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UV cross-link mapping of the substrate-binding site of an RNase P ribozyme to a target mRNA sequence

AHMED F. KILANI[1] and FENYONG LIU[1,2]

[1]Program in Infectious Diseases and Immunity, University of California, Berkeley, California 94720, USA
[2]Program in Comparative Biochemistry, University of California, Berkeley, California 94720, USA

ABSTRACT

RNase P ribozyme cleaves an RNA helix that resembles the acceptor stem and T-stem structure of its natural ptRNA substrate. When covalently linked with a guide sequence, the ribozyme can function as a sequence-specific endonuclease and cleave any target RNA sequences that base pair with the guide sequence. Using a site-directed ultraviolet (UV) cross-linking approach, we have mapped the regions of the ribozyme that are in close proximity to a substrate that contains the mRNA sequence encoding thymidine kinase of human herpes simplex virus 1. Our data suggest that the cleavage site of the mRNA substrate is positioned at the same regions of the ribozyme that bind to the cleavage site of a ptRNA. The mRNA-binding domains include regions that interact with the acceptor stem and T-stem and in addition, regions that are unique and not in close contact with a ptRNA. Identification of the mRNA-binding site provides a foundation to study how RNase P ribozymes achieve their sequence specificity and facilitates the development of gene-targeting ribozymes.

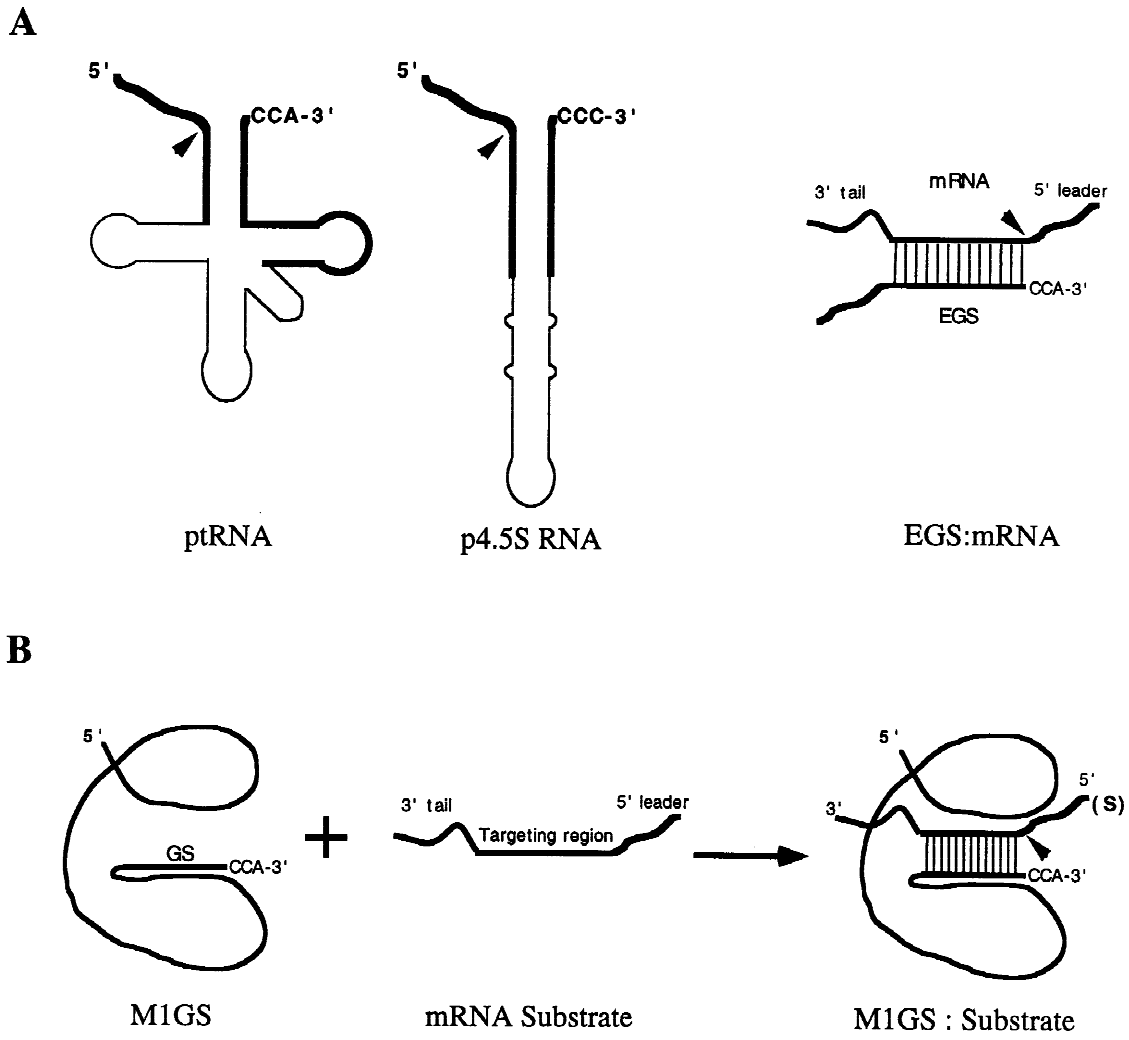
Keywords: gene targeting; ribozyme; RNase P; UV cross-linking

INTRODUCTION

RNase P is a ribonucleoprotein complex responsible for the 5′ maturation of tRNAs (Frank & Pace, 1998; Altman & Kirsebom, 1999). It catalyzes a hydrolysis reaction to remove a 5′ leader sequence from tRNA precursors (ptRNA) and several small RNAs (e.g., p4.5S RNA) (Fig. 1A). In *Escherichia coli*, RNase P consists of a catalytic RNA subunit (M1 RNA) and a protein subunit (C5 protein) (Frank & Pace, 1998; Altman & Kirsebom, 1999). In the presence of a high concentration of salt, such as 100 mM Mg^{2+}, M1 RNA acts as a catalyst and cleaves pre-tRNAs in vitro in the absence of C5 protein (Guerrier-Takada et al., 1983; Gopalan et al., 1995). The addition of C5 protein dramatically increases the rate of cleavage by M1 RNA in vitro and is required for RNase P activity and cell viability in vivo (Gopalan et al., 1995). Extensive studies have established models for the secondary and three-dimensional structures of RNase P catalytic RNAs (Haas et al., 1994; Chen et al., 1998; Massire et al., 1998). These models provide a framework to identify the putative active site and substrate-binding site, and to study the catalytic mechanism of RNase P catalytic RNAs.

The natural substrates for RNase P in *E. coli* include ptRNAs and the precursor to 4.5S RNA, which is required for protein translocation (Bothwell et al., 1976; Miller et al., 1994) (Fig. 1A). RNase P has also been shown to be involved in the processing of 10Sa RNA and several other RNAs (Alifano et al., 1994; Komine et al., 1994; Hartmann et al., 1995). All of these substrates can fold into a structure equivalent to the top portion of a ptRNA molecule (i.e., a 5′ leader sequence, an acceptor stem-like structure, and a 3′ CCA sequence) (Fig. 1A). M1 RNA and RNase P recognize these substrates in a structure-specific rather than sequence-specific manner. Systematic deletion analyses of a pre-tRNA molecule were carried out to determine the minimal requirements for substrate recognition by M1 RNA and RNase P (McClain et al., 1987; Forster & Altman, 1990; Li et al., 1992). These studies have revealed that a small model substrate that contains a structure equivalent to the acceptor stem, the T-stem, the 3′ CCA sequence, and the 5′ leader sequence of a

Reprint requests to: Dr. Fenyong Liu, School of Public Health, 140 Warren Hall, University of California, Berkeley, California 94720, USA; e-mail: liu_fy@uclink4.berkeley.edu.



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Substrate	5' leader	Targeting region	3' tail
tk46S1	5'-GACCCCUGCCAUCAACACGC	GUCUGCGUUCGAC	CAGGCUGCGCGGU-3'
tk46S2	5'-GACCCCCGCCACCAACAUUU	GCCCGCGCCCGAC	CAGGCCGCGGA-3'
tk46S3	5'-GACCCCCGCCACCAACACCC	GUUUGCGCCCGAC	CAGGCCGCGGA-3'
tk46S4	5'-GACCCCCGCCACCAACACCC	GCCCGCGCCCUUU	CAGGCCGCGGA-3'
tk46S5	5'-GACCCCCGCCACCAACACCC	GCCCGCGCCCGAC	CUUUCCGCGGA-3'

FIGURE 1. **A**: Schematic representation of natural substrates (ptRNA and p4.5S RNA) and a small model substrate (EGS:mRNA) for ribonuclease P and M1 RNA from *E. coli*. The structural components common to both precursor tRNA and 4.5S RNA are highlighted. The site of cleavage by RNase P or M1 RNA is marked with a filled arrow. **B**: Schematic diagram of an M1GS RNA construct to which a target RNA (S) has hybridized. **C**: 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. In **A** and **B** the stem structure formed between the target RNA and either the EGS RNA or M1GS RNA is shown as 13 bp; it can be varied from 5 to 19 bp as described previously (Liu & Altman, 1995).

ptRNA molecule can be cleaved efficiently by M1 RNA (Fig. 1A, boldface regions). This was consistent with the results of ultraviolet (UV) cross-linking (Guerrier-Takada et al., 1989; Burgin & Pace, 1990; Harris et al., 1997; Chen et al., 1998), chemical footprinting, and interference experiments (Kahle et al., 1990; Knap et al., 1990; LaGrandeur et al., 1994), which indicated that RNase P interacts with the acceptor stem, T-stem, T-loop, and 3′ CCA sequence. The binding site of M1 RNA to the 3′ CCA sequence has also been determined (Kirsebom & Svard, 1994; Oh & Pace, 1994). More recently, nucleotides G_{332} and A_{333} have been found to be in close proximity to the nucleotide immediately 5′ to the cleavage site in the leader sequence of a pre-tRNA (Kufel & Kirsebom, 1996; Christian et al., 1998).

Studies on substrate recognition by M1 RNA and RNase P have also led to the development of a general strategy in which M1 RNA and RNase P can be used as gene-targeting tools to cleave any specific mRNA sequences. In the small model substrate of M1 RNA (Fig. 1A, boldface regions), the 5′ proximal sequence (the 5′ leader and 5′ half of acceptor stem sequence) base pairs to the 3′ half of sequence (the 3′ proximal acceptor stem sequence) (Forster & Altman, 1990). This 3′ proximal sequence, called an external guide sequence (EGS), can base pair with the targeted sequence and guide M1 RNA to cleave the substrate (Fig. 1A). Thus, any RNA sequence can be targeted for cleavage by M1 RNA and RNase P, provided that an EGS is designed to hybridize with the target RNAs (Fig. 1A) (Forster & Altman, 1990). Accordingly, M1 RNA can be converted into a sequence-specific ribozyme, M1GS RNA, by linking the ribozyme covalently to a guide sequence (GS) (Fig. 1B) (Frank et al., 1994; Liu & Altman, 1995). More recently, we have shown that M1GS RNA can cleave the mRNA encoding thymidine kinase (TK) of human herpes simplex virus (HSV-1) both in vitro and in tissue culture cells infected with HSV-1 (Liu & Altman, 1995).

RNA enzymes are being developed as promising gene-targeting reagents to cleave specifically RNA sequences of choice (Sarver et al., 1990; Yu et al., 1993). Targeted cleavage of mRNA by RNase P ribozyme provides a unique approach to inactivate any RNA of known sequence expressed in vivo. However, further studies to investigate the mechanism of how an M1GS RNA cleaves an mRNA substrate are necessary in order to improve the catalytic efficiency and sequence specificity of the ribozyme. In this study, we have determined the regions of M1GS RNA that are in close contact with the TK mRNA substrate by site-specific UV cross-link mapping. Identification of the mRNA binding site of the ribozyme will serve as the starting point to delineate the mechanism of how M1GS RNA recognizes its target substrates and achieves its sequence specificity.

RESULTS

Cleavage of the TK mRNA substrates that contain photoactive groups by RNase P ribozymes

Substrate tk46S1 contains a 5′ TK mRNA sequence of 46 nt that has been shown to be accessible to ribozyme binding in mammalian cell cultures (Fig. 1C) (Liu & Altman, 1995). This substrate consists of three sequence elements: (1) a 5′ leader sequence of 20 nt, (2) a targeting sequence of 13 nt that base pair with the GS of an M1GS ribozyme, and (3) a 3′ tail sequence of 13 nt (Figs. 1B and 1C). Accordingly, the regions of the M1GS ribozyme (designated as M1-S1 RNA) that bind to tk46S1 can be divided into three parts that interact specifically with each one of these three elements. These regions can be mapped by UV cross-linking studies with tk46S1 that is incorporated with a photoactive group. 4-thio-uridine, which has been extensively used in UV cross-linking studies to investigate RNA–RNA and RNA–protein interactions (Favre, 1990; Wyatt et al., 1992), was used as the photoactive agent. Upon exposure to UV light, this photoactive agent can cross-link to the nearby regions within a distance of 5–10 Å (Favre, 1990; Wyatt et al., 1992). The RNA substrate containing 4-thio-uridine (designated as tk46S1-thio) was synthesized in vitro by T7 RNA polymerase in the presence of 4-thio-uridine triphosphate (4-thio-UTP) and the photoactive group was incorporated at every one of the eight uridine positions of the tk46S1 sequence (Fig. 1C). These positions are located at the 5′ leader sequence, targeting sequence, and 3′ tail sequence. Mapping of the regions of M1-S1 ribozyme cross-linked with substrate tk46S1-thio should reveal the entire mRNA binding site of the ribozyme.

To identify the regions of M1GS RNAs that interact with a particular part of tk46S1 (e.g., the 5′ leader sequence), the photoactive groups were also incorporated into only a specific region of the substrate (Fig. 1C). Substrates tk46S2, tk46S3, tk46S4, and tk46S5 were constructed to contain three uridines at the positions 5′ and 3′ adjacent to the cleavage site, and 5′ and 3′ adjacent to the 3′ end of the targeting sequence, respectively (Fig. 1C). RNase P RNAs from *E. coli* (M1 RNA) and from *Bacillus subtilis* (P RNA) were used to construct mRNA-targeting ribozymes, M1GS and PGS RNA, by linking the 3′ termini of these catalytic RNAs with a guide sequence. Different guide sequences were used to construct M1GS and PGS RNAs (e.g., M1-S1, M1-S2, etc.) to target the corresponding substrates (i.e., tk46S1, tk46S2, etc.). Because substrates tk46S2 and tk46S5 have the same targeting sequence (Fig. 1C), ribozyme M1-S2 that targeted tk46S2 was also used for tk46S5. Among all known bacterial RNase P RNAs, the *B. subtilis* RNase P RNA has the most disparate secondary structure

compared with M1 RNA from *E. coli* (Haas et al., 1994). UV cross-linking studies with both M1GS and PGS RNAs should reveal whether the mRNA binding domains are highly conserved among all RNase P RNAs.

Cleavage of substrates tk46S1, tk46S2, tk46S3, tk46S4, and tk46S5 by the M1GS and PGS ribozymes with the corresponding guide sequences was carried out (Fig. 2; data not shown). As expected, cleavage of tk46S1 by M1-S1 ribozyme yielded a 5′ product of 20 nt and a 3′ product of 26 nt, respectively (Fig. 2) (Liu & Altman, 1995). The cleavage of tk46S3 and tk46S5 by both M1GS and PGS RNAs also yielded two products that comigrated with those from cleavage of tk46S1 (Fig. 2, lanes 5–6, 8–9). Further characterization of the cleavage products indicated that the cleavage of these two substrates occurs at the same location as that of tk46S1 (data not shown). Kinetic analyses were also carried out under single-turnover conditions to determine the cleavage rate of these substrates by the ribozymes. The catalytic efficiencies, indicated as the values of k_{cat}/K_m (average of three experiments), were within twofold difference regardless of which substrates were used and whether the substrate contained the photoactive group or not (Table 1). Thus, neither difference in their primary nucleotide sequences nor the presence of the photoactive group significantly affected the cleavage of these substrates by M1GS or PGS RNAs.

TABLE 1. Kinetic parameters of the cleavage reactions governed by different M1GS or PGS RNAs under single-turnover conditions.

		k_{cat}/K_m ($\mu M^{-1}min^{-1}$)	
Substrate	4-thio-U	M1GS	PGS
tk46S1	−	0.30 ± 0.05	0.31 ± 0.05
	+	0.30 ± 0.05	0.23 ± 0.05
tk46S2	−	0.35 ± 0.05	0.18 ± 0.04
	+	0.23 ± 0.04	0.16 ± 0.04
tk46S3	−	0.26 ± 0.04	0.24 ± 0.04
	+	0.25 ± 0.04	0.16 ± 0.03
tk46S4	−	0.39 ± 0.05	0.19 ± 0.04
	+	0.24 ± 0.04	0.19 ± 0.04
tk46S5	−	0.38 ± 0.05	0.30 ± 0.05
	+	0.35 ± 0.05	0.25 ± 0.05

Cleavage assays were performed in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) at 50 °C. The values were the average of three experiments and exhibited a variation of less than 25%.

The regions of ribozymes cross-linked with substrate tk46S1

Ribozymes M1-S1 and P-S1 RNA were incubated with substrate tk46S1-thio that was uniformly labeled with [^{32}P]-GTP. The reaction mixtures were then irradiated with UV light at 365 nm for different periods of time. The cross-linked products were separated in denaturing polyacrylamide gels (Fig. 3). Two major clusters (designated as c1 and c2) and a minor cluster (designated as c3) of cross-linking species were found (Fig. 3, lane 1). The major and minor species accounted for about 5–20% and 2% of the total amount of substrates used in the experiments, respectively. These species were not found in control experiments that were not treated with UV irradiation (Fig. 3, lanes 2 and 4) or that were carried out with substrate tk46S1 that did not contain the photoactive group (Fig. 3, lane 3). Cross-links c2 and c3 were found when the substrate tk46S1-thio was irradiated in the absence of the ribozyme, indicating that these species were derived from cross-linking of the substrate (Fig. 3, lane 5). In contrast, cross-link c1 was observed only when the substrate was irradiated in the presence of ribozyme M1-S1 (Fig. 3, lane 1). This complex was not observed when M1-S1 was replaced with either M1 RNA that does not contain a guide sequence or M1ICP4, which targets another HSV-1 mRNA, the ICP4 mRNA (Fig. 3, lanes 6 and 7). These results indicated that cross-link c1 was 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 nonspecific binding of the substrate to other regions of the ribozyme.

It is possible that the c1 conjugates were derived from cross-links either between tk46S1 and its bound M1-S1 (*cis*-cross-link) or the ribozyme in another enzyme–substrate complex (*trans*-cross-link). To exclude the latter possibility, it is necessary to demonstrate that substrate cleavage takes place within the active ribozyme–substrate complex and is not due to *trans* cleavage by other ribozyme–substrate complexes.

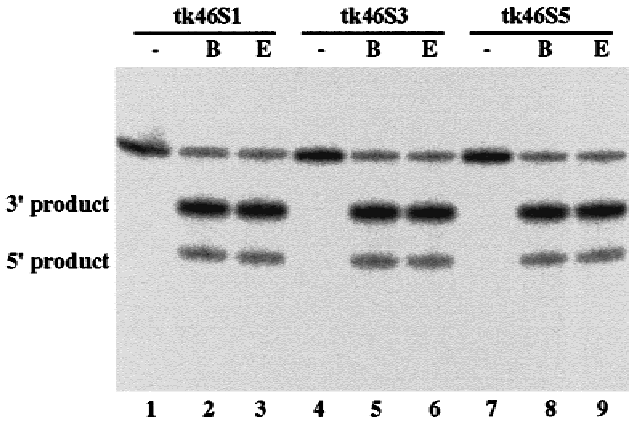

FIGURE 2. Cleavage of mRNA substrates by M1GS and PGS RNAs. A dashed line indicates that ribozymes were not included in the incubation mixture. Substrates (10 nM) were either incubated alone (lanes 1, 4, and 7) or with 5–30 nM of M1GS (E, lanes 3, 6, and 9) or PGS (B, lanes 2, 5, and 8) ribozymes. Cleavage reactions were carried out for 30 min in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) in the presence of tk46S1 (lanes 1–3), tk46S3 (lanes 4–6), and tk46S5 (lanes 7–9), respectively. Cleavage products were separated in 15% polyacrylamide gels containing 8 M urea.

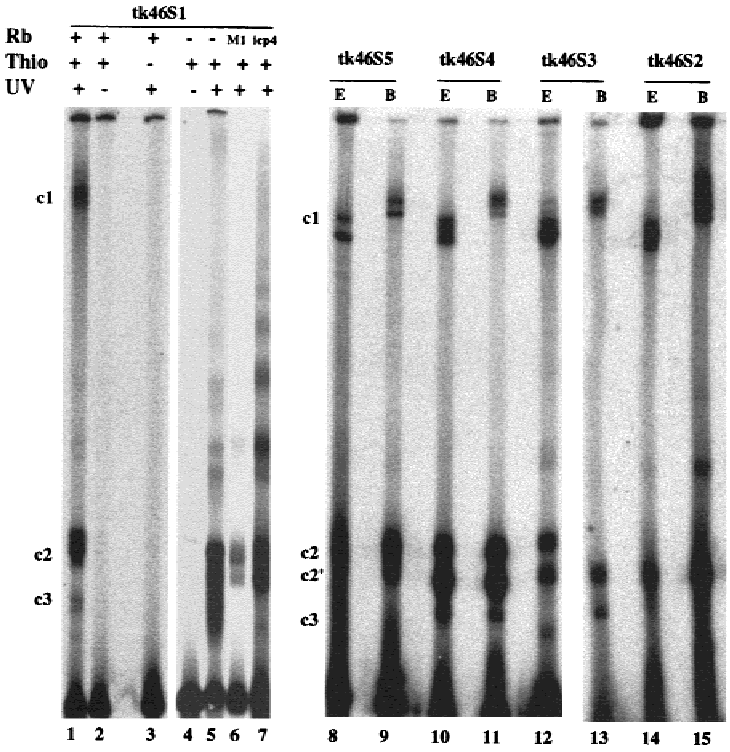

FIGURE 3. Autoradiograph of the cross-linked products between the substrates and ribozymes. Radiolabeled tk46S1 was incubated alone (lanes 4 and 5), with M1-S1 RNA (lanes 1–3), M1 RNA (M1, lane 6), or M1ICP4 RNA (icp4, lane 7) in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$). Then, the reaction mixtures were loaded on denaturing gels directly (lanes 2 and 4) or after UV irradiation (lanes 1, 3, 5, 6, and 7). The substrate used in lane 3 did not contain the photoactive group. Radiolabeled substrates (5–100 nM) that contained thio-U were cross-linked with 5–100 nM of either M1GS (E, lanes 8, 10, 12, and 14) or PGS ribozymes (B, lanes 9, 11, 13, and 15). All the cross-linked conjugates were separated in 4% polyacrylamide gels that contained 8 M urea. The letters at the side of the photographs specify the different cross-linked species.

To investigate whether this is the case, we have determined the effect of dilution upon the apparent reaction rate constant, k_{app}, for the ribozyme–substrate complexes. In a *cis*-cleavage reaction, k_{app} is expected to be insensitive to dilution of the reactants (M1GS-tk46 complexes). Equimolar amounts of M1-S1 and tk46S1 were mixed and the M1GS-tk46S1 complexes were separated from the unbound substrates using G-50 Sephadex gel filtration columns. The complexes were first diluted in different concentrations and then further incubated to allow cleavage. The apparent rate constant, k_{app}, for M1GS-tk46S1 complexes is independent of the concentrations of the complexes within the range of 2–70 nM tested (Table 2). These observations suggest that substrate cleavage takes place predominantly in the bound complexes (*cis*-cleavage) rather than being catalyzed by ribozymes from other complexes (*trans*-cleavage). This suggestion is consistent with our observation that the amount of c1 complex was similar between reaction mixtures that were not diluted and those that were diluted 100-fold before UV irradiation (data not shown). Less than 5% of the substrates remained uncleaved after a 160-min incubation (data not shown), suggesting that 95% of the enzyme–substrate complexes can be folded into the active conformation and no more than 5% of complexes are in a nonactive (possibly misfolded) conformation.

TABLE 2. The apparent rate constants (k_{app}) of the ribozyme–substrate complexes at different concentrations.

Concentration (nM)	k_{app} (min^{-1})
70	0.59 ± 0.11
48	0.51 ± 0.09
12	0.41 ± 0.12
6	0.56 ± 0.09
2	0.52 ± 0.10

The ribozyme–tk46S1 complexes were separated from the unbound substrates by G-50 Sephadex gel filtration columns and diluted in different concentrations before the rate of cleavage was assayed. The cleavage products were separated in 15% polyacrylamide gels and quantitated with a STORM840 phosphorimager.

To determine the sites of the ribozymes cross-linked with tk46S1-thio, all three clusters of the cross-linked species were 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 and P RNA as the primers. Reverse transcriptase terminates 1 nt 3′ to cross-link sites in the RNA template (Favre, 1990; Wyatt et al., 1992). Four oligonucleotides (AK1–AK4 and AK10–AK13) were used in experiments with M1GS and PGS RNA, respectively, to cover the full-length ribozyme sequences. An example of the primer-extension experiments for cross-link product c1 is shown in Figure 4. Comparison of the extension products obtained from cross-linked RNA species (Fig. 4, lane 6) with those from the control RNA conjugates (Fig. 4, lane 5) and sequencing reactions (Fig. 4, lanes 1–4) identifies the individual cross-linked

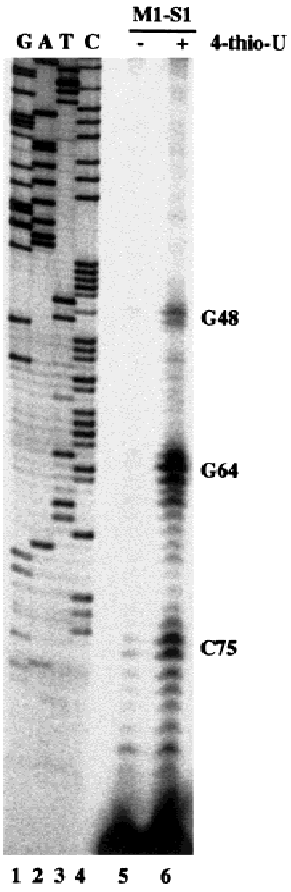

FIGURE 4. Identification of the nucleotides of the ribozymes that were cross-linked with the substrate by primer-extension analyses. UV cross-linking was carried out with the reaction mixture that contained substrate tk46S1 and ribozyme M1-S1. Primer-extension experiments with radiolabeled primer AK1(85–102) were carried out using RNA samples generated in the presence of thio-U-containing substrates (+, lane 6). In lane 5, the ribozyme was UV irradiated in the presence of the substrate that did not contain the photoactive group and the RNA mixtures were purified by ethanol precipitation and directly subjected to primer extension analyses. Sequencing ladder (lanes 1–4) was generated with T7 sequenase kit using pFL117 as the template and AK1(85–102) as the sequencing primer.

nucleotides in the ribozyme. 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 cross-link species in the control experiments were found to comigrate with c1 (Fig. 3). The RNA mixtures were purified by ethanol precipitation and directly subjected to primer-extension analyses. The cross-linking sites are listed in Table 3 and shown in Figure 5A (for M1GS RNA), and Figure 5B (for PGS RNA). No primer extension products were detected when using c2 and c3 as the templates regardless of which primers were used (data not shown), consistent with our conclusion that these two conjugates do not contain ribozyme sequences and are probably the cross-linking products of the substrate (Fig. 3, lane 5).

The regions of ribozymes cross-linked with RNA substrates that contain the photoactive groups at different locations

To map the regions of the ribozymes that specifically interact with the 5′ leader sequence, targeting region, and the 3′ tail sequence, substrates tk46S2, tk46S3, tk46S4, and tk46S5 (Fig. 1C) were incubated with the M1GS and PGS ribozymes. The reaction mixtures were UV irradiated and the cross-linked products were separated in denaturing polyacrylamide gels (Fig. 3, lanes 8–15). Similarly, two to three major (c1, c2, and c2′) and a minor cluster (i.e., c3) of cross-linking species were observed (Fig. 3, lanes 8–15). The major and minor cross-linked conjugates accounted for about 5–20% and 2% of the total amount of substrates used in the experiments, respectively. The cross-linked species were excised from the gels and purified. Primer extension experiments were carried out using these RNA species as the templates. The cross-linking sites in the ribozymes determined by these experiments are listed in Table 3 and shown in Figure 5C (M1GS RNA) and Figure 5D (PGS RNA). The salient features of these results are as follows.

(1) Regions of the ribozymes cross-linked to the nucleotides close to the cleavage site

The regions of ribozymes cross-linked to both substrates tk46S2 and tk46S3 include positions 246–248 (domain J5/15), 327–329 (J2/18), and 255–258 (J15/16) of M1 RNA (or the homologous regions of P RNA) (Table 3). (See Fig. 5 for the nomenclature and location of the domains.) Moreover, the amounts of the cross-linked products at the J2/18 domain decreased and those at J5/15 and J15/16 increased when the RNA substrate used in the experiments was changed from tk46S2 to tk46S3 (Table 3). The J5/15 and J2/18 regions have been shown to cross-link to the +1 position of a tRNA whereas the J15/16 domain consists of the binding site to the CCA sequence of a ptRNA (Burgin & Pace, 1990; Kirsebom & Svard, 1994; LaGrandeur et al., 1994). These results indicated that the cleavage site of the mRNA substrate is recognized by the same regions of the ribozymes that interact with the cleavage site of a ptRNA.

The regions of M1GS ribozymes cross-linked with substrate tk46S3 but not tk46S2 included positions 4–10 (P1), 69–71 (P4), and 345 (J2/4). The regions of M1GS RNA cross-linked to tk46S2 but not tk46S3 included positions 45–53 (P3), 164–172 (P12), 179–188 (J12/13 and P13), and 280–285 (P17). Therefore, these regions at P3, P12/P13, and P17 are in close contact with the 5′ leader sequence adjacent to the cleavage site. This conclusion is further supported by the results that the domains of PGS RNA homologous to the corresponding M1 RNA regions were also

TABLE 3. Analyses of the cross-linked products formed between the substrates and ribozymes.

Substrate	Positions of thio-U	Cross-linking sites	
		E. coli RNase P RNA	*B. subtilis* RNase P RNA
tk46S 1	7, 12, 22, 24, 28, 29, 39, 46	47–49 (P3), 63–70, 73–77 (P4), 169, 173 (P12), 190, 193, 197, 202 (P13), 204–207 (P14), 230 (J11/14), 234–235 (P11), 237–242 (P7), 246–250 (J5/15), 252–253 (J15/16), 261–262 (P16), 281–282, 284 (P17), 315 (P18), 329–331 (J2/18), 336 (P2), 355 (P4)	6 (P1), 25–26 (P3), 48, 51, 53–54, 56 (P4), 63 (P5.1), 90 (P7), 128 (P9), 137, 148–149, 154, 156 (P10.1), 204 (P12), 255, 260 (L15), 279, 288 (L15.1), 308 (P15.2), 319–320 (J2/15.2), 331, 337, 341, 349–351 (P19), 370–371, 374 (J2/4)
tk46S2	18, 19, 20	45-53 (P3), 74–75 (P4), 164–172 (P12), 179–180 (J12/13), 280–285 (P17), 327–329 (J2/18), 181–188* (J12/13), 246–248* (J5/15), 257–258* (J15/16)	28–30, 33–37 (P3), 56–57 (P4), 152–154 (P10.1a), 169–171 (L10.1), 199–201 (P12), 318–319 (J2/15.2), 165–166* (L10), 245–246* (J5/15), 260–261* (L15), 306–308* (P15.2)
tk46S3	22, 23, 24	4–10 (P1), 69–71 (P4), 246–248 (J5/15), 255-256 (J15/16), 345 (J2/4), 327–329* (J2/18)	9–15 (P1), 51–55 (P4), 245–246 (J5/15), 257–258 (L15), 113* (P9), 318–319* (J2/15.2)
tk46S4	31, 32, 33	123–124 (P11), 224–227 (J11/14), 348* (J2/4), 63–64* (P4)	220–223 (J11/12), 233-235 (P10/P11), 47–48* (P4)
tk46S5	35, 36, 37	189–193 (P13), 224–227 (J11/14), 183–184* (J12/13), 350* (J2/4)	137–139 (P10.1), 141–143 (L10.1), 159–165 (P10.1a), 220–223 (J11/12), 308–312* (P15.2)

The cross-linking reactions were carried out in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$). The numbers and letters in parenthesis represent the nucleotide positions and domains of the ribozymes, respectively (Chen et al., 1998; Massire et al., 1998) (Fig. 5). P represents the helix regions, J represents the junction regions between two helix sequences, and L represents the loop regions (Haas et al., 1994; Chen et al., 1998; Massire et al., 1998).

*Weak cross-link.

found to be cross-linked to these substrates (Table 3 and Fig. 5). For example, P3, P12, and P10.1a/P10.1 of P RNA, which have been proposed to be homologous to P3, P12, and P13/P14 of M1 RNA, respectively (Chen et al., 1998; Massire et al., 1998), were found to be cross-linked with substrate tk46S2 but not tk46S3 (Fig. 5).

(2) Regions of the ribozymes cross-linked to the 3′ terminus of the targeting region

The regions of the M1GS ribozyme cross-linked with substrate tk46S4 included positions 63–64 (P4), 123–124 (P11), and 224–227 (J11/14), whereas those of PGS RNA were positions 47–48 (P4), 233–235 (P10/P11), and 220–223 (J11/12). J11/12 of P RNA is the structure homologous to J11/14 of M1 RNA (Haas et al., 1994; Chen et al., 1998; Massire et al., 1998). The regions of M1GS ribozymes cross-linked to substrate tk46S5 included positions 183–184 (J12/13), 189–193 (P13), and 224–227 (J11/14), whereas those of PGS ribozymes were primarily located at P10.1a/P10.1 and J11/12. Regions P10.1a/P10.1 have been proposed to be the homologous structures of P13/P14 (Loria & Pan, 1996; Massire et al., 1998).

The regions of the ribozymes that cross-linked to both tk46S3 and tk46S4 included the P4 domain whereas those cross-linked to both tk46S4 and tk46S5 were in J11/14 of M1 RNA (or J11/12 of P RNA). The amounts of cross-linked products at P4 and P1 decreased and those at P10/P11 increased when the photoactive agents moved from the 5′ region (in tk46S3) to the 3′ region of the targeting sequence (in tk46S4) (Table 3). These results suggested that the 5′ and 3′ regions of the targeting sequence are in close contact with P4 and P1, and with P10/P11 and J11/14, respectively. Similar amounts of cross-linked products at J11/14 were observed in experiments with tk46S4 and tk46S5, indicating that this region is in close contact to the junction region between the targeting sequence and the 3′ tail sequence. P13 and J12/13 of M1 RNA (P10.1a/P10.1 of P RNA) were only found to be cross-linked to tk46S5 but not tk46S4, suggesting that these regions are in close contact with the 3′ tail sequence.

Catalytic activity of the cross-linked complexes between M1GS ribozymes and the mRNA substrates

If the substrates were cross-linked to the active site of the ribozymes, they should be cleaved by the enzymes in the complexes upon incubation under optimal RNase P cleavage conditions (e.g., 100 mM $MgCl_2$). To determine whether this was the case, 5′ end-labeled tk46S5

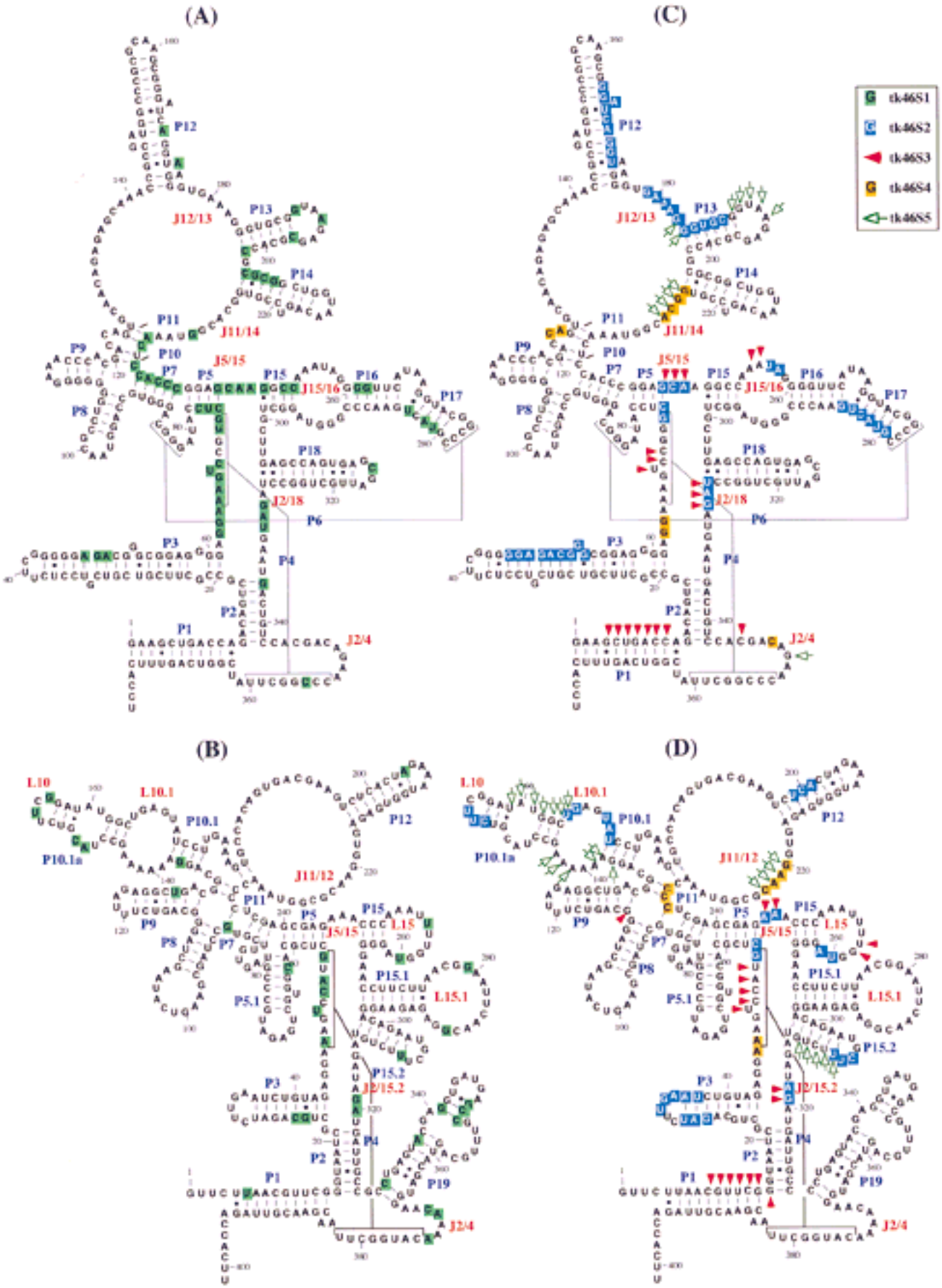

FIGURE 5. (*Legend on facing page.*)

was cross-linked with M1-S2. The cross-linked products were purified and then incubated for 2 h in the presence of 100 mM $MgCl_2$ to allow cleavage to proceed. The cleavage products were separated in denaturing gels (Fig. 6). The 5′ cleavage product of tk46S5 was released from the complex whereas the 3′ product, which included the photoactive groups, was retained in the complex (Fig. 6, lanes 1–5). In another set of experiments, the 3′ end-labeled tk46S2 was allowed to cross-link to M1-S2 and the tk46S2-ribozyme conjugates were first purified and then incubated in the presence of 100 mM $MgCl_2$. As shown in Figure 6, the 3′ cleavage product of tk46S2 that did not contain the photoactive groups was released (lanes 5–10). More than 95% of substrates in these complexes were cleaved after a 2-h incubation (Fig. 6, lanes 5 and 9), suggesting that the majority of the substrates were cross-linked to the active conformation of the ribozymes. Similar results were also observed when the cross-linked complexes were diluted 100-fold before incubation under the in vitro optimal cleavage conditions, supporting the notions that the cleavage of the substrates occurred within the cross-linked complexes and was not catalyzed by ribozymes from other adjacent cross-linked complexes (data not shown).

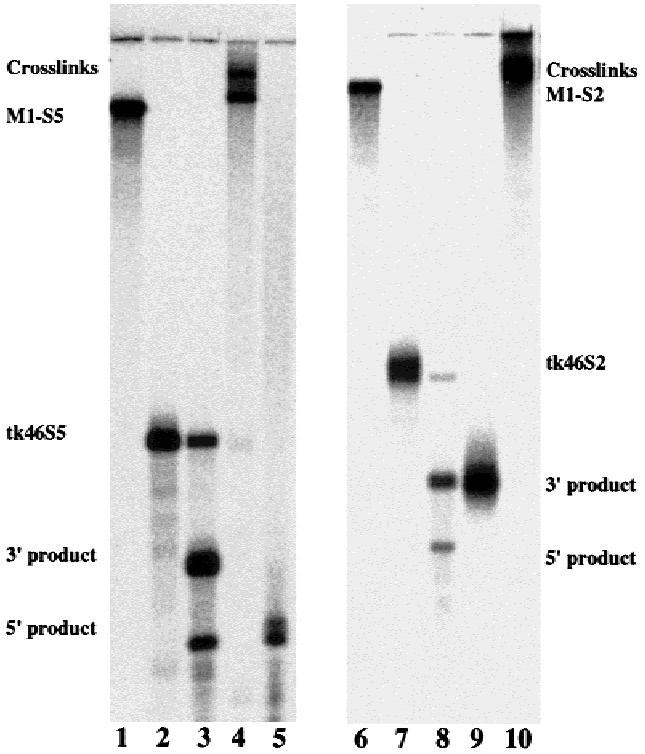

FIGURE 6. Catalytic activity of the cross-linked complexes. 5′-labeled tk46S5 (lanes 1–5) and 3′-labeled tk46S2 (lanes 6–10) were cross-linked with M1-S2. The purified cross-linked conjugates were either first incubated in buffer A (lanes 5 and 9) or directly loaded on denaturing gels (lanes 4 and 10). The cleavage products comigrated with the corresponding products generated from cleavage of tk46S5 and tk46S2 (lanes 3 and 8). The radiolabeled ribozymes are shown in lanes 1 and 6 and the substrates are shown in lanes 2 and 7.

DISCUSSION

RNA enzymes are being developed as promising gene-targeting reagents to cleave specifically RNA sequences of choice (Castanotto et al., 1994; Poeschla & Wong-Staal, 1994). For example, both hammerhead and hairpin ribozymes have been shown to inhibit HIV replication by specifically cleaving viral mRNA sequences in infected cells (Sarver et al., 1990; Yu et al., 1993), and a ribozyme derived from a group I intron has been used to repair mutant mRNAs in cells (Lan et al., 1998). Thus, ribozymes can be used as a tool in both basic and clinical research, such as in studies of tumorigenesis and antiviral gene therapy. In this study, we have mapped the regions of M1GS RNA that are in close contact with the TK mRNA substrate by a site-specific UV cross-linking approach. Active cross-linked enzyme–substrate complexes were isolated and the regions of the ribozymes cross-linked with the substrates were determined.

It is possible that the detected cross-links are either results of nonspecific 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 cases and that the cross-links we detected reflect the active conformation of the ribozymes. First, no specific M1GS–substrate cross-linked complexes were found when M1 RNA or M1ICP4 RNA, which did not contain a guide sequence or contained a guide sequence that targets HSV-1 ICP4 mRNA, respectively, were allowed to react with the TK mRNA substrates (Fig. 3, lanes 6 and 7), suggesting that the cross-links were not results of nonspecific binding of the substrates to the ribozymes. Second, the cross-linked conjugates still exhibited catalytic activity and more than 95% of the substrates in the conjugates were cleaved in the presence of magnesium (Fig. 6,

FIGURE 5. (*facing page*.) Schematic representation of the nucleotides of M1GS (**A** and **C**) and PGS (**B** and **D**) ribozymes that were found to be cross-linked to substrates tk46S1 (**A** and **B**), tk46S2, tk46S3, tk46S4, and tk46S5 (**C** and **D**) by primer-extension analyses. P represents the helix regions, J represents the junction regions between two helix sequences, and L represents the loop regions (Haas et al., 1994; Chen et al., 1998; Massire et al., 1998). This is a summary of several independent experiments. Only the results that were reproducible are included.

lanes 5 and 9), indicating that most of the enzymes in the cross-linked complexes maintained their active conformations. Similar results were also observed when the cross-linked complexes were diluted 100-fold before incubation under optimal cleavage conditions in vitro, supporting the notion that the cleavage of the substrates occurred within the cross-linked complexes and was not catalyzed by ribozymes from other cross-linked complexes (data not shown). Third, when the substrate was allowed to be bound to a ribozyme through base pairing to the guide sequence, the apparent rate of cleavage (k_{app}) of the substrate appeared to be similar over a 35-fold range of concentrations of the enzyme–substrate complex (Table 2). These results suggested that the TK mRNA substrate was cleaved predominantly by the bound ribozymes (*cis*-cleavage) and not by the ribozymes from other complexes (*trans*-cleavage). Therefore, the conjugated species observed are primarily derived from the cross-link between the substrate and its bound ribozyme. Finally, the efficient cross-links found at the homologous positions in both *E. coli* and *B. subtilis* RNase P ribozymes strongly suggest that the cross-linking results represent the general features of the ribozyme–mRNA substrate complex.

The active site of M1GS RNA

The active site of the M1GS ribozyme is defined as the region responsible for catalytic cleavage, and, therefore, should be in close proximity to the cleavage site in an active enzyme–substrate complex. Three regions, positions 246–248 (J5/15), 327–329 (J2/18), and 255–258 (J15/16) of M1 RNA (or the homologous regions of P RNA), were found to be commonly cross-linked to both tk46S2 and tk46S3. The amount of cross-linked products at positions 327–329 with substrate tk46S2 was more than that with substrate tk46S3 whereas fewer cross-links were found at positions 246–248 in the presence of tk46S2 than in the presence of tk46S3. These observations suggested that the 5′ and 3′ adjacent regions of the cleavage site interact with the J2/18 and J5/15 regions (M1 RNA), respectively. These two regions have previously been shown to be cross-linked to the +1 site of a tRNA molecule (Burgin & Pace, 1990). More recently, region J2/18 was found to be in close proximity to the −1 position of a ptRNA (Kufel & Kirsebom, 1996; Christian et al., 1998). Meanwhile, the ribozyme-binding site to the CCA sequence of a ptRNA has been mapped to be at the J15/16 region (e.g., nt 254–255 and 292–294) (Kirsebom & Svard, 1994; LaGrandeur et al., 1994; Oh & Pace, 1994). Therefore, the cleavage site of the TK mRNA substrate is positioned at the same regions of the RNase P ribozyme that are in close contact with the cleavage site of a ptRNA.

The ribozyme binding site to the targeting sequence

The ribozyme sequences cross-linked to the 5′ region of the targeting sequence (i.e., substrate tk46S3) but not the leader sequence (i.e., tk46S2) include positions 69–71 and 345 (P4) and 4–10 (P1) of M1 RNA (or the homologous regions of P RNA). The ribozyme sequences cross-linked to the 3′ region of the targeting sequence (i.e., tk46S4) are primarily at positions 123–124 (P10/P11) and 224–227 (J11/14) of M1 RNA. These results suggest that P4 and P1 constitute the binding site to the 5′ region of the targeting sequence whereas P10/P11 and J11/14 bind to the 3′ region. The positions at P10/P11 have been shown to interact with the T-stem of a tRNA (Nolan et al., 1993). However, little is known about the regions of the ribozyme that interact with the 5′ half of the acceptor stem of a ptRNA, the natural equivalent of the 5′ targeting sequence of the TK mRNA substrate (Burgin & Pace, 1990; Kufel & Kirsebom, 1996; Christian et al., 1998). It is conceivable that the P4 and P1 domains might also be a part of the binding site to the 5′ half of the acceptor stem of a ptRNA. This hypothesis is consistent with the previous observations that intramolecular UV cross-linking of M1 RNA in the presence and absence of a tRNA substrate indicated that the P4 and P1 domains are close to the active site of the ribozyme and the cleavage site of a ptRNA (Harris et al., 1994; Chen et al., 1998). Moreover, this hypothesis is consistent with the orientations of these domains in the current three-dimensional models of M1 and P RNAs (Chen et al., 1998; Massire et al., 1998).

Positions 224–227 in J11/14 of M1 RNA (or J11/12 of P RNA) were found to be cross-linked to both tk46S4 and tk46S5. This observation indicates that these 4 nt interact with the junction region between the targeting sequence and the 3′ tail sequence and is consistent with previous results that a nearby position, A_{230} of P RNA, interacts with the T-stem of ptRNA (Pan et al., 1995).

The ribozyme domains that bind to the 5′ leader and the 3′ tail sequence

Recent UV cross-linking studies indicated that the −1 position at the 5′ leader sequence is in close proximity to nucleotide G_{332} and A_{333}, (Kufel & Kirsebom, 1996; Christian et al., 1998). However, little is known about the regions of the ribozymes that interact with the rest of the leader sequence of a ptRNA substrate. The regions that cross-linked to substrate tk46S2 but not tk46S3 include P3 and P12/P13 of M1 RNA (or the homologous regions of P RNA) (Table 3), suggesting that these regions interact with the 5′ leader sequence adjacent to the cleavage site. The regions cross-linked to substrate tk46S5 but not tk46S4 include J12/13 and P13 of M1 RNA and their homologous counterparts (i.e., P10.1/P10.1a) of P RNA (Table 3). This result sug-

gests that these regions are the ribozyme-binding sites for the 3′ tail sequence immediately adjacent to the targeting region. One of the interesting observations here is that a low level of cross-link was commonly detected at J12/13 and P13 of M1 RNA (or P10.1/10.1a of P RNA) in experiments with tk46S2 and tk46S5 (Table 2). Therefore, these 5′ leader and 3′ tail sequences may be in close proximity to the same ribozyme regions and to each other.

Comparison of the mRNA binding site of the ribozyme to its tRNA binding site

Our results indicate that the cleavage site of the mRNA substrate interacts with the same regions of the ribozyme that bind to the cleavage site of a ptRNA. Moreover, the regions of the ribozyme that bind to the targeting region of the mRNA substrate (i.e., P4, P1, and P10/P11) are also a part of the binding domains interacting with the acceptor and T-stem of a tRNA, the natural equivalent of the RNA helix formed between the targeting region and the guide sequence (Chen et al., 1998; Massire et al., 1998). 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.

Our results also indicate that P3, P12, and P13/P14 (or P10.1a/10.1 of P RNA) interact with the 5′ leader and 3′ tail regions. P3 is close to the active site and probably interacts with the 5′-leader sequence of a ptRNA (Chen et al., 1998). However, P12/P13/P14 of M1 RNA (or P12/P10.1/P10.1a of P RNA) are believed not to be in close proximity to the tRNA substrate (Chen et al., 1998; Massire et al., 1998). In M1 and P RNA structures folded either in the presence of a ptRNA or in the absence of any substrates, cross-links were not found between the P12 or J12/13 domains and any regions of the ribozyme or a ptRNA. Thus, the ribozyme interacts with the mRNA substrate by utilizing binding motifs (e.g., P12/P13/P14) different from those used to interact with a ptRNA. Such a mechanism as an enzyme using a single active center to catalyze reactions with different substrates is commonly used by protein enzymes and might also be generally used by RNA catalysts (Guerrier-Takada & Altman, 1992; Pan & Jakacka, 1996; Altman & Kirsebom, 1999; Cech & Golden, 1999). Our results are consistent with the hypothesis that M1 RNA utilizes multiple binding sites to interact with different natural substrates such as ptRNA and p4.5S RNA (Westhof & Altman, 1994; Kufel & Kirsebom, 1996).

Cleavage of the mRNA substrate yields a 5′ product that immediately leaves the ribozyme upon cleavage, and a 3′ product that still base pairs with the guide sequence (Nepomuceno E, Liou K, Kilani AF, Liu F, unpubl. data). Although the cross-linking was carried out at 4 °C to minimize the extent of cleavage, cleavage might still occur during our experimental procedures. It is possible that some of the cross-links detected may also represent those between the ribozymes and the 3′ cleavage products. We believe that the same regions of M1GS RNA interact with the targeting sequence of both the substrate and the 3′ product. This hypothesis is consistent with previous findings that the same regions of M1 RNA were found to be in proximity to the acceptor stem of both a ptRNA and mature tRNA (Chen et al., 1998; Massire et al., 1998). To identify the nucleotides that specifically interact with the product, cross-linking experiments can be carried out using products that contain the photoactive group. In our studies, a cluster of three photoactive groups were incorporated into most of the substrates to identify the nucleotides of the ribozymes that are in close proximity to a particular region (i.e., the 3-nt positions) of the substrate. To identify the nucleotides cross-linked to a single position of the substrate, cross-linking experiments can be carried out by using the substrate that contains a single 4-thio uridine. These studies will further our understanding of how RNase P ribozyme recognizes the mRNA substrate and facilitate the construction of ribozymes that exhibit optimal substrate binding and high sequence specificity.

MATERIALS AND METHODS

Chemicals, enzymes, and plasmids

All chemical reagents were either purchased from Sigma or Aldrich Co. (St. Louis, Missouri). 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. Plasmid pDW66 (a gift from Dr. Norman Pace) (Frank & Pace, 1999) and pFL117 contain the DNA sequences coding for P RNA and M1 RNA driven by the T7 RNA polymerase promoter (Liu & Altman, 1995), respectively. Plasmid pFL120 contains the DNA sequence that encodes the RNA substrate tk46S1 driven by the T7 RNA polymerase promoter (Liu & Altman, 1995). The DNA sequences that encode M1GS and PGS ribozymes were constructed by PCR using *Pvu*II-digested pFL117 and pDW66 as the templates and oligonucleotide AF25 (5′-GGAATTCTAATACGACTCACTATAG-3′) as the 5′ primer. The 3′ PCR primers for the DNA sequences that encode ribozymes M1-S1, M1-S2, M1-S3, and M1-S4 are oligonucleotides TK31 (5′-GTGGTGTCTGCGTTCGACTATGAC CATG-3′), S5-GS (5′-GTGGTGCCCGCGCCCGACTATGACCATG-3′), S3-GS (5′-GTGGTGTTTGCGCCCGACTATGACCATG-3′), and S4-GS (5′-GTGGTGCCCGCGCCCTTTTATGACCATG-3′), respectively. The DNA sequences that encode ribozymes P-S1, P-S2, P-S3, and P-S4 were also constructed in a similar way.

To construct plasmid pS5, which contained the DNA sequence coding for substrate tk46S5, DNA sequence was generated by PCR using pFL120 as the template and oligonucleotides AF25 and S5 (5′-CGCGGATCCGCGGAAAGGTCGGGCGCGGGCGCGTGTTGGTGGCGGGGGTC-3′) as the 5′ and 3′ primers, respectively, and was subsequently

cloned into pUC19 vector. The DNA sequences in plasmids pS2, pS3, and pS4, which encode substrates tk46S2, tk46S3, and tk46S4, 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, pS3, and pS4, were S2 (5′-CGCGGATCCGCGGCCTGGTCGGGCGCGGGCAAATG TTGGTGGC-3′), S3 (5′-CGCGGATCCGCGGCCTGGTCGG GCGCAAACGCGTGTTGG-3′), and S4 (5′-CGCGGATCC GCGGCCTGAAAGGGCGCGGGC-3′), respectively. The ribozymes and RNA substrates were synthesized in vitro from these DNA templates by T7 RNA polymerase. The substrates that contained the photoactive 4-thio-uridine were synthesized in the presence of 4-thio-uridine triphosphate (Amersham, Arlington Heights, Illinois).

UV cross-linking and primer extension experiments

UV cross-linking was carried out essentially as described by Wyatt et al. (1992). Radiolabeled RNA substrates (5–100 nM) were incubated with ribozymes (5–100 nM) in buffer A (50 mM Tris, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) for 5 min at 25 °C. Then, the reaction mixture was exposed to UV light (365 nm) on ice for 5–30 min. RNA samples were recovered from the cross-linking reactions by ethanol precipitation. The cross-linked species were separated in denaturing gels and visualized by autoradiography. The conjugates were then excised from the gels and further purified.

The oligonucleotide primers used for primer extension experiments were radiolabeled at their 5′ termini by T4 polynucleotide kinase in the presence of γ-[^{32}P]-ATP. The oligonucleotides used for experiments with M1GS RNA were AK1(85–102) (5′-CGTTACCTGGCACCCTG-3′), AK2(174–191) (5′-CCGCACCCTTTCACCCT-3′), AK3(265–282) (5′-ACGGGCCGTACCTTATG-3′), and AK4 (360–377) (5′-AGG TGAAACTGACCGATA-3′). The oligonucleotides used for experiments with PGS RNA were AK10(85–102) (5′-GACT TCGCTAGGCACGAA-3′), AK11(175–192) (5′-GTCACTGT GGCACTTTCA-3′), AK12(280–297) (5′-CCTTCTCTCCGT TGAATT-3′), and AK13(384–401) (5′-AAGTGGTCTAACGT TCTG-3′). Primers (100,000 cpm) and different amounts of purified cross-linked 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 Inc., Madison, Wisconsin), 50 mM Tris, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 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 nM 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 (Molecular Dynamics, Sunnyvale, California). 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 and pDW66 DNA as the templates and in the presence of T7 sequenase and α-[^{35}S]-dATP (Amersham Inc., Arlington Heights, Illinois). The sequencing primers were the same oligonucleotides used in the primer-extension analyses of the ribozymes.

Kinetic analyses of the reactions catalyzed by the selected ribozymes

The cleavage reactions of the substrates by different ribozymes were carried out in buffer A as described previously (Liu & Altman, 1995; Kim et al., 1997). To measure the rate of cleavage of the substrate–ribozyme complexes, equimolar amounts of substrates and ribozymes were incubated in buffer B (50 mM Tris, pH 6.0, 100 NH_4Cl, 10 mM $MgCl_2$) at 25 °C for 5 min to allow binding. After binding, the mixture was transferred to ice and the bound ribozyme–substrate complexes were isolated using G-50 Sephadex gel filtration columns (Boehringer Mannheim, Indianapolis, Indiana). The eluate was examined in 5% polyacrylamide nondenaturing gels for the presence of labeled ribozyme–substrate complexes and the concentrations of the complexes were quantitated by a STORM840 phosphorimager. Then ribozyme–substrate complexes were diluted in different concentrations (2–70 nM) and incubated in buffer A at 37 °C. Aliquots were withdrawn from reaction mixtures at regular intervals (from 0 to 160 min). The cleavage products were separated on 15% denaturing gels, autoradiographed, and quantitated with a STORM840 phosphorimager (Molecular Dynamics, Sunnyvale, California). The apparent rate constant, k_{app}, was calculated by the slope of the plot of $\ln(S_0/S_t)$ versus time, where S_0 equals the initial substrate concentration and S_t equals the substrate concentration at a given time point. The values were the average of three experiments.

Assays to determine the observed reaction rate (k_{obs}) and the values of k_{cat}/K_m under single-turnover conditions were performed as described previously (Kim et al., 1997). Briefly, analyses were performed with a trace amount of radioactive substrate and an excess of ribozyme. The concentration of radioactive substrate was less than 0.5 nM and the concentrations of ribozyme tested ranged from 1 to 100 nM. Variations of the amount of substrate did not affect the k_{obs} at fixed excess ribozyme concentrations and the reactions followed pseudo-first-order kinetics. The values of the overall cleavage rate (k_{cat}/K_m) were calculated by the slope of a least-squares linear regression (KaleidaGraph, Synergy Software, Reading, Pennsylvania) 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 cross-links, the cross-linked species were purified from denaturing gels, either diluted 100-fold first or directly incubated at 37 °C in buffer A for different periods of time (from 5 min to 2 h). The cleavage products were separated on 8% polyacrylamide denaturing gels and quantitated with a Molecular Dynamics STORM840 Phosphorimager.

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