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Requirements for cleavage by a modified RNase P of a small model substrate

Fenyong Liu+ and Sidney Altman*

Department of Biology, Yale University, New Haven, CT 06520, USA

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ABSTRACT

M1 RNA, the catalytic RNA subunit of RNase P from *Escherichia coli,* has been covalently linked at its 3′ terminus to oligonucleotides (guide sequences) that guide the enzyme to target RNAs through hybridization with the target sequences. These constructs (M1GS RNAs) have been used to determine some minimal features of model substrates. As few as 3 bp on the 3′ side of the site of cleavage in a substrate complex and 1 nt on the 5′ side are required for cleavage to occur. The cytosines in the 3′ terminal CCA sequence of the model substrates are important for cleavage efficiency but not cleavage site selection. A purine (base-paired or not) at the 3′ side of the cleavage site is important both for cleavage site selection and efficiency. M1GS RNAs provide both a simple system for characterization of the reaction governed by M1 RNA and a tool for gene therapy.

INTRODUCTION

RNase P is a ribonucleoprotein complex responsible for the maturation of the 5′ termini of tRNAs (1,2). It catalyzes a hydrolysis reaction that removes a 5′ leader sequence from tRNA precursors (pretRNA) and several other small RNAs. In *Escherichia coli*, RNase P consists of a catalytic RNA subunit [M1 RNA: 377 nucleotides (nt)] and a protein subunit (C5 protein: 119 amino acids) (1,2). In the presence of a high concentration of salt, such as 100 mM Mg^{2+}, M1 RNA acts as a catalyst and cleaves pretRNAs *in vitro* in the absence of C5 protein (3). 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* (4).

Studies of substrate recognition by M1 RNA and RNase P (1,5–10) have led to the development of a general strategy of gene targeting in which M1 RNA and RNase P can be used as tools to cleave any specific mRNA sequence. In a small model substrate for M1 RNA [Fig. 1A (boldface regions) and Fig. 1B], the 5′ proximal sequence (the 5′ leader and 5′ proximal acceptor stem sequence) base pairs to the 3′ proximal sequence (the 3′ proximal acceptor stem sequence) (5). This 3′ proximal sequence is called an external guide sequence (EGS) because it can base pair with the targeted sequence and guide M1 RNA to cleave the substrate (Fig. 1B). Subsequent studies carried out in our laboratory have demonstrated that the mRNAs encoding *E.coli* β-galactosidase and *S.aureus* nuclease A can be cleaved by M1 RNA and *E.coli* RNase P *in vitro* with custom-designed EGSs (6). One limitation of this method is the relatively weak binding of the target RNA to M1 RNA (8). In order to increase cleavage efficiency and strengthen substrate binding, we constructed a derivative of M1 RNA, M1GS RNA, by linking a guide sequence (GS) covalently to the 3′ end of M1 RNA (Fig. 1C) (11). Similar constructs have also been described recently in studies of M1 RNA–pretRNA conjugated molecules (12–17).

M1GS RNA provides a simple model system to study the catalytic mechanism of and substrate recognition by M1 RNA. In the studies reported here, the minimal requirements for cleavage by M1GS RNA of a small model substrate were determined. We showed that M1GS RNA can cleave a RNA substrate of eight nucleotides which forms 7 base pairs (bp) with the guide sequence. These studies suggest that the minimal structural motif recognized by M1 RNA is a short RNA double-stranded helix, consistent with previous observations that the 3′ CCA sequence, a 5′ leader sequence, and the nucleotide at the 3′ side of the cleavage site are critical for cleavage efficiency (5–8).

MATERIALS AND METHODS

Construction of RNA substrates

DNA templates for *in vitro* transcription of RNA substrates phe7 (identical to phe7-G), phe3, phe7-A, phe7-C and phe7-U were constructed (18) by annealing the T7 promoter-containing oligonucleotide, OliT7 (5′-TAATACGACTCACTATAG-3′) with oligonucleotides:
Oli58 (5′-TCCGGGCGGTCCTATAGTGAGTCGTATTAA-3′),
Oli51 (5′-GGCGGTCCTATAGTGAGTCGTATTAA-3′),
Oli84 (5′-TCCGGGTGGTCCTATAGTGAGTCGTATTAA-3′),
Oli85 (5′-TCCGGGGGGTCCTATAGTGAGTCGTATTAA-3′) and
Oli86 (5′-TCCGGGAGGTCCTATAGTGAGTCGTATTAA-3′)
respectively. The RNA substrates were synthesized by T7 RNA polymerase (Promega Inc., Madison, WI) from these DNA templates, according to the manufacturer's instructions. RNA substrate phe7-1 (5′-CGCCCGGA-3′) was synthesized chemically with a 380B DNA synthesizer (Applied Biosystems). All RNA substrates synthesized either chemically or enzymatically were subjected to gel purification in 15% polyacrylamide gels that contain 7 M urea.

To generate radioactive RNA substrates, substrates were either uniformly labeled with [α-^{32}P]GTP by T7 RNA polymerase, 5′-end-labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide

* To whom correspondence should be addressed

+Present address: Program in Infectious Diseases, School of Public Health, University of California, 140 Earl Warren Hall, Berkeley, CA 94720, USA

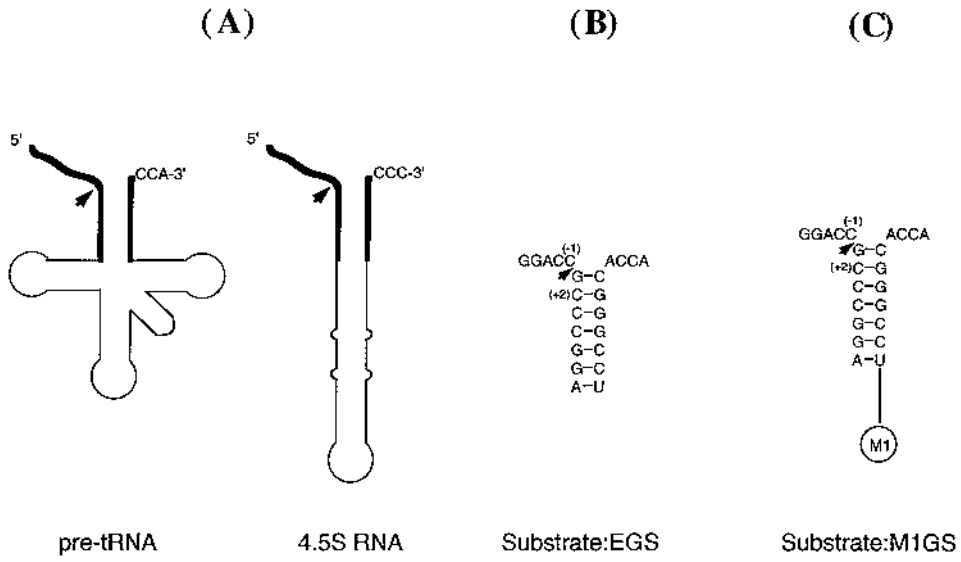

Figure 1. (A) Schematic representation of natural substrates (precursor tRNA and 4.5S RNA) for ribonuclease P and M1 RNA from*E.coli*. The structural components common to both precursor tRNA and 4.5S RNA are highlighted; they are equivalent to 7 bp of the acceptor stem of a tRNA. The site of cleavage by RNase P or M1 RNA is marked with a filled arrow. (**B**) Schematic diagram of a complex formed between a substrate (phe7) and an EGS (PHE). (**C**) Schematic diagram of an M1GS RNA construct (M1PHE) to which a target RNA (S) (phe7) has hybridized. In (B) and (C), the stem structure formed between the target RNA and either the EGS RNA or M1GS RNA is shown as 7 bp to mimic the structure of the tRNA acceptor stem; it can be varied from 3 to 19 bp as described in the text and in previous studies (6,11). The positions 3′ and 5′ adjacent to the scissile bond are designated as the +1 and –1 sites respectively. Accordingly, the position 3′ adjacent to the +1 site is called the +2 site. The sequence shown here are from the acceptor stem region of $tRNA^{Phe}$ (7).

kinase or 3′-end-labeled with [^{32}P]pCp in the presence of T4 RNA ligase.

Construction of ribozymes

Plasmids pTK117, pM1Δ(1–54), pM1Δ(1–163), pM1Δ(62–108), pM1Δ(94–281), pM1Δ(156–290) and pM1Δ(169–377) are derivatives of pUC19, in which the DNA sequences coding for M1 RNA and mutant M1 RNAs with deletions from nt 1 to 54, 1 to 163, 61 to 108, 94 to 281, 156 to 290 or 169 to 377 are under the control of the T7 RNA polymerase promoter (19). The DNA templates for M1PHE (identical to M1PHE-c), M1PHE-C, M1PHE-CC, M1PHE-CCA (identical to M1PHE-CCA-c), M1PHE-a, M1PHE-g, M1PHE-u, M1PHE-CCA-a, M1PHE-CCA-g and M1PHE-CCA-u were constructed by the polymerase chain reaction (PCR). In the PCR, the DNA sequence for M1 RNA in plasmid pTK117 was used as the template and OliT7 was used as the 5′ primer oligonucleotide. The 3′ primers which contain the appropriate guide sequences were:
Oli52 (5′-TGGT**GCCCGGA- CTC**TATGACCATG-3′),
Oli57 (5′-GGT**GCCCGGACTC**TATGACCATG-3′),
Oli56 (5′-GT**GCCCGGACTC**TATGACCATG-3′),
Oli55 (5′-TGCCCGGACTCTATGACCATG-3′),
Oli83 (5′-TGGT**TCCCGGACTC**TATGACCATG-3′),
Oli82 (5′-TGG-T**CCCCGGACTC**TATGACCATG-3′),
Oli81 (5′-TGGT**ACCCGGACTC**TATGACCATG-3′),
Oli89 (5′-T**TCCCGGACTC**- TATGACCATG-3′),
Oli88 (5′-T**CCCCGGACTC**TATGACCATG-3′) and
Oli87 (5′-T**ACCCGGACTC**TATGACCATG-3′).
The 3′ proximal sequences of 10 nt serve as the primers for the PCR with the pUC19 sequence. The underlined sequences and the bold sequences correspond to the 3′ ACCA sequence and the guide sequences respectively.

The DNA templates for ribozymes Δ(1–54)M1PHE, Δ(1–163)M1PHE, Δ(62–108)M1PHE, Δ(94–281)M1PHE, Δ(156–290)M1PHE and Δ(169–377)M1PHE were constructed by PCR in which the DNA sequence coding for M1 RNA in pM1Δ(1–54), pM1Δ(1–163), pM1Δ(62–108), pM1Δ(94–281), pM1Δ(156–290) and pM1Δ(169–377) was used as the template respectively. The primers were OliT7 and OliTK13.

In all the M1GS ribozymes described here, the linker sequence connecting the 5′ end of the guide sequence and the 3′ end of M1 RNA is a 24 nt-long sequence of pUC19 (5′-TATGACCATGATTACGCCAAGCTT-3′). The enzymatic activity of M1GS RNA is not affected significantly when the length of the linker sequence varies from 24 to 50 nt (11,17).

The corresponding RNA enzymes were synthesized from the DNA templates by T7 RNA polymerase. The synthesized RNA was subjected to gel purification in 4% polyacrylamide gels which contain 7 M urea. Subsequently, RNA enzyme samples were eluted from the gel slices and precipitated in the presence of ethanol. Eventually the RNA enzymes were resuspended into buffer C (50 mM Tris, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$). Prior to the assays for RNA enzymatic activity, these RNA enzymes were heated to 75°C for 3 min, and then allowed to renature by cooling slowly to room temperature.

Assays for cleavage by M1GS RNA

RNA enzyme (20 nM) and substrate (50 nM), either uniformly labeled or end-labeled, were incubated for 30 min at 37 or 50°C in buffer A (50 mM Tris, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) or buffer B (50 mM Tris, pH 7.5, 100 mM NH_4Cl) that contains $MgCl_2$ at various concentrations. Reactions were stopped by the addition of 8 M urea and the cleavage products were then separated on 20% polyacrylamide gels that contain 7 M

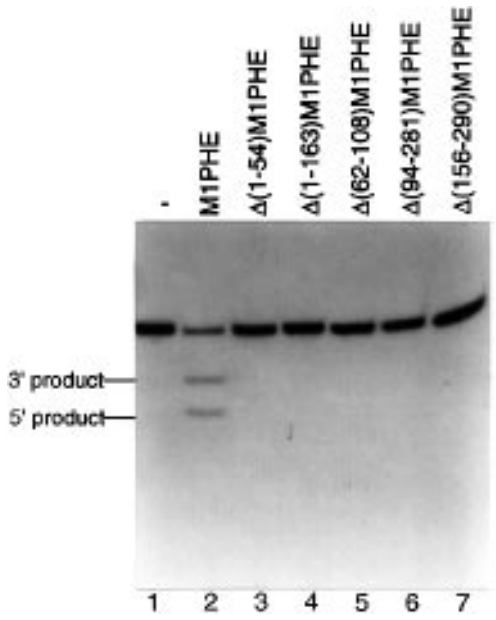

Figure 2. Cleavage of substrate phe7 by different M1GS RNAs. RNA substrate phe7 was incubated either alone (lane 1) or with M1PHE (lane 2) or different M1GS RNAs that contain the guide sequence PHE7 and, in addition, a deletion in the catalytic domain of M1 RNA (lanes 3–7). Substrate phe7 (20 nM) was incubated with 20 nM ribozyme in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) at 50°C. Cleavage products were separated in 20% polyacrylamide gels containing 8 M urea. Further experimental details are given in the Materials and Methods.

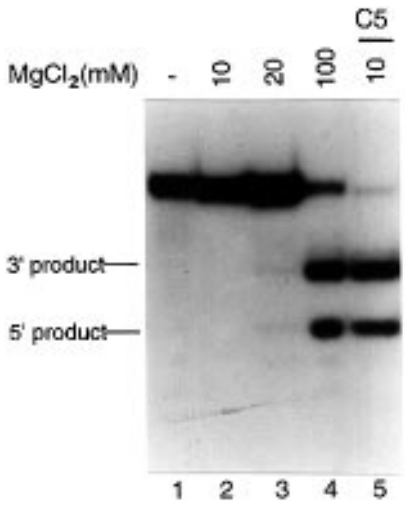

Figure 3. Cleavage of phe7 RNA by M1GS RNAs in the presence of different concentrations of Mg^{2+} and in the presence of C5 protein. No RNA enzyme was added to the reaction mixture in lane 1. RNA substrate (20 nM) was incubated with 20 nM enzyme as specified. Reactions were carried out at 37°C either in buffer B (50 mM Tris, pH 7.5, 100 mM NH_4Cl) that contained different concentrations of $MgCl_2$ as specified (lanes 2–4) or in the presence of C5 protein and 10 mM $MgCl_2$ (lane 5).

urea. C5 protein was purified from *E.coli* as described previously (20). The RNase P holoenzyme from *E.coli* was assembled by mixing M1 RNA and C5 protein at a molar ratio of 1:20.

Assays to determine kinetic parameters under single and multiple-turnover conditions were performed as described previously (3,21). Cleavage was assayed at various concentrations of substrate (in 2–20-fold excess over enzyme concentration), both above and below the K_m for the substrate. Aliquots were withdrawn from the reaction mixtures at regular intervals and the cleavage products were separated in polyacrylamide–urea gels. Quantitation was carried out with a phosphorimager (Fuji). The values of K_m and k_{cat} were obtained from Lineweaver–Burk double reciprocal plots. In single turnover experiments, trace amounts of substrates were used and the concentrations used were much lower than the K_m. Rate of cleavage was assayed at various concentrations of enzyme (10–200-fold excess over substrate concentration). The observed rate constant of cleavage (k_{obs}) was determined from the slope of the linear regression of a plot of ln (S_t/S_o) versus time, where S_o is the initial amount of the substrate and S_t is the amount of substrate remaining at a given time, and the value of k_{cat}/K_m was obtained from the equation $k_{cat}/K_m = k_{obs}/[E]$ where [E] is the concentration of the enzyme.

RESULTS

Construction of a M1GS ribozyme and its minimal substrate

DNA encoding a guide sequence that contains the 3′ half of the acceptor stem of $tRNA^{Phe}$ from *E.coli* was covalently linked to the 3′ end of the DNA sequence that encodes M1 RNA. The linker sequence connecting the 5′ end of the guide sequence and the 3′ end of M1 RNA is a sequence (24 nt) derived from pUC19. The RNA transcript of this construct, M1PHE, cleaves a substrate, phe7, of 12 nt which contains a sequence 7 nt long that is the 5′ half of the acceptor stem of $tRNA^{Phe}$ (Fig. 1C). The complex formed between M1PHE and phe7 contains a helix of 7 bp and resembles the acceptor stem of $tRNA^{Phe}$ (Fig. 1C). Cleavage of this substrate generated two cleavage products as expected (Fig. 2, lanes 1 and 2).

A common catalytic mechanism is shared by M1 RNA and M1GS RNA

Studies with M1 RNA–pretRNA conjugates have shown that the interactions between M1 RNA and its conjugated pretRNA substrate are similar to those observed when the ribozyme and the substrate are separated (13–15). However, it is also important to establish that the cleavage reactions catalyzed by M1GS RNA share the same characteristics with those catalyzed by M1 RNA. Four sets of experiments were carried out to examine the features of the M1PHE cleavage reactions.

First, a set of M1GS RNAs were constructed by linking the guide sequence covalently to the 3′ end of several M1 RNA deletion mutants (such as M1 RNA mutants with a deletion from nt 1 to 54, 1 to 163, 62 to 108, 94 to 281, 156 to 290 and 169 to 377) (19,22). These mutants did not exhibit RNase P catalytic activity with pre-tRNA substrates when assayed *in vitro* (19,22). As shown in Figure 2 (lanes 3–7), the M1GS RNAs derived from these mutants were unable to cleave substrate phe7, indicating that mutations in the catalytic domain of M1 RNA also abolish the enzymatic activity of M1GS RNA.

In the second set of experiments, the effects of the concentrations of divalent ion (Mg^{2+}) on the enzymatic activity of M1GS RNA were investigated. While ribozyme M1PHE cleaved phe7 in low concentrations of Mg^{2+} (10–20 mM $MgCl_2$) (11,17), M1PHE

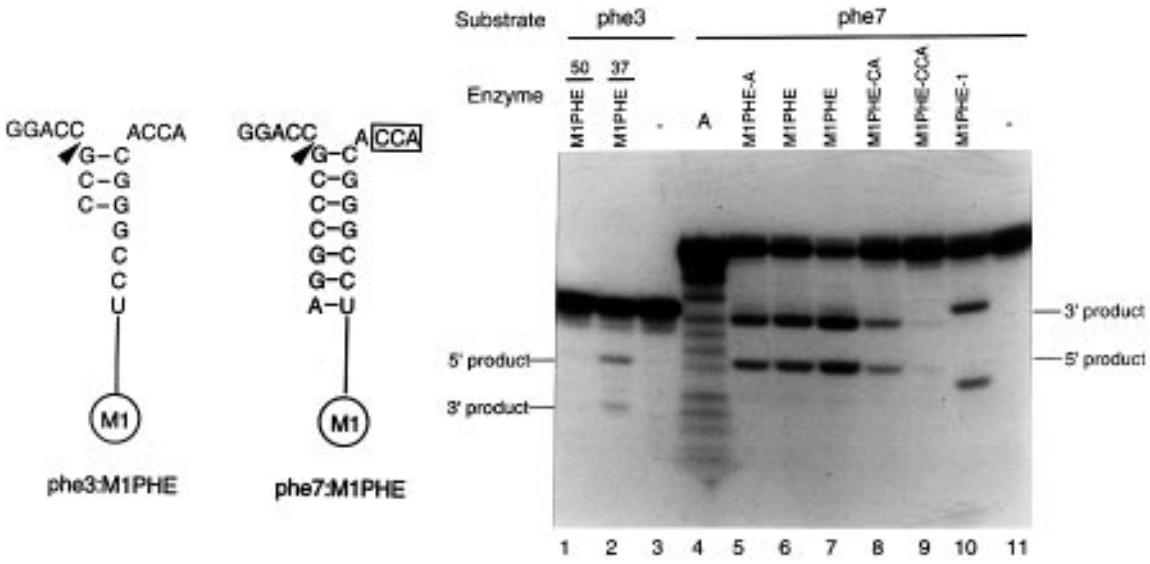

Figure 4. Cleavage of substrates phe3 (lanes 1–3) and phe7 (lanes 5–11) by different M1GS RNAs. The left panel shows the complexes formed between M1PHE and substrate phe3, and M1PHE and phe7 respectively. The 3′ CCA sequence is boxed. RNA substrate phe7 was incubated either alone (lane 11), or with M1PHE (lanes 6 and 7) or with different M1GS RNAs that contain the guide sequence PHE and, in addition, a deletion in the 3′ CCA sequence (lanes 5, 8 and 9). In lane 6, half the amount of ribozyme M1PHE was added compared to that in lane 7. Lane 10 shows the result of an experiment in which cleavage of substrate phe7 by ribozyme M1PHE-1 is determined by a guide sequence that targets the –1 position of the substrate phe7: two cleavage products, of 4 and 8 nt respectively are produced. In the reaction shown in lane 4, the substrate was subjected to alkaline treatment to create a molecular weight ladder. In lanes 1–3, only trace amount of substrate phe 3 was used. Cleavage reactions were carried out in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) at 50°C (lanes 1, 3 and 5–11) or at 37°C (lane 2). Cleavage products were separated in 20% polyacrylamide gels containing 8 M urea.

exhibited much more significant enzymatic activity in high concentrations of Mg^{2+} (100 mM) (Fig. 3, lanes 2–4). A similar dependence on Mg^{2+} was observed in the cleavage reactions catalyzed by M1 RNA with pretRNA substrates and small model substrates (3,23,24). It has been reported that the optimal concentration of Mg^{+} for M1 RNA activity is reduced as the ionic strength (i.e., the concentration of monovalent ions) in the assay buffer increases (24). Similar results were also obtained in reactions catalyzed by M1GS RNA (11,16,17).

The cleavage we observed can, in principle, be catalyzed either by the same M1GS RNA molecule that base pairs to substrate phe7 (*cis*-cleavage) or by another M1GS RNA molecule that does not bind to the substrate (*trans*-cleavage). However, efficient *trans*-cleavage can only be observed in high concentrations of Mg^{2+}. It has been shown previously that at low concentration of Mg^{2+}, cleavage of a substrate by an M1GS RNA is much more efficient than cleavage of the same substrate by M1 RNA in the presence of a separated EGS (11,16,17). These results indicated that *trans*-cleavage is very much diminished and *cis*-cleavage is responsible for the results we report here. Similar observations have also been reported in the studies of the cleavage of M1 RNA–pretRNA conjugates (13–15).

The third set of experiments indicated that the cleavage activity of M1PHE ribozyme was greatly stimulated by C5 protein (Fig. 3, lane 5), as is that of M1 RNA (3).

Finally, the cleavage of phe7 by M1PHE occurred at the +1 position in the substrate, yielding two cleavage products of 5 and 7 nt respectively (Fig. 4, lane 6). The cleavage site is identical to that in the reaction with $pretRNA^{Phe}$ and M1 RNA (7). Ribozyme M1PHE-1, the guide sequence of which targets the cytosine at the fourth position (–1 site) instead of the guanine at the fifth position (+1 site) (Fig. 1C), cleaves phe7 at the –1 site, yielding two cleavage products of 4 and 8 nt (Fig. 4, lane 10). Further analysis of the cleavage products indicated that cleavage results in a 5′ phosphoryl and 3′ hydroxyl group, as does cleavage of pretRNA substrates by M1 RNA (data not shown). These observations showed that the cleavage mechanism of M1GS RNA is similar to that of M1 RNA.

The minimal requirements for cleavage of a model substrate by M1GS RNA

The complex formed between M1PHE and substrate phe7 resembles a structure equivalent to an acceptor stem, a 3′ CCA sequence and a 5′ leader sequence of a pre-tRNA molecule (Fig. 1B and C). By systematically deleting parts of the 5′ leader sequence, the 3′ CCA sequence and the helix structure of the substrate–EGS complex, the minimal requirements for cleavage by M1GS RNA of a model substrate were determined. This was achieved in three sets of experiments.

The first set of experiments was designed to study the effect of the 3′ CCA sequence on cleavage activity of M1GS RNA. Deletion of this sequence in substrate $ptRNA^{Tyr}$ moderately reduces the cleavage rate by M1 RNA (22,25) (Table 1). A ribozyme, M1PHE-CCA, was constructed in which the guide sequence did not contain the 3′ CCA sequence. Cleavage of substrate phe7 by this ribozyme was at least 250-fold slower than that by M1PHE (Fig. 4, compare lanes 7 and 9 and Table 1). These results were consistent with previous observations that the 3′ CCA sequence is important for cleavage of a helix-like model substrate, pAT-1, by M1 RNA and RNase P (5,7,8). The less severe effects of the 3′ CCA deletion observed with a pre-tRNA substrate could be explained as the loss of interactions between M1 RNA and the substrate due to the deletion of segments of the

tRNA structure. Binding of this substrate by M1 RNA can still be achieved by interactions between M1 RNA and the other, remaining, domains of the pretRNA molecule.

Table 1. Kinetic parameters of cleavage reactions governed by different M1GS RNAs

Substrate	Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m
phe7	M1PHE	0.07 ± 0.01	0.17 ± 0.02	2.6 ± 0.2
phe7	M1PHE-A	0.1 ± 0.02	0.14 ± 0.02	1.4 ± 0.1
phe7	M1PHE-CA	0.09 ± 0.02	0.01 ± 0.002	0.1 ± 0.04
phe7	M1PHE-CCA	0.41 ± 0.04	0.004 ± 0.001	0.01 ± 0.004
phe7-1	M1PHE	–	–	0.05 ± 0.009
phe3	M1PHE	–	–	0.01 ± 0.002

Cleavage assays were performed in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) at either 50°C (with phe7 and phe7-1) or 37°C (with phe3). Further experimental details are described in the Materials and Methods.

To characterize the role of each nucleotide in the 3′ CCA sequence, a set of M1GS ribozymes with a deletion of each nucleotide in the 3′ CCA sequence was constructed and tested for cleavage of phe7 (Fig. 4 and Table 1). The salient features of the results are as follows. (i) Cleavage catalyzed by all M1GS RNA constructs tested here was always at the correct position (i.e., between the –1 and +1 sites), indicating that the 3′ CCA sequence is not essential either for cleavage or cleavage site selection. (ii) Deletion of the 3′ terminal adenine led merely to a 2-fold reduction of the cleavage rate. In contrast, deletion of either of the cytosines resulted in a 10-fold reduction of the cleavage rate.

In the second set of experiments, an RNA substrate, phe7-1, was synthesized chemically in which the 5′ leader sequence was replaced with a single nucleotide (Fig. 5). Subsequently, this substrate was labeled at the 3′ end with [^{32}P]pCp in the presence of T4 RNA ligase. Cleavage of this substrate by M1PHE yielded two cleavage products of 7 and 1 nt respectively, as expected (Fig. 5). Further kinetic analysis revealed that the cleavage rate with this substrate is ~50-fold slower than that of phe7 by M1PHE (Table 1). This observation indicated that the 5′ leader sequence, except for the nucleotide at the –1 position, is not required for cleavage or cleavage site selection but is important for cleavage efficiency. These results are consistent with observations of cleavage reactions with pretRNA and pAT-1-like model substrates catalyzed by M1 RNA (26,27).

Previous studies indicated that the structure equivalent to the acceptor stem in tRNA-like model substrates for M1 RNA can be as small as 4 bp (28). However, whether 4 bp is the minimal motif required for M1 RNA recognition was not proven as M1 RNA could have also interacted with other domains of those substrates used previously (28). Therefore, in the third set of experiments, an RNA substrate, phe3, was constructed in which a part of the 5′ acceptor stem sequence has been deleted (Fig. 4). Accordingly, phe3 can only form a helix of 3 bp with the guide sequence of M1PHE. Cleavage of phe3 by M1PHE occurred at the expected position (Fig. 4, lane 2). The reaction was much more efficient at 37°C than at 50°C (Fig. 4, compare lanes 2 and 1) since the base-paired complex is less stable at the higher temperature. Further kinetic analyses revealed that the cleavage of phe3 by M1PHE (37°C) is at least 200-fold slower than that of phe7 by the same ribozyme at 50°C (Table 1).

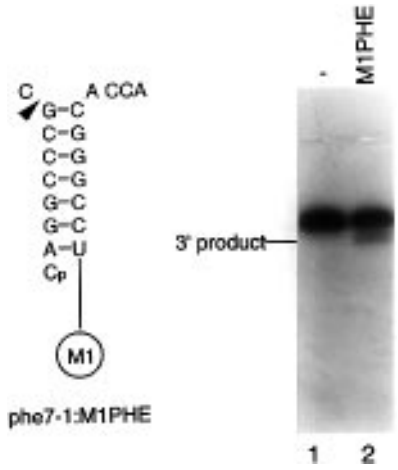

Figure 5. Cleavage of substrate phe7-1 by M1GS RNA. The left panel shows the complex formed between ribozyme M1PHE and substrate phe7-1. Substrate phe7-1 was synthesized chemically (380B DNA synthesizer; Applied Biosystems) and labeled at its 3′ end with [^{32}P]pCp with T4 RNA ligase (Pharmacia). Substrate phe7-1 (1 nM) was incubated either alone (lane 1) or with M1PHE (20 nM) (lane 2). Cleavage reactions were carried out in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) at 50°C. Cleavage products were separated in 20% polyacrylamide gels containing 8 M urea. The 3′ cleavage (8 nt) product migrates slightly faster than the intact substrate (9 nt) as shown.

Required features of the nucleotide at the site of cleavage by M1GS RNA

The site of cleavage by RNase P of a pretRNA is nearly always at the junction between a single- and a double-stranded region. Guanine at position +1 is optimal for recognition by the enzyme (1,29). However, it is not clear whether the interaction between M1 RNA and the nucleotide at position +1 is alone sufficient for positioning the scissile band in the active center. Nor is it known if the identity of the nucleotide at position +1 in a minimal, model substrate is important for M1 RNA recognition of the cleavage site. A set of RNA substrates was derived from phe7 in which the guanine at the +1 position was replaced with the other three bases (Fig. 1C). Accordingly, a set of ribozymes was also derived from M1PHE in which the cytosine in the guide sequence of the ribozyme that base pairs with the guanine of phe7 was replaced with the other three bases. RNA substrates and ribozymes were incubated together and cleavage products were separated in denaturing gels. The salient features of the results (Fig. 6 and Table 2) are as follows. (i) Optimal cleavage was observed when the nucleotide at the +1 site was base-paired. For example, significant cleavage was found in the reactions with phe7-G and M1PHE-C (lane 5), phe7-A and M1PHE-U (lane 7), phe7-C and M1PHE-G (lane 13) and phe7-U and M1PHE-A (lane 19). (ii) Selection of the cleavage site was influenced by the identity of the nucleotide at the +1 site. When there was a purine at this position, cleavage at the correct position (i.e., between –1 and +1 position) occurred regardless of whether the +1 position was not base-paired [e.g., cleavage of phe7-G by M1PHE-A and M1PHE-G (lanes 3 and 4) and phe7-A by M1PHE-G (lane 8)]. In contrast, cleavage occurred at the +2 position when a pyrimidine was at the +1 position and was not base-paired [e.g., cleavage of phe7-C by M1PHE-U, M1PHE-A and M1PHE-C

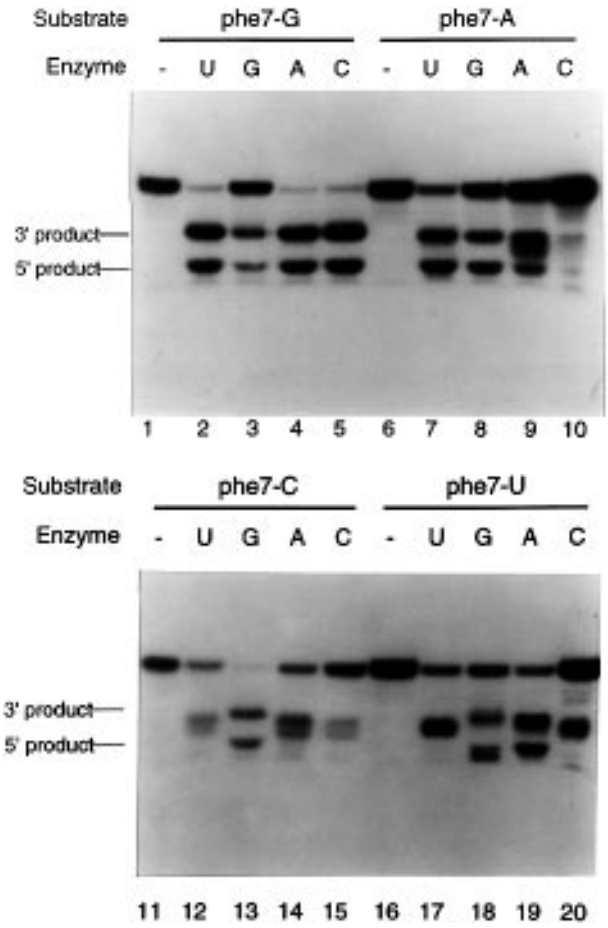

Figure 6. Cleavage of various small substrates by different M1GS RNAs. RNA substrates (10 nM each for phe7-G, phe7-A and phe7-U, and 4 nM for phe-C) were incubated either alone (lanes 1, 6, 11 and 16) or with different ribozymes (lanes 2–5, 7–10, 12–15 and 16–20; see text for description of ribozymes). The amount of ribozyme (40 nM) used in the reaction with substrate phe7-C is four times more than that with other substrates. Cleavage reactions were carried out in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM NH_4Cl, 100 mM $MgCl_2$) at 50°C. Cleavage products (indicated in the figure) were separated in 20% polyacrylamide gels containing 8 M urea.

(lanes 12, 14 and 15) and phe7-U by M1PHE-U and M1PHE-C (lanes 17 and 20)]. These observations suggest that a purine nucleotide is a determinant for cleavage site selection by M1 RNA. (iii) The identity of the nucleotide at the +1 site was also important for cleavage efficiency. For example, the cleavage rate of the reaction with phe7-G and M1PHE-C was ~10-fold higher than that with phe7-C and M1PHE-G. This observation also explains why very little cleavage at the +2 position (cytosine) of the substrates tested here was found when the base pair at the +1 site was disrupted.

DISCUSSION

M1GS RNA as a model system to study the catalytic mechanism of and substrate recognition by M1 RNA

M1 RNA–pretRNA conjugated constructs have been described recently (13–16,30) and have been used to identify the phosphates in M1 RNA important for catalysis (31). Similar strategies have also been used to construct a sequence-specific ribozyme, M1GS RNA: a part of the substrate (the guide sequence) has been linked to M1 RNA (11–13,16,17) in this latter case. Subsequently, M1GS RNA has been shown to cleave its targeted substrates including mRNAs, both *in vitro* and in cells in tissue culture (11,12,16,17). In this report, we attempted to use M1GS RNA as a model system to investigate the substrate requirements for cleavage by M1 RNA. We have demonstrated that: (i) mutations in the catalytic domain of M1 RNA abolish M1GS RNA catalytic activity; (ii) cleavage by M1GS RNA, similar to that by M1 RNA, can be greatly stimulated in the presence of C5 protein; and (iii) M1GS RNA is highly active in high, but not low, ionic strength. These observations are in agreement with the findings of previous studies of M1 RNA, indicating that M1GS RNA and M1 RNA share a similar active conformation and catalytic mechanism (11–15,17) as described further below.

Table 2. The overall cleavage rates [$k_{cat}(min^{-1})/K_m(\mu M)$] of the reactions governed by different M1GS RNAs with different substrates under single-turnover conditions

Substrate/enzyme	M1PHE-c	M1PHE-u	M1PHE-g	M1PHE-a
phe7-G	3.0 ± 0.3 (+1)	1.6 ± 0.2 (+1)	0.5 ± 0.08 (+1)	2.9 ± 0.3 (+1)
phe7-A	0.5 ± 0.08 (+1)	1.5 ± 0.2 (+1)	0.5 ± 0.08 (+1)	0.2 ± 0.02 (+1, +2)
phe7-C	0.1 ± 0.02 (+2)	0.1 ± 0.02 (+2)	0.4 ± 0.06 (+1)	0.1 ± 0.02 (+2)
phe7-U	0.1 ± 0.02 (+2)	0.2 ± 0.02 (+2)	0.6 ± 0.08 (+1, -1)	0.9 ± 0.1 (+1)

The number in parenthesis represents the location of the cleavage site. 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. Further experimental details are described in the Materials and Methods.

Minimal features of substrates for M1 RNA

An important question regarding the catalytic mechanism of M1 RNA is how this ribozyme recognizes numerous substrates of different sequences and structures. Previously, systematic deletion analyses of a pretRNA molecule were carried out to determine minimal substrate requirements for M1 RNA (5–8). Tethering substrates to M1 RNA has provided an additional unique approach to the study of substrate recognition (11,13,14). In this report, we demonstrated that an RNA substrate, 8–12 nt long, can be cleaved by M1 RNA, provided that a guide sequence which can form a 3–7 bp duplex with the substrate is linked to M1 RNA. Thus, part of the minimum substrate requirement for cleavage by M1 RNA is an RNA duplex as short as 3 bp.

The 3′ CCA sequence and 5′ leader sequence (upstream of nt –1) of substrates are important for cleavage efficiency but not required for cleavage. The importance of each nucleotide in the 3′ CCA sequence was further examined. These studies indicated that the entire 3′ CCA sequence, but especially the CC sequence, is important for efficient cleavage of the model substrates we used. These observations are consistent with the notions that interactions between M1 RNA and this sequence bring the cleavage site in proximity to the active site (9,10).

It is noteworthy that the cleavage rate of the minimal substrate phe7 by M1 RNA in the presence of an EGS is at least 10-fold slower than that of the same substrate by M1PHE, and is at least

100-fold slower than that of pretRNA by M1 RNA (unpublished experiments). Therefore, it is to be expected that in *E.coli* possible substrates that contain very short RNA helices are processed at extremely low rates, if at all.

Cleavage site selection by M1GS RNA

Disruption of the RNA duplex at the 3′ side of the site of cleavage in M1GS RNA significantly diminishes cleavage efficiency. The identity of the base at the +1 position is also a determinant for cleavage site selection. When a purine is at the +1 position, cleavage occurs correctly (i.e., between the –1 and +1 site), regardless of whether the +1 position is base-paired. In contrast, aberrant cleavage occurs when a pyrimidine is at the +1 position and is not base-paired. Furthermore, cleavage of a substrate with a purine at the +1 position is more efficient than that with a pyrimidine of +1. Therefore, a purine nucleotide seems to be the dominant determinant for cleavage site selection by M1GS RNA. This observation is consistent with the notion that guanine at the +1 position, which has been found in most pretRNAs, serves as the guide nucleotide for M1 RNA and RNase P cleavage (29).

Proposed mechanism of substrate recognition by M1 RNA

It has been shown that M1 RNA binds to both the 3′ CCA and 5′ leader sequence, and brings the cleavage site in proximity to the catalytic center of M1 RNA (9,10,22). This conclusion is consistent with our results that deletion of the 5′ leader sequence (except for the nucleotide at –1) segment of substrates and the 3′ CCA sequence lead to a reduction of 50- and 250-fold in cleavage rate respectively. Our results also demonstrated that the interactions between the helix and M1 RNA are sufficient to position the cleavage site into the catalytic center.

The helix binding domain of M1 RNA has not been defined. In the case of the well-studied group I intron ribozyme, the ribozyme registers the RNA helix that contains the substrate through interactions between 2′ hydroxyl groups of the substrate and the ribozyme (32) and determines the cleavage site by recognizing a wobble G–U pair (33). However, it is likely that the helix binding domain of M1 RNA interacts in a critical way with the functional groups of bases (34) in substrates for RNase P, as well as with the 2′ OH groups at several positions (8,35), since the identity of the nucleotide at position +1 is important, Additionally, results of chemical footprinting experiments show that denaturation of the acceptor stem (to make bases more accessible) occurs during the cleavage reaction (36). Such a denaturation step can only occur after binding to M1 RNA, which recognizes initially the intact helix structure of a model substrate or the acceptor stem portion of a tRNA.

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