides used for experiments with M1GS RNA were AK1(85–102) (5'-CGTTACCTGGCACCTG-3'), AK2(174–191) (5'-CCGCACCCTTTCACCT-3'), AK3(265–282) (5'-ACGGCCGTACCTTATG-3') and AK4(360–377) (5'-AGGTGAACTGACCGATA-3'). 100 000 c.p.m. of primers and different amounts of purified crosslinked products were incubated at 90°C for 2 min to allow denaturing of the RNA structure and then immediately placed on ice. The reaction mixtures were then incubated at 42°C in the presence of 20 U of AMV reverse transcriptase (Promega, Madison, WI), 50 mM Tris (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM DTT, and 1 mM each of dATP, dGTP, dCTP and dTTP for 2 h. The RNA templates were then hydrolyzed with 5 mM NaOH at 68°C for 1 h. The cDNA samples were purified by phenol/chloroform extraction and recovered by ethanol precipitation. The primer extension products were separated in 8% denaturing gels and quantitated with a STORM840 phosphorimager. For control experiments, ribozymes were UV irradiated either in the absence of the substrates or in the presence of the substrates that did not contain the photoactive group. The RNA mixtures were purified by ethanol precipitation and directly subjected to primer extension analyses. Sequencing reactions were carried out using pFL117 as the template in the presence of T7 sequenase and [α -³⁵S]dATP (Amersham). The sequencing primers were the same oligonucleotides used in the primer extension analyses of the ribozymes. The secondary structure of M1GS RNA was mapped in buffer F or G using RNase T1 and V, as described previously (36,40).

RESULTS

Cleavage of the TK mRNA substrates by M1GS ribozymes in the presence of C5

A model mRNA substrate for a M1GS ribozyme can be considered to consist of three sequence elements: (i) a 5' leader

sequence, (ii) a targeting sequence that base pairs with the guide sequence of a M1GS ribozyme, and (iii) a 3' tail sequence (Fig. 1A and B). By introducing a photoactive agent into these three different sequences of a mRNA substrate, we can use a UV crosslinking approach to map the regions of M1GS RNA that are in close proximity to these nucleotides of the substrate. 4-Thio-uridine, which has been extensively used in UV crosslinking studies to investigate RNA–RNA and RNA–protein interactions (42,43), was used as the photoactive agent. Upon exposure to UV light, this photoactive agent can crosslink to the nearby regions within a distance of 5–10 Å (43). tk46, which contained a 5' TK mRNA sequence of 46 nt, was used as the model substrate here. This 46 nt long region of TK mRNA has been shown to be accessible to ribozyme binding in mammalian cells (33). Significant inhibition of TK mRNA and protein expression was observed in cells that expressed a M1GS ribozyme targeting to this region (33).

In order to identify the regions of a M1GS RNA that interact with a particular part of tk46 (e.g. the 5' leader sequence), the photoactive groups can be incorporated into a specific region of the substrate (Fig. 1B). Substrates tk46S2, tk46S3 and tk46S5 were derived from tk46 and contained three uridines at the positions 5' and 3' adjacent to the cleavage site, and 5' adjacent to the 3' end of the targeting sequence, respectively (Fig. 1B). M1 RNA was used to construct the mRNA-targeting ribozyme, M1GS RNA, by linking the 3' terminus of the catalytic RNA to a guide sequence. Different guide sequences were used to construct M1GS RNAs (e.g. M1-S2 and M1-S3) to target the corresponding substrates (e.g. tk46S2 and tk46S3). Since substrates tk46S2 and tk46S5 had the identical targeting sequence which represented the guide sequence-binding region (Fig. 1B), ribozyme M1-S2 was used to target both tk46S2 and tk46S5. UV crosslinking studies with substrates tk46S2, tk46S3 and tk46S5 should reveal the regions of the ribozyme that are in close proximity to the different sequence elements of the substrate.

Cleavage of substrates tk46S2, tk46S3 and tk46S5 by M1GS RNAs in the presence of C5 was carried out, and an example of the cleavage analyses is shown in Figure 2. As expected, cleavage of tk46S3 by M1-S3 ribozyme under high salt conditions (i.e. 100 mM MgCl₂) in the absence of C5 protein yielded a 5' product and a 3' product (Fig. 2, lane 2) (35). The cleavage of tk46S3 by M1-S3 in the presence of C5 protein also yielded two products that comigrated with those from the cleavage of tk46S3 in the absence of C5 (Fig. 2, lane 3). Further characterization of the cleavage products indicated that the cleavage of tk46S3 in the presence of C5 occurred at the same location as that in the absence of the protein cofactor (data not shown). Similar results were also observed in the reactions with substrates tk46S2 and tk46S5.

Kinetic analyses were carried out in the absence and presence of C5 protein in buffer H (50 mM Tris pH 6.5, 100 mM NH₄Cl, 20 mM MgCl₂) in order to determine the cleavage rate of these substrates by the ribozymes. The catalytic efficiencies for the ribozyme–C5 complexes, indicated as the values of k_{cat}/K_m (average of three independent experiments), were within 2-fold difference regardless of whether the substrate contained the photoactive group or not (Table 1). Thus, the presence of the photoactive group did not greatly affect the cleavage of these substrates in the presence of C5 (Fig. 2, lanes 3 and 5; Table 1). Similar results were also found in the reactions without C5

Figure 2. Cleavage of substrate tk46S3 by M1GS RNA. Substrates (2 nM) were incubated alone (lanes 1 and 4), with 2 nM of M1-S3 (lanes 3 and 5) or 20 nM of M1-S3 ribozyme (lanes 2, 6 and 7). Cleavage reactions were carried out for 30 min either in the presence of S3 (lanes 1–3) or S3-thio (lanes 4–7), either in buffer E (50 mM Tris-HCl pH 7.5, 100 mM NH₄Cl, 100 mM MgCl₂) (lanes 1 and 2), buffer F (50 mM Tris-HCl pH 7.5, 100 mM NH₄Cl, 20 mM MgCl₂) (lanes 3–5) or buffer G (50 mM Tris-HCl pH 7.5, 100 mM NH₄Cl, 20 mM CaCl₂) (lanes 6 and 7), and either at 25 (lane 7) or 37°C (lanes 1–6). The reactions were performed in the absence (lanes 1 and 2) and presence of 40 nM (lanes 3–5) or 400 nM of C5 protein (lanes 6 and 7). Cleavage products were separated in 15% polyacrylamide gels containing 8 M urea.

(Table 1). Moreover, a comparison of the rates of cleavage of these substrates in the absence and presence of C5 revealed that the presence of the protein cofactor increased the value of k_{cat}/K_m of the reactions by at least 100-fold (Table 1).

Table 1. Kinetic parameters of the cleavage reactions governed by different M1GS RNAs either in the absence or presence of C5 protein

Substrate	4-thio-U	k_{cat}/K_m ($\mu\text{M}^{-1} \text{min}^{-1}$)	
		–C5	+C5
tk46S2	–	0.05 ± 0.01	6.2 ± 1.3
	+	0.05 ± 0.01	5.9 ± 1.2
tk46S3	–	0.06 ± 0.01	7.2 ± 1.4
	+	0.05 ± 0.01	6.6 ± 1.5
tk46S5	–	0.04 ± 0.01	6.1 ± 1.5
	+	0.05 ± 0.01	6.8 ± 1.5

In brief, cleavage assays were performed in buffer H (50 mM Tris pH 6.5, 100 mM NH₄Cl, 20 mM MgCl₂) at 37°C. The values were the average of three experiments and exhibited a variation of <25%.

To identify the regions of the ribozyme that specifically interact with the substrates but not with the cleavage products, UV crosslinking experiments were carried out under reaction

conditions that inhibited cleavage of the mRNA substrate. In order to reduce the rate of cleavage while preserving the interactions between the ribozyme and the substrate in an active ribozyme–substrate complex, two changes were introduced to the mRNA cleavage reactions: CaCl₂ was used instead of MgCl₂ as the source of divalent ions and the reactions were carried out at 25 instead of 37°C. It has been shown that divalent ions are essential for M1 catalytic activity (3) and M1 RNA cleaves a ptRNA and a model mRNA substrate at least 30 times slower in the presence of CaCl₂ than in the presence of MgCl₂ (36,44). Indeed, in the presence of CaCl₂, cleavage of substrate tk46S3 in the presence of C5 occurred and the rate of cleavage was at least 40-fold slower than that in the presence of MgCl₂ (Fig. 2, lane 6). The reaction rate was further reduced at low temperatures (Fig. 2, lane 7). No cleavage products were detected under these changed conditions (Fig. 2, lane 7), while substantial amounts of products were found under the optimal cleavage conditions (Fig. 2, lanes 3 and 5). Similar results were also observed in the reactions without C5 (data not shown). The overall secondary structure of the M1GS ribozymes in the cleavage buffer that contained CaCl₂, mapped by nuclease cleavage, was very similar to that in the buffer that contained MgCl₂ (Fig. 1C).

Ribozyme–substrate crosslinked complexes in the absence and presence of C5

M1GS ribozymes were mixed with substrates tk46S2-thio, tk46S3-thio and tk46S5-thio that were uniformly labeled with [α -³²P]GTP. The reaction mixtures were incubated in buffer G (50 mM Tris pH 7.5, 100 mM NH₄Cl, 20 mM CaCl₂) in the absence and presence of C5 and then irradiated with UV light at 365 nm. The crosslinked products were separated in denaturing polyacrylamide gels. An example of the crosslinked products formed between M1-S2 and tk46S5-thio is shown in Figure 3A. A major cluster (designated as cluster A) and a minor cluster (designated as cluster B) of crosslinked species were found (Fig. 3A, lanes 1 and 2). The major and minor cluster accounted for ~5–20% and 2% of the total amount of substrates used in the experiments, respectively. These clusters were not found in control experiments that were not treated with UV irradiation or that were carried out with substrate tk46S5 that did not contain the photoactive group (data not shown). The crosslinked species in cluster B were found when substrate tk46S5-thio was irradiated in the absence of the ribozyme, indicating that these species were derived from crosslinking of the substrate (Fig. 3, lane 3). In contrast, the conjugated complexes in cluster A were observed only when the substrate was irradiated in the presence of ribozyme M1-S2 (Fig. 3, lane 1). These complexes were not observed when M1-S2 was replaced with either M1 RNA which did not contain a guide sequence or M1ICP4 which targeted another HSV-1 mRNA, the ICP4 mRNA (Fig. 3, lanes 4 and 5). These results suggested that the conjugated species in cluster A (i.e. c51 and c52) were specifically derived from the ribozyme–substrate complexes formed by base pairing interactions of the substrate to the guide sequence of the ribozyme, rather than from those formed through non-specific binding of the substrate to other regions of the ribozyme. Similar results were also observed when tk46S2-thio and tk46S3-thio were used as the substrates.

The mobility of the crosslinked complexes in a denaturing gel correlates with the patterns of crosslink in which specific

Figure 3. Autoradiograph of the crosslinked products between the substrates and ribozymes. (A) Radiolabeled tk46S5-thio (5–20 nM) was incubated alone (–, lane 3), with equimolar amounts of M1-S2 RNA (M1-S2, lanes 1 and 2), M1 RNA (M1, lane 5) or M1ICP4 RNA (M1ICP4, lane 4) in buffer G (50 mM Tris–HCl pH 7.5, 100 mM NH₄Cl, 20 mM CaCl₂) either in the absence (lane 2) or presence of a 20-fold excess amount of C5 protein (lanes 1 and 3–5). Then, the reaction mixtures were subjected to UV irradiation. (B) 5–20 nM of radiolabeled substrates tk46S2 (tk46S2-thio, lanes 6 and 7), tk46S3 (tk46S3-thio, lanes 8 and 9) and tk46S5 (tk46S5-thio, lanes 10 and 11) that contained thio-U were crosslinked with 5–20 nM ribozymes either in the absence (–, lanes 7, 9 and 11) or presence of a 20-fold excess amount of C5 protein (+, lanes 6, 8 and 10). All the crosslinked conjugates were separated in 4% polyacrylamide gels that contained 8 M urea. The letters at the side of the photographs specify the different crosslinked species.

regions of the ribozyme are crosslinked to particular substrate positions within the enzyme–substrate complexes. It is expected that a difference in the crosslink patterns between the ribozyme and the substrate may lead to a change in the mobility of the complexes. The presence of C5 did not appear to affect the mobility and yield of the crosslinked species formed between M1-S3 and tk46S3-thio. Two major ribozyme–substrate conjugated complexes (i.e. c31 and c32) were found in the reactions in the presence of C5 and comigrated with

the two complexes found in the absence of the protein cofactor (Fig. 3, lanes 8 and 9). In contrast, when ribozyme M1-S2 was crosslinked to tk46S2, a single crosslinked species (i.e. c23) was predominately found in the presence of C5. Complex c23 exhibited an electrophoretic mobility different from that of the two conjugated species (i.e. c21 and c22) which were generated in the absence of the protein cofactor (Fig. 3, lanes 6 and 7). In the absence of C5, crosslinking between M1-S2 and tk46S5 yielded two ribozyme–substrate conjugated species (c51 and c52) (lanes 2 and 11). However, the presence of C5 protein increased the relative yield of the c52 complex, which accounted for >80% of the total amount of the ribozyme–substrate conjugated complexes (lanes 1 and 10).

The regions of the ribozymes crosslinked with RNA substrates in the absence and presence of C5

To determine the sites of the ribozymes crosslinked to the substrates, each species within the clusters A and B of the crosslinked complexes was excised from the polyacrylamide gels. Primer extension experiments in the presence of reverse transcriptase were carried out using these species as the templates and oligonucleotides complementary to M1 RNA as the primers. Reverse transcriptase usually terminates 1 nt 3' to crosslink sites in the RNA template (42,43). The primers used in the primer extension analysis were AK1, AK2, AK3 and AK4, which were complementary to the positions 85–102, 174–191, 265–282 and 360–377 of M1 RNA, respectively. These primers were used in the experiments in order to cover the full-length ribozyme sequence. Figure 4 shows the examples of the primer extension experiments for the conjugated complexes formed between M1-S2 and tk46S2-thio (Fig. 4A and C), M1-S3 and tk46S3-thio (Fig. 4B), and M1-S2 and tk46S5-thio (Fig. 4D and E) in the absence and presence of C5. A comparison of the extension products obtained from crosslinked RNA species (e.g. Fig. 4A, lanes 6–8) with those from the control RNA conjugates (e.g. Fig. 4A, lane 5) and sequencing reactions (e.g. Fig. 4A, lanes 1–4) identifies the nucleotides in the ribozyme that are potentially crosslinked with the substrate. For control experiments, ribozymes were UV irradiated either in the absence of the substrates or in the presence of the substrates that did not contain the photoactive group. No reproducible crosslinked species in the control experiments were found to comigrate with the species of cluster A (e.g. c21) formed in the reactions between M1GS ribozymes and the substrates that contained the photoactive groups (data not shown). The RNA mixtures in the control experiments were purified by ethanol precipitation and directly subjected to primer extension analyses. No primer extension products were detected when using the species in cluster B as the template, regardless of which primers were used. These observations were consistent with our conclusion that the conjugates within this cluster did not contain ribozyme sequences and were probably the crosslinking products of the substrate (Fig. 3, lane 3). Primer extension experiments using other minor species in the cluster A (i.e. c24, c33 and c53) as templates did not yield any reproducible termination products at a specific ribozyme position, suggesting that these species might represent non-specific crosslinks or artifacts. The crosslinking sites of the ribozymes within the conjugates are listed in Table 2 and shown in Figure 5. The salient features of these results are as follows.

Table 2. Analyses of the crosslinked products formed between the substrates and ribozymes in the absence and presence of C5

Substrate	C5 protein	Complexes	Crosslinking sites of M1GS RNA
tk46S2	–	c21	74–75 (P4), 285 (P17), 327–329 (J2/18), 247–248 ^a (J5/15), 257–258 ^a (J15/16)
	–	c22	48–49 (P3), 332–333 (J2/18), 247–248 ^a (J5/15)
	+	c23	138 (J11/12), 168–169 (P12), 292–293 (J15/16), 327–329 (J2/18), 332–333 (J2/18)
tk46S3	±	c31	69 (P4), 247–248 (J5/15), 345 (J2/4)
	±	c32	7–10 (P1), 247–248 (J5/15), 255–256 (J15/16), 327–329 ^a (J2/18)
tk46S5	±	c51	123–124 (P11), 189–190 (P13), 350 (J2/4)
	±	c52 ^b	189–190 (P13), 224–226 (J11/14)

The crosslinking reactions were carried out in buffer G (50 mM Tris–HCl pH 7.5, 100 mM NH₄Cl, 20 mM CaCl₂). The numbers and letters in parentheses represent the nucleotide positions and regions of the ribozymes, respectively, according to the predicted secondary structure of M1 RNA (4,54) (Fig. 5). P, helix regions; J, junction regions between two helix sequences; L, loop regions.

^aWeak crosslink.

^bIn the presence of C5, c52 accounts for >80% of the total crosslinked complexes.

Figure 4. Identification of the nucleotides of the ribozymes that were crosslinked with the substrates by primer extension analyses. UV crosslinking was carried out with the reaction mixture that contained the substrates and M1GS ribozymes, and conjugated species as listed in Table 2 were purified (e.g. c21). The templates used for the primer extension experiments with one of the four primers (AK1–AK4) were the crosslinked complexes formed between M1-S2 and tk46S2-thio (A and B), M1-S3 and tk46S3-thio (C), and M1-S2 and tk46S5-thio (D and E) in the absence (lanes 6, 7, 14, 16, 24, 25, 31, 32, 38 and 39) and presence of C5 [(+C5), lanes 8, 15, 17, 23, 33 and 40]. In the control lanes (control, lanes 5, 13, 22, 30 and 41), the ribozyme was UV irradiated either in the absence or presence of C5 with the substrate that did not contain the photoactive group and the RNA mixtures were purified by phenol/chloroform extraction and ethanol precipitation, and directly subjected to primer extension analyses. Sequencing ladder (lanes 1–4, 9–12, 18–21, 26–29 and 34–37) was generated with T7 sequenase kit using pFL117 as the template and one of the four primer extension oligonucleotides (AK1–AK4) as the sequencing primer. The primer extension products were separated in 8% denaturing gels and analyzed with a STORM840 phosphorimager.

(i) *The sites of the ribozymes crosslinked to tk46S3.* Two M1-S3–tk46S3-thio conjugated species (i.e. c31 and c32) were predominantly found in both the absence and presence of C5. Analyses of each individual species revealed that the sites of the ribozyme crosslinked to tk46S3-thio in the presence of C5 were identical to those found in the absence of the protein cofactor. These regions include positions 7–10 (region P1), 69 (P4), 247–248 (J5/15), 255–256 (J15/16), 327–329 (J2/18) and 345 (J2/4) of M1 RNA (Table 2) (see Fig. 5 for the nomenclature and location of the regions). These results are consistent with our observations that the ribozyme–substrate conjugate complexes generated in the presence of C5 exhibited similar electrophoretic mobility and radioactive intensity as those generated in the absence of C5. These results suggested that the presence of C5 protein did not greatly affect the interactions between the ribozyme and the positions of the substrate that contained the photoactive group (i.e. the targeting sequence positions 3' immediately to the cleavage site).

(ii) *The sites of the ribozymes crosslinked to tk46S2.* Analyses of the single M1-S2–tk46S2-thio conjugated species (c23) found in the presence of C5 revealed that the crosslinked regions of M1-S2 included positions 138 (J11/12), 168–169 (P12), 292–293 (J15/16), 327–329 (J2/18) and 332–333 (J2/18) (Table 2). In contrast, the crosslinked sites of M1-S2 in the c21 and c22 complexes, both of which were found only in the absence of the protein cofactor, included nt 48–49 (P3), 74–75 (P4), 247–248 (J5/15), 257–258 (J15/16), 285 (P17), 327–329 (J2/18) and 332–333 (J2/18). Thus, in the presence of C5, some of the ribozyme regions that interact with the 5' leader sequence adjacent to the cleavage site were different from those regions of the ribozymes found in the absence of the protein cofactor. In particular, the P12 and J11/12 regions were found to be in close proximity to the substrate in the presence of C5 but not in the absence of the protein cofactor. Meanwhile, the P3, P4 and P17 regions were in close contact

Figure 5. Schematic representation of the nucleotides of M1GS ribozymes that were found to be crosslinked to substrates tk46S2 (A), tk46S3 (B) and tk46S5 (C) by primer extension analyses. The predicted secondary structure of M1 RNA is presented as described previously (4,54). P, helix regions; J, junction regions between two helix sequences; L, loop regions. This is a summary of at least three independent experiments. Only the results that were reproducible are included.

with the substrate in the absence but not the presence of the protein cofactor.

(iii) *The regions of the ribozymes crosslinked to substrate tk46S5.* In the absence of C5, crosslink between M1-S2 and tk46S5-thio yielded two major conjugated species (c51 and c52). The regions of the M1GS ribozyme crosslinked with substrate tk46S5-thio in the absence of C5 included positions 123–124 (P11), 189–190 (P13), 224–226 (J11/14) and 350 (J2/4). However, in the presence of C5, the c52 species accumulated and accounted for >80% of the ribozyme–substrate crosslinked complexes. The crosslink sites of the ribozyme in the c52 complex included positions 189–190 (P13) and 224–226 (J11/14). These results suggest that the presence of C5 favors the interactions of the J11/14 and P13 regions of the ribozyme to the substrate positions in the 3' tail sequence which contained the photoactive groups.

Catalytic activity of the crosslinked complexes

If the ribozyme–substrate complexes were folded into an active conformation during the UV crosslinking analysis, the crosslinked conjugates would be expected to represent the active complexes. The substrates in the conjugates should be cleaved by the enzyme within the same complex when the complexes were incubated under optimal RNase P cleavage conditions (e.g. in the presence of MgCl_2). To determine whether this was the case, 3' end-labeled tk46S2-thio was crosslinked with M1-S2 either in the absence or presence of C5 and the crosslinked complexes were purified. The complexes

were then incubated in the presence of 20 mM MgCl_2 in buffer F to allow cleavage either in the absence or presence of C5. A radiolabeled 3' cleavage product, which does not contain the photoactive group, is expected to be released from the c23 complexes after the cleavage while a non-radiolabeled 5' product containing the photoactive group is expected to be retained with the crosslinked ribozymes. Indeed, a radiolabeled RNA species that comigrated with the 3' product from the cleavage of tk46S2-thio was found in the reactions of the c23 complexes (Fig. 6, compare lanes 3 and 5). In another set of experiments, the 5' end-labeled tk46S5-thio was allowed to crosslink to M1-S2 in the absence and presence of C5. The tk46S2-ribozyme conjugates (e.g. c52) were first purified and then incubated in the presence of 20 mM MgCl_2 in the absence and presence of C5. A radiolabeled 5' cleavage product, which does not contain the photoactive group, is expected to be released from the c52 complexes after the cleavage while a non-radiolabeled 3' product containing the photoactive group is expected to be retained with the crosslinked ribozymes. Indeed, as shown in Figure 6, a radiolabeled RNA species that comigrated with the 5' product from the cleavage of tk46S5-thio was found in the reactions of the c52 complexes (compare lanes 8 and 10). More than 95% of substrates in the c23 (lane 5) and c52 complexes (lane 10) were cleaved after a 1 h incubation. Similar results were also observed for the c21, c22, c31, c32 and c51 complexes. These observations suggested that the majority of the crosslinks occurred in the native structure of the M1GS-TK mRNA complexes and most of the enzymes in the crosslinked complexes could adopt an active conformation.

Figure 6. Catalytic activity of the crosslinked complexes. 3' labeled tk46S2-thio (lanes 1–5) and 5' labeled tk46S5-thio (lanes 6–10) were crosslinked with M1-S2 either in the absence (lanes 6–10) or presence of C5 protein (lanes 1–5). The purified crosslinked conjugates were either first incubated in buffer F in the absence (lane 10) and presence of C5 (lane 5), or directly loaded on denaturing gels (lanes 4 and 9). The cleavage products comigrated with the corresponding products generated from cleavage of tk46S2-thio in the presence of C5 (lane 3) and tk46S5-thio in the absence of the protein cofactor (lane 8). The radiolabeled ribozymes were shown in lanes 1 and 6 while the substrates were shown in lanes 2 and 7. All the samples were purified by phenol/chloroform extraction and ethanol precipitation, then separated in 4% polyacrylamide gels that contained 8 M urea, and finally analyzed by a phosphorimager.

Similar results were also observed when the crosslinked complexes were diluted 100-fold before incubation under the optimal *in vitro* cleavage conditions, supporting the notion that cleavage of the substrates occurred within the crosslinked complexes and was not catalyzed by ribozymes from other adjacent crosslinked complexes (data not shown).

DISCUSSION

In this study, we used a UV crosslinking approach to identify the regions of M1GS RNA that are in close contact with the TK mRNA either in the absence or presence of C5 protein. Crosslinked enzyme–substrate complexes were isolated and the regions of the ribozymes that were potentially crosslinked to the substrates were determined. These crosslinked regions are most likely in close contact with the nucleotide positions of

the substrates that contain the photoactive agent. An alteration of the sites of crosslinks upon binding of the protein cofactor implies a change in the conformation of the ribozyme, which now allows different ribozyme regions to be in close proximity to the substrate.

It is possible that the detected crosslinks are either the result of non-specific binding of the mRNA substrates to the ribozymes or represent minor misfolded inactive conformations that are in rapid equilibrium with the native structure. However, several lines of evidence strongly suggest that these are not the case, and that the detected crosslinks reflect the native structure of the ribozyme–substrate complexes. First, no specific M1GS–substrate crosslinked complexes were found when M1 RNA or M1ICP4 RNA were allowed to react with the TK mRNA substrates, regardless of the absence or presence of C5 (Fig. 3, lanes 4 and 5). M1 and M1ICP4 ribozymes did not contain a guide sequence or contained a guide sequence that targeted HSV-1 ICP4 mRNA, respectively. Therefore, these observations suggest that the crosslinks did not result from the non-specific binding of the substrates to the ribozymes. Second, the crosslinked conjugates, when incubated in the presence of magnesium, still exhibited catalytic activity and >95% of the substrates in the conjugates were cleaved (Fig. 6, lanes 5 and 10). These results suggest that most of the enzymes in the crosslinked complexes still were in a native structure and could adopt an active conformation. Similar results were also observed when the crosslinked complexes were diluted 100-fold before incubation under optimal *in vitro* cleavage conditions, supporting the notion that cleavage of the substrates occurred within the crosslinked complexes and were not catalyzed by ribozymes from other crosslinked complexes (data not shown). Thus, the majority of the crosslinks appeared to occur within the native structure of the M1GS–TK mRNA complexes and represent the interactions between the RNase P ribozyme and the mRNA model substrate in an active ribozyme–substrate complex. Third, the crosslinking results (Table 2, Fig. 5) were obtained from at least three independent experiments and only the reproducible results were included.

Another concern is whether the observed contacts between the ribozyme and the substrate are specific since the experiments were carried out in buffer F (20 mM MgCl₂) instead of buffer E (100 mM MgCl₂) and the reactions in the absence of C5 may be disfavored in the presence of low ionic strength. The rationale to carry out our experiments at low (20 mM MgCl₂) but not high ionic strength (100 mM MgCl₂) is 3-fold. First, tethering of the EGS sequence to M1 RNA obviates the requirement of high ionic strength to achieve optimal activity. In the absence of C5, efficient cleavage of a mRNA substrate (see Fig. 2) (33) or a tethered ptRNA substrate (16,32,45) has been observed in the presence of 10–20 mM MgCl₂. These results suggested that optimal folding of the ribozyme and formation of the active complex between a ribozyme and a tethered substrate can be achieved under low ionic strength conditions. This notion is further supported by our observations that >95% of substrates in the crosslinked complexes were cleaved when the complexes were incubated in buffer F (Fig. 6). These observations suggest that the majority of the crosslinks appeared to occur in the native structure of the M1GS–TK mRNA complexes and most of the enzymes in the crosslinked complexes could adopt an active conformation. Second, the binding affinity of C5 to M1 RNA is weakened in

the presence of 100 mM MgCl₂ (46,47), raising the possibility of whether the contacts between M1 and C5 observed under these conditions represent specific interactions in a native M1–C5 active complex. Third, in the absence of C5, most of the crosslinks observed in the presence of 20 mM MgCl₂ were also found in the presence of 100 mM MgCl₂ (35), further suggesting that the ribozyme–substrate contacts at low ionic strength are similar to those at high ionic strength.

The effect of C5 on the contacts between the ribozyme and the targeting sequence positions adjacent to the cleavage site and the 3' tail sequence positions adjacent to the targeting sequence

The presence of C5 protein did not appear to lead to a change in the ribozyme regions that are in close proximity to the nucleotides immediately 3' to the cleavage site in the substrate (i.e. tk46S3). Substrate tk46S3 crosslinked to the same regions of the ribozyme both in the absence and presence of C5. The crosslinked regions include P1, P4, J2/4, J5/15, J15/16 and J2/18. These regions have been found to be in close proximity to the cleavage site of a ptRNA and a model mRNA substrate (18,35,48,49). Therefore, in both the absence and presence of C5, the regions of the ribozymes that are in close proximity to the nucleotides 3' immediately to the cleavage site of substrate tk46S3 appear to be similar to those that interact with the cleavage site of a ptRNA.

When the ribozymes were crosslinked with tk46S5-thio, the presence of C5 increased the relative yield of one inter-molecular crosslinked complex (i.e. c52) over the others. In c52, the regions of the ribozyme crosslinked to the substrate included P13 and J11/J14 while those in c51 were in P11, P13 and J2/4. Therefore, C5 protein appeared to select or favor a specific structural arrangement within the ribozyme–substrate complex so that the J11/14 region was in close proximity to the 3' tail sequence of the substrate and could possibly interact directly with the substrate. J11/14 has been shown to be one of the regions that potentially interact with the 3' tail sequence of a model mRNA substrate in the presence of high concentrations of divalent ions (high salt conditions) (35,36). However, it is believed that this region is not in close proximity to the active site of the ribozyme or the ptRNA substrate in a ribozyme–ptRNA complex (6,7). Our results suggest that the binding site of the ribozyme to the 5' region of the 3' tail sequence in the presence of C5 includes the P13 and J11/14 regions. One of the possible explanations of why the protein cofactor selectively increased the yield of a specific crosslink complex (i.e. c52) is that the interactions of the crosslinks (e.g. J11/14) within this complex may lead to better substrate recognition and higher cleavage efficiency. Indeed, a ribozyme variant with base substitution mutations at positions 224 and 225 of the J11/14 region exhibited at least 10-fold higher efficiency to cleave a mRNA substrate in the absence of C5 (50). Further studies using ribozyme variants with mutations in these regions will determine whether they exhibit higher cleavage efficiency and provide insight into the construction of highly active ribozymes.

The effect of C5 on the contacts between the ribozyme and the 5' leader sequence positions adjacent to the cleavage site

Fierke and co-workers have recently shown that C5 specifically enhances the interactions between RNase P and the 5' leader

sequence of a ptRNA (19,27,28). However, little is known about whether the 5' leader sequence of a mRNA model substrate makes contact differently with M1 RNA in the presence of C5. Our results suggest that, in the presence of C5, the ribozyme regions that are close to the 5' leader sequence of a mRNA substrate are different from those found in the absence of the protein cofactor. The common crosslinked regions among c21, c22 and c23 included J2/18 and J15/16. In contrast, regions J11/12 and P12 were found to be crosslinked to tk46S2-thio only in the presence of C5 while regions P3, P4, P17 and J5/15 were crosslinked only in the absence of the protein cofactor. P3, P4, P17 and J5/15 are in close proximity to the active site and a ptRNA in the ribozyme–substrate complex, as proposed in the current models for the three-dimensional structure of M1 RNA (6,7). In contrast, J11/12 and P12 are not in close proximity to a ptRNA in the ribozyme–ptRNA complexes (6,7). Thus, our results raise the possibility that the structure of the ribozyme, upon binding of C5, is altered to allow the nucleotides immediately 5' to the cleavage site of substrate tk46S2-thio to crosslink with J11/12 and P12 instead of the P3, P4 and P17 regions. Previous studies have suggested that C5 protein functions to maintain the active structure of the catalytic RNA in the holoenzyme complexes (1,2). Nuclease and Fe(II)-EDTA cleavage analyses indicated that, upon binding of C5, the P3, P4 and P12 regions became less susceptible to nuclease degradation and Fe(II)-EDTA cleavage (40,51,52), suggesting that these regions are either involved in a conformational change or are directly bound by the protein. Recently, C5 protein has been shown to be in close proximity to the P3 and P4 regions of M1 RNA in the holoenzyme complex (53). It is possible that the presence of C5 may physically prevent the formation of the crosslinks between these regions (e.g. P3 and P4) and the substrate by interacting directly with the ribozyme or the substrate. Moreover, binding of C5 protein to the ribozyme may also trigger a conformational change so that J11/12 and P12 regions, but not P3 and P4, are now in close proximity to the 5' leader sequence of the model mRNA substrate.

Comparison of the contacts of the ribozyme with a model mRNA to those with a ptRNA substrate

Our results indicate that the sequences adjacent to the mRNA cleavage site are in close proximity to the same regions of the ribozyme which bind to the sequences close to the cleavage site of a ptRNA both in the presence and absence of C5. Moreover, the regions of the ribozyme (i.e. P4, P1, J5/15, etc.) that bind to the targeting region of the mRNA substrate (i.e. tk46S3) are also a part of the binding domains interacting with the acceptor and T-stem of a ptRNA, the natural equivalent of the RNA helix formed between the targeting region and the guide sequence (6,7). These results are consistent with the notion that the ribozyme utilizes its binding domains for the acceptor and T-stem of a ptRNA to interact with the mRNA substrate (35,36).

Our results also suggest that P11, J2/4, P13 and J11/14 are close to the 3' tail region (i.e. tk46S5). P11 and J2/4 regions are in close proximity to the acceptor and T-stem of a ptRNA (6,7). However, P13 and P11/14 are believed to be not in close proximity to the ptRNA substrate. The interactions between the 3' tail sequence and J11/14 appear to be important for cleavage of a mRNA but not a ptRNA. A ribozyme variant with base substitution mutations at positions 224 and 225 of

the J11/14 region exhibited at least 10-fold higher efficiency in cleaving a mRNA but not a ptRNA substrate (50). Thus, the ribozyme appears to interact with the mRNA substrate by utilizing binding motifs (e.g. P13 and J11/14) different from those used to interact with a ptRNA.

Most of the ribozyme regions (i.e. J15/16 and J2/18) that are found to be close to the 5' leader sequence adjacent to the mRNA cleavage site, both in the absence and presence of C5, are also those that interact with the 5' leader sequence adjacent to the cleavage site of a ptRNA. In contrast, regions J11/12 and P12 were found to be crosslinked to tk46S2-thio only in the presence of C5, while regions P3, P4, P17 and J5/15 were crosslinked only in the absence of the protein cofactor. The latter four regions are in close proximity to the 5' leader sequence adjacent to the cleavage site in the current three-dimensional structure of M1 RNA while J11/12 and P12 are not. Thus, our results suggest that binding of C5 to the ribozyme may trigger a conformational change so that J11/12 and P12 are now in close contact with the 5' leader sequence of the mRNA substrate.

In this study, a cluster of three photoactive groups were incorporated into the substrates in order to identify the nucleotides of the ribozymes that are in close proximity to a particular region (i.e. the three nucleotide positions) of the substrate. To identify the nucleotides crosslinked to a single position of the substrate, crosslinking experiments can be carried out by using substrates that contain a single 4-thio-uridine. Studies of the effects of C5 protein on the interactions between a M1GS ribozyme and a model mRNA substrate provide a model system to understand how the ribozyme functions to cleave a mRNA substrate *in vivo*, in the presence of cellular proteins. While C5 protein does not appear to affect the binding of the ribozyme to the targeting sequence adjacent to the cleavage site, differences in the contacts between the ribozyme and the 5' leader and 3' tail sequence were found in the presence of the protein cofactor. It will be interesting to determine whether these differences will account for the better substrate recognition and the increased cleavage efficiency observed in the presence of the protein cofactor. If this is the case, a ribozyme mutant, which, in the absence of C5, interacts with a mRNA substrate under low salt conditions in a similar way as the wild-type ribozyme-C5 complexes, is expected to exhibit optimal catalytic activity to cleave a mRNA substrate under physiological conditions in the absence of the protein cofactor. Alternatively, ribozyme variants, which, upon binding a cellular protein, exhibit similar interactions to a mRNA substrate as those observed in the presence of C5, may function effectively *in vivo*. Further studies to generate these ribozyme variants and to investigate the mechanism of how they interact with a mRNA substrate will facilitate the construction of ribozymes that exhibit optimal substrate binding and high sequence specificity *in vivo*.

ACKNOWLEDGEMENTS

We are grateful to Dr Venkat Gopalan and Mr Tim Eubank of Ohio State University for kindly providing the C5 protein and helpful discussions. Thanks also go to Phong Trang and Arash Nassi for technical assistance. A.W.H. acknowledges the support of a summer fellowship from University of California at Berkeley. A.F.K. is a recipient of a Russell M. Grossman

Medical Research Fellowship and a President's Graduate Dissertation Fellowship from University of California. K.L. is a Robert and Colleen Haas Undergraduate Scholar at University of California at Berkeley. F.L. is a Pew Scholar in Biomedical Sciences and a recipient of Regent's Junior Faculty Fellowship (University of California) and a Basil O'Connor Award (March of Dimes). The research has been supported by grants from State of California AIDS research program, March of Dimes Foundation, and NIH (GM54875 and AI41927).

REFERENCES

- Altman, S. and Kirsebom, L.A. (1999) In Gesteland, R.F., Cech, T.R. and Atkins, J.F. (eds), *The RNA World*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 351–380.
- Frank, D.N. and Pace, N.R. (1998) *Annu. Rev. Biochem.*, **67**, 153–180.
- Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. and Altman, S. (1983) *Cell*, **35**, 849–857.
- Haas, E.S., Brown, J.W., Pitulle, C. and Pace, N.R. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2527–2531.
- Haas, E.S., Armbruster, D.W., Vucson, B.M., Daniels, C.J. and Brown, J.W. (1996) *Nucleic Acids Res.*, **24**, 1252–1259.
- Chen, J.L., Nolan, J.M., Harris, M.E. and Pace, N.R. (1998) *EMBO J.*, **17**, 1515–1525.
- Massire, C., Jaeger, L. and Westhof, E. (1998) *J. Mol. Biol.*, **279**, 773–793.
- Stams, T., Niranjanakumari, S., Fierke, C.A. and Christianson, D.W. (1998) *Science*, **280**, 752–755.
- Kirsebom, L.A. and Svard, S.G. (1994) *EMBO J.*, **13**, 4870–4876.
- Guerrier-Takada, C., Lumelsky, N. and Altman, S. (1989) *Science*, **246**, 1578–1584.
- Kahle, D., Wehmeyer, U. and Krupp, G. (1990) *EMBO J.*, **9**, 1929–1937.
- Pan, T., Loria, A. and Zhong, K. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 12510–12514.
- Siew, D., Zahler, N.H., Cassano, A.G., Strobel, S.A. and Harris, M.E. (1999) *Biochemistry*, **38**, 1873–1883.
- Burgin, A.B. and Pace, N.R. (1990) *EMBO J.*, **9**, 4111–4118.
- Knap, A.K., Wesolowski, D. and Altman, S. (1990) *Biochimie*, **72**, 779–790.
- Pomeranz Krummel, D.A. and Altman, S. (1999) *RNA*, **5**, 1021–1033.
- Hardt, W.D., Warnecke, J.M., Erdmann, V.A. and Hartmann, R.K. (1995) *EMBO J.*, **14**, 2935–2944.
- Harris, M.E., Nolan, J.M., Malhotra, A., Brown, J.W., Harvey, S.C. and Pace, N.R. (1994) *EMBO J.*, **13**, 3953–3963.
- Kurz, J.C., Niranjanakumari, S. and Fierke, C.A. (1998) *Biochemistry*, **37**, 2393–2400.
- LaGrandeur, T.E., Huttenhofer, A., Noller, H.F. and Pace, N.R. (1994) *EMBO J.*, **13**, 3945–3952.
- Loria, A. and Pan, T. (1997) *Biochemistry*, **36**, 6317–6325.
- Christian, E.L. and Harris, M.E. (1999) *Biochemistry*, **38**, 12629–12638.
- Christian, E.L., McPheeters, D.S. and Harris, M.E. (1998) *Biochemistry*, **37**, 17618–17628.
- Kufel, J. and Kirsebom, L.A. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 6085–6090.
- Liu, F. and Altman, S. (1994) *Cell*, **77**, 1083–1100.
- Gopalan, V., Talbot, S.J. and Altman, S. (1995) In Nagai, K. and Mattaj, J.W. (eds), *RNA-Protein Interactions*. Oxford University Press, Oxford, UK, pp. 103–126.
- Niranjanakumari, S., Stams, T., Crary, S.M., Christianson, D.W. and Fierke, C.A. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 15212–15217.
- Crary, S.M., Niranjanakumari, S. and Fierke, C.A. (1998) *Biochemistry*, **37**, 9409–9416.
- Peck-Miller, K.A. and Altman, S. (1991) *J. Mol. Biol.*, **221**, 1–5.
- McClain, W.H., Guerrier-Takada, C. and Altman, S. (1987) *Science*, **238**, 527–530.
- Forster, A.C. and Altman, S. (1990) *Science*, **249**, 783–786.
- Frank, D., Harris, M. and Pace, N.R. (1994) *Biochemistry*, **33**, 10800–10808.
- Liu, F. and Altman, S. (1995) *Genes Dev.*, **9**, 471–480.
- Liu, F. and Altman, S. (1996) *Nucleic Acids Res.*, **24**, 2690–2696.
- Kilani, A.F. and Liu, F. (1999) *RNA*, **5**, 1235–1247.
- Trang, P., Hsu, A.W. and Liu, F. (1999) *Nucleic Acids Res.*, **27**, 4590–4597.
- Castanotto, D., Rossi, J.J. and Sarver, N. (1994) *Adv. Pharmacol.*, **25**, 289–317.
- Poeschla, E. and Wong-Staal, F. (1994) *Curr. Opin. Oncol.*, **6**, 601–606.
- Rossi, J.J. (1999) *Chem. Biol.*, **6**, R33–R37.
- Kim, J.J., Kilani, A.F., Zhan, X., Altman, S. and Liu, F. (1997) *RNA*, **3**, 613–623.

41. Fedor, M.J. and Uhlenbeck, O.C. (1992) *Biochemistry*, **31**, 12042–12054.
42. Wyatt, J.R., Sontheimer, E.J. and Steitz, J.A. (1992) *Genes Dev.*, **6**, 2542–2553.
43. Favre, A. (1990) In Morrison, H. (ed.), *Bioorganic Photochemistry: Photochemistry and Nucleic Acids*. John Wiley and Sons, New York, NY, pp. 379–425.
44. Smith, D., Burgin, A.B., Haas, E.S. and Pace, N.R. (1992) *J. Biol. Chem.*, **267**, 2429–2436.
45. Kikuchi, Y., Sasaki-Tozawa, N. and Suzuki, K. (1993) *Nucleic Acids Res.*, **21**, 4685–4689.
46. Talbot, S.J. and Altman, S. (1994) *Biochemistry*, **33**, 1406–1411.
47. Talbot, S.J. and Altman, S. (1994) *Biochemistry*, **33**, 1399–1405.
48. Kazakov, S. and Altman, S. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9193–9197.
49. Kufel, J. and Kirsebom, L.A. (1998) *RNA*, **4**, 777–788.
50. Kilani, A.F., Trang, P., Jo, S., Hsu, A., Kim, J., Nepomuceno, E., Liou, K. and Liu, F. (2000) *J. Biol. Chem.*, **275**, 10611–10622.
51. Loria, A., Niranjanakumari, S., Fierke, C.A. and Pan, T. (1998) *Biochemistry*, **37**, 15466–15473.
52. Vioque, A., Arnez, J. and Altman, S. (1988) *J. Mol. Biol.*, **202**, 835–848.
53. Biswas, R., Ledman, D.W., Fox, R.O., Altman, S. and Gopalan, V. (2000) *J. Mol. Biol.*, **296**, 19–31.
54. Haas, E.S. and Brown, J.W. (1998) *Nucleic Acids Res.*, **26**, 4093–4099.