Artificial self-cleaving molecules consisting of a tRNA precursor and the catalytic RNA of RNase P

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ABSTRACT

We synthesized two types of chimeric RNAs between the catalytic RNA subunit of RNase P from *Escherichia coli* (M1 RNA) and a tRNA precursor (pre-tRNA); one had pre-tRNA at the 3' side to the M1 RNA (M1 RNA-pre-tRNA). The second had pre-tRNA at the 5' side of the M1 RNA (pre-tRNA-M1 RNA). Both molecules were self-cleaving RNAs. The self-cleavage of M1 RNA-pre-tRNA occurred at the normal site (5'-end of mature tRNA sequence) and proceeded under the condition of 10 mM Mg^{2+} concentration. This reaction at 10 mM Mg^{2+} was an intramolecular reaction (cis-cleavage), while, at 40 mM and 80 mM Mg^{2+}, trans-cleavage partially occurred. The self-cleavage rate was strictly affected by the distance between the M1 RNA and the pre-tRNA in the molecule. The self-cleavage of pre-tRNA-M1 RNA occurred mainly at three sites within the mature tRNA sequence. This cleavage did not occur at 10 mM Mg^{2+}. Use of M1 RNA-pre-tRNA molecule for the *in vitro* evolution of M1 RNA is discussed.

INTRODUCTION

RNase P is an endoribonuclease responsible for the 5'-processing of all tRNAs (1). This enzyme cleaves tRNA precursors to make mature 5'-ends of tRNAs and is represented in all cell types so far examined (1). In eubacteria this enzyme is a ribonucleoprotein and the RNA component is the catalytic subunit of the enzyme. The RNA alone, without the protein subunit, can cleave tRNA precursor accurately *in vitro* in the presence of high concentration of Mg^{2+} (2). RNase P RNA is only one naturally occurring trans-acting ribozyme, namely a true enzyme, among the ribozymes described to date. Other ribozymes (*e.g.*, group I introns and hammerhead and hairpin ribozymes) are originated from naturally occurring self-splicing or self-cleaving (cis-acting) RNAs, although they can be modified artificially to give true enzymes *in vitro* (3, 4). On the contrary, we have here tried to synthesize self-cleaving RNAs consisting of RNase P RNA and tRNA precursor. Since both, the enzyme and the substrate, are RNAs, it is easy to synthesize chimeric molecules between the enzyme and the substrate by *in vitro* transcription of the chimeric DNA using bacteriophage RNA polymerases (5). This artificial

molecule is expected to be a self-cleaving molecule. By studying this self-cleaving reaction, especially an optimal distance between the RNase P RNA and the tRNA precursor for self-cleavage, we can also expect to know some features of the enzyme-substrate interaction.

We tethered *Drosophila* initiator methionine tRNA precursor sequence (pre-tRNA) to M1 RNA, the catalytic RNA subunit of RNase P of *Escherichia coli*. We constructed two types of molecules; one had pre-tRNA at the 3' side to the M1 RNA (M1 RNA-pre-tRNA). The second had pre-tRNA at the 5' side of the M1 RNA (pre-tRNA-M1 RNA). Both RNAs were self-cleaving RNAs. In this paper, we report the characterization of these self-cleaving reactions. Suitability of these molecules for *in vitro* evolution experiments of M1 RNA will also be discussed.

MATERIALS AND METHODS

Enzymes and chemicals

T7 RNA polymerase was obtained from Toyobo (Osaka). Restriction endonucleases were from Toyobo, Takara Shuzo (Kyoto), or New England Biolab. Other enzymes and chemicals were purchased from commercial sources.

Constructions of plasmids

To obtain chimeric RNAs between a tRNA precursor and the M1 RNA, we constructed two *in vitro* transcribable plasmids, pYAY-DtY and pDtY-YAY, that consist of fragments from pYAY and pDtY (6, 7). The M1 RNA and pre-tRNA sequences in these plasmids were from plasmids pYAY and pDtY, respectively. The plasmids, pYAY and pDtY were digested with *Bam* HI and *Hin* dIII, respectively, and the sites were filled in with dNTPs and DNA polymerase I Klenow fragment. These linearized plasmids were again digested with *Sph* I. The M1 RNA containing fragment from pYAY and the pre-tRNA containing fragment from pDtY were ligated together to give pYAY-DtY. This plasmid was used as a template for the preparation of M1 RNA-pre-tRNA. Using other restriction enzyme recognition sites instead of *Bam* HI site of pYAY, various addition or deletion mutants in the region between M1 RNA and pre-tRNA sequences were also obtained. On the other hand, the *Hin* dIII-*Xba* I fragment containing M1 RNA sequence from pYAY was treated
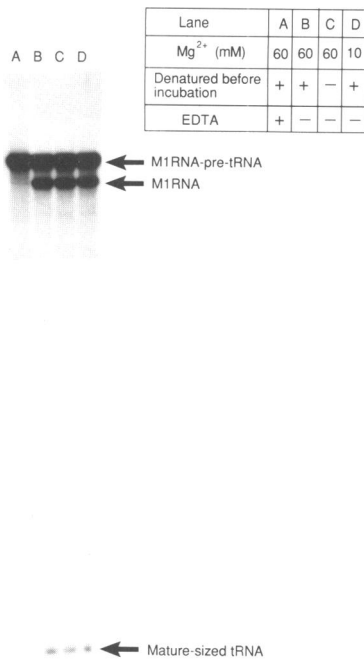
by dNTPs and DNA polymerase I Klenow fragment to obtain the blunt ends and cloned into the similarly blunt-ended *Eco* RI site of pDtY to give pDtY-YAY. This plasmid was used as a template for pre-tRNA-M1 RNA.

Preparation of RNAs

RNAs were prepared by *in vitro* transcription. The plasmid pYAY-DtY or pDtY-YAY was linearized with *Pst* I or *Eco* RI, and subjected to the transcription reaction with T7 RNA polymerase to synthesize the transcript, M1 RNA-pre-tRNA or pre-tRNA-M1 RNA, respectively. The transcription reactions and purification of RNAs were done as described (7). RNAs were internally labeled with [α-^{32}P] UTP during transcription reaction. When terminally labeled RNAs were required, non-radioactive RNAs synthesized by transcription were labeled at 5'-end with [γ-^{32}P] ATP and T4 polynucleotide kinase as described (7).

Self-cleavage reactions

The standard reaction mixture contained 50 mM Tris−HCl (pH 7.6), 100 mM NH$_4$Cl, 10−80 mM MgCl$_2$, 5%(wt/vol) polyethylene glycol, 1−10 nM 5'-end- or internally [^{32}P] labeled M1 RNA-pre-tRNA or pre-tRNA-M1 RNA in a total volume of 10 μl. Mixtures were incubated at 37°C for 0.5−2 hr and reactions were stopped by addition of 2 μl of 0.5 M EDTA. When the pre-denaturation of RNAs was done, the mixture containing 83 mM Tris−HCl (pH 7.6) and the RNA in a total volume of 6 μl was incubated at 95°C for 2 min and chilled in ice, then the other components were added to give the standard reaction condition and incubated as described above. The products were separated by electrophoresis through 5% polyacrylamide/8 M urea gels and were detected by autoradiograhy.

Cloning, nucleic acid manipulations, and analytical methods

DNA manipulations, cloning techniques, and labeling and other analytical methods for RNA were performed essentially as described (8, 9). Determination of self-cleavage sites was as described (10). Quantitative analyses of the self-cleavage reactions were performed by counting photo-stimulated luminescence of the product bands in a radiogram of the gel using a Bio-Image Analyzer BAS2000 (Fuji Film Co.; ref. 11).

RESULTS

Self-cleavage of M1 RNA-pre-tRNA and pre-tRNA-M1 RNA

We have synthesized M1 RNA-pre-tRNA and pre-tRNA-M1 RNA as described in Materials and Methods and tested those self-cleaving activities. Both molecules were self-cleaving molecules. Figures 1−3 show the nucleotide sequences and the cleavages of these molecules.

M1 RNA-pre-tRNA cleaved at the normal site (5'-end of the mature tRNA sequence; Figure 1A). The cleavage-site was confirmed by analyses of cleavage reactions using terminally labeled M1 RNA-pre-tRNA as well as labeling and terminal sequence analyses of the cleavage products. The mature-sized tRNA produced from the non-radioactive M1 RNA-pre-tRNA could be labeled at its 5'-end using T4 polynucleotide kinase and [γ-^{32}P] ATP, only after treatment with calf intestinal alkaline phosphatase (data not shown). This suggests that the mature-sized tRNA product had 5'-phosphoryl end. From the wandering spots

Figure 1. Nucleotide sequences and self-cleavage sites of chimeric molecules between M1 RNA and tRNA precursor. The region of M1 RNA is depicted by an ellipsoid. The arrows 1−4 indicate the cleavage sites. (A) M1 RNA-pre-tRNA. (B) pre-tRNA-M1 RNA.

analysis of this product and the results described above (data not shown), it was proved that the cleavage occurred exactly at the 5'-end of the mature tRNA sequence to produce 5'-phosphoryl end and 3'-hydroxyl end.

M1 RNA-pre-tRNA cleaved by incubation in the standard reaction mixture containing only 10 mM Mg^{2+} as a sole metal ion (Figure 2, lane D), although the M1 RNA requires 60 mM or higher concentration of Mg^{2+} for the cleavage of the separated pre-tRNA substrate (2). This indicates that 10 mM Mg^{2+} is enough to promote the cleavage reaction by M1 RNA. Since denaturation of this molecule before incubation did not affect the cleavage (Figure 2, lane D), 10 mM Mg^{2+} is also enough for proper folding for cleavage of this molecule.

Pre-tRNA-M1 RNA cleaved at several sites (Figures 1B and 3). Three fragments visible in Figure 3 indicate cleavages shown in Figure 1B. These sites were also confirmed by similar methods as described in the case of M1 RNA-pre-tRNA (data not shown). Almost no cleavage at 5'-end of the mature tRNA sequence occurred (Figure 3, lane C), while this cleavage was slightly observed when free M1 RNA (M1 RNA itself) was added in the reaction mixture (Figure 3, lane A). Denaturation of this RNA affects the cleavage reaction (compare Figure 3, lane C with lane D). Self-cleavage of pre-tRNA-M1 RNA did not occur at 10 mM Mg^{2+} (Figure 3, lane B), in contrast to the case of M1 RNA-pre-tRNA (Figure 2, lane D). After this, we examined the self-cleavage reaction of M1 RNA-pre-tRNA more closely.

Lane	A	B	C	D
Mg^{2+} (mM)	60	60	60	10
Denatured before incubation	+	+	−	+
EDTA	+	−	−	−

Lane	A	B	C	D	E
Mg^{2+} (mM)	60	10	60	60	60
Denatured before incubation	+	+	−	+	+
EDTA	−	−	−	−	+
Free-M1RNA	+	−	−	−	−

Figure 2. Self-cleavage of M1 RNA-pre-tRNA. M1 RNA-pre-tRNA was synthesized by *in vitro* transcription using [α-^{32}P] UTP. The RNA was incubated at 37°C for 1 hr in the standard reaction mixture as described in Materials and Methods. Mg^{2+} concentrations and pre-denaturation conditions were as indicated in the figure. In lane A, a stop solution (EDTA) was added to the reaction mixture before incubation. Products were separated by electrophoresis in a 5% polyacrylamide-8M urea gel, an autoradiogram of which is shown.

Figure 3. Self-cleavage of pre-tRNA-M1 RNA. [5'-end ^{32}P] Pre-tRNA-M1 RNA was incubated and analyzes as described in the legend of Figure 2. Electrophoresis was done in a 15% polyacrylamide-8M urea gel. An autoradiogram of Bio-Image-Analyzer BAS2000 is shown. OH$^-$ ladder was partial alkaline digest of the RNA and was prepared as described (7). The arrows 1−4 correspond to those of Figure 1B. In lane A, 1 μg of M1 RNA (transcript of pYAY; ref. 7) was added to the reaction mixture.

Effect of divalent cations on self-cleavage of M1 RNA-pre-tRNA

Self-cleavage of M1 RNA-pre-tRNA in the standard reaction mixture containing 10 or 40 mM Mg^{2+} proceeded linearly at least for 2 hr at which 20 or 40% of the RNA cleaved, respectively (data not shown). We tested effect of Mg^{2+} concentration on the rate of self-cleavage (Figure 4). No self-cleavage of M1 RNA-pre-tRNA was observed at 0−4 mM Mg^{2+} and the rate of cleavage increased with increasing concentration of Mg^{2+}, reaching a maximum at around 15−40 mM (Figure 4). When M1 RNA-pre-tRNA was synthesized by *in vitro* transcription in the T7 RNA polymerase buffer, which contains 6 mM MgCl$_2$, no cleavage of the RNA was observed during the transcription reaction. However, 2% of M1 RNA-pre-tRNA cleaved in 30 min at 6 mM Mg^{2+} (Figure 4). This difference may be due to a difference between the compositions of two buffers, the transcription buffer and the M1 RNA buffer.

Mn^{2+} and Ca^{2+} were capable of supporting self-cleavage reaction. The cleavage yield of 5% or 2.4% was obtained by incubation for 30 min at 40 mM of Mn^{2+} or Ca^{2+}, respectively. Co^{2+} was inert in promoting the self-cleavage reaction in the range of 10−40 mM.

Cleavage of M1 RNA-pre-tRNA at 10 mM Mg^{2+} is an intramolecular reaction

M1 RNA is originally a true catalytic RNA that acts in trans, although M1 RNA-pre-tRNA is a 'self-cleaving' RNA. To test whether or not this cleavage is a 'cis-cleavage' in that the M1

RNA moiety of an M1 RNA-pre-tRNA molecule acts on the pre-tRNA moiety of the same molecule, effect of concentration of the RNA on the reaction rate was examined. If this cleavage is an intermolecular 'trans-cleavage' reaction, the rate of cleavage is strongly affected by concentration of the RNA and would be expected to exhibit second-order kinetics (12, 13). Figure 5 shows that the rate of cleavage at 10 mM Mg^{2+} was not affected by concentration of the RNA. This indicates that cleavage of M1 RNA-pre-tRNA at 10 mM Mg^{2+} is an intramolecular reaction. Interestingly, however, the cleavage was partially affected by the RNA concentration at 40 and 80 mM Mg^{2+} (Figure 5). This indicates that the M1 RNA moiety of an M1 RNA-pre-tRNA molecule or a free M1 RNA produced by self-cleavage reaction of M1 RNA-pre-tRNA can act at least in part on the other M1 RNA-pre-tRNA molecules.

Effect of distance between the M1 RNA and the pre-tRNA in the molecule on the self-cleavage of M1 RNA-pre-tRNA

M1 RNA-pre-tRNA has 29-nucleotide long spacer (Figure 1A: In this paper, the term 'spacer' is restricted to the joining region between the mature tRNA and M1 RNA). To examine effect of the spacer length of M1-RNA-pre-tRNA on self-cleavage, several nucleotides were deleted or added within the spacer region of M1 RNA-pre-tRNA and self-cleavages of these variants were tested. Fortuitously, we found that the original molecule that has 29 nucleotides spacer is the most active among these variants tested (Figure 6). The spacer length of the molecule is rather strict for self-cleavage activity. Almost no cleavage occurred, when the molecule had 39-nucleotide long spacer (Figure 6). Also, insertion of 100 nucleotides or more into the spacer region inhibited completely the self-cleavage reaction of M1 RNA-pre-tRNA in the range of 10−60 mM Mg^{2+} (data not shown).
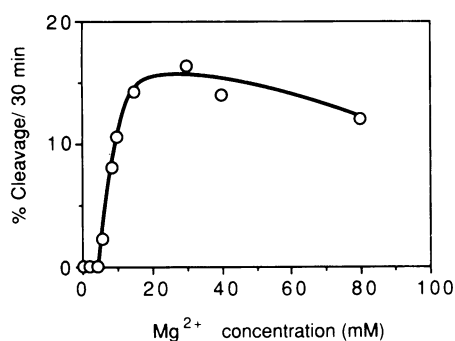
Figure 4. Effect of Mg^{2+} concentration on self-cleavage of M1 RNA-pre-tRNA. Internally [^{32}P] labeled M1 RNA-pre-tRNA (20 nM) was incubated at 37°C in the standard reaction mixture containing 0, 2, 4, 6, 8, 10, 15, 30, 40, or 80 mM Mg^{2+}. The self-cleavage rate was determined as described in Materials and Methods.
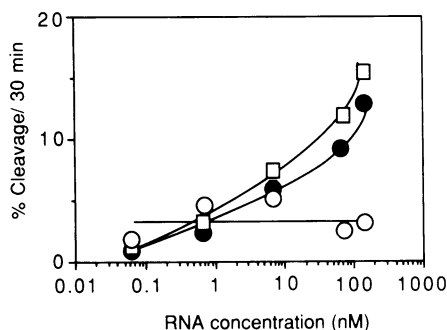

Figure 5. Effect of RNA concentration on the rate of self-cleavage. Internally [^{32}P] labeled M1 RNA-pre-tRNA at 0.068, 0.68, 6.8, 68.0, or 136 nM was incubated at 37°C in the standard reaction mixture containing 10 (open circles), 40 (open squares), or 80 (filled circles) mM Mg^{2+}. The self-cleavage rate was determined as described in Materials and Methods.

DISCUSSION

We have synthesized new type of self-cleaving RNAs. M1 RNA-pre-tRNA is the first molecule that is autocatalytically hydrolyzed to generate a 5'-phosphoryl end and a 3'-hydroxyl end. In a preliminary experiment, we have recently observed the self-cleavage of M1 RNA-model substrate (14) construct. This indicates that any other pre-tRNA sequences instead of *Drosophila* initiator methionine tRNA can probably substitute for the pre-tRNA moiety of the molecule and various self-cleaving M1 RNA-pre-tRNA molecules can be constructed.

M1 RNA-pre-tRNA molecule cleaves at the 5' end of mature tRNA sequence to produce mature-sized tRNA (Figures 1A and 2). Pre-tRNA-M1 RNA is also a self-cleaving RNA but this RNA cleaves at three sites in the mature tRNA sequence (Figures 1B and 3). These three sites have already been known to be cleaved by M1 RNA and this is a unique property of *Drosophila* initiator methionine tRNA (15). Since these unusual M1 RNA cleavages of this tRNA have been known to occur by altered conformations of this tRNA (15), the tRNA moiety of pre-tRNA-M1 RNA is thought to take exclusively the altered conformations but not the common L-shaped structure of tRNA. Also, denaturation of this RNA before incubation weakened the self-cleavage (Figure 3,
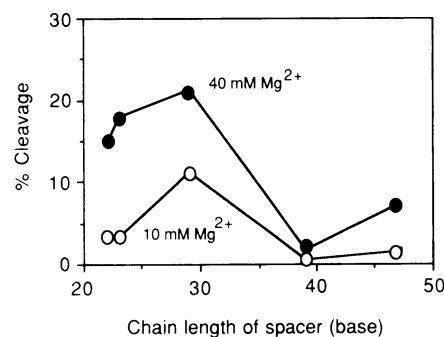

Figure 6. Effect of distance between the 3'-end of M1 RNA and the 5'-end of mature tRNA sequences in the M1 RNA-pre-tRNA molecule on self-cleavage. The spacer length of the original M1 RNA-pre-tRNA was 29-nucleotide long (Figure 1A). Several nucleotides were deleted or added within the spacer region and the mutants having 22, 23, 39, and 47 nucleotide long spacers were constructed. Self-cleavages of these molecules were tested. Internally [^{32}P] labeled mutant RNAs and the original RNA were incubated at 37°C for 1 hr in the standard reaction mixture containing 10 (open circles) or 40 (filled circles) mM Mg^{2+}. The cleavages (%) were determined as described in Materials and Methods.

lane D), whereas the denaturation of M1 RNA-pre-tRNA did not affect the self-cleavage (Figure 2, lanes B and D). Therefore, joining pre-tRNA to 5' side of M1 RNA sequence also seems to inhibit proper folding of M1 RNA moiety of this molecule under the condition used. We have not examined effect of the spacer length of pre-tRNA-M1 RNA on cleavage nor another tRNA sequence instead of *Drosophila* initiator methionine tRNA. Although these examinations may be profitable to know the enzyme-substrate interaction of the M1 RNA reaction and RNA folding properties as well, we have more closely examined self-cleaving reaction of M1 RNA-pre-tRNA as a first step.

Self-cleavage of M1 RNA-pre-tRNA occurs with 6 mM Mg^{2+} as the only metal ion (Figure 4). It has been reported that M1 RNA requires 60 mM Mg^{2+} for efficient substrate cleavage (2) and that high salt concentrations facilitate substrate binding to the RNA enzyme in the absence of protein, probably by functioning as counterions (16, 17). As shown in Figure 4, the optimum concentration of Mg^{2+} for self-cleavage of M1 RNA-pre-tRNA is 15−30 mM in our standard reaction mixture (which also contains 50 mM Tris−HCl (pH 7.6), 100 mM NH_4Cl, 5%(wt/vol) polyethylene glycol). This condition has also been used as an optimal condition for the reaction of RNase P holoenzyme (2, 16). Since the substrate pre-tRNA is covalently bound to the enzyme in M1 RNA-pre-tRNA molecule, high concentration of Mg^{2+} may be no longer required for cleavage reaction. At 0−4 mM Mg^{2+}, cleavage of M1 RNA-pre-tRNA did not occur (Figure 4). It has been reported that RNase P RNA does not require Mg^{2+} to bind pre-tRNA (17, 18). It is possible that M1 RNA-pre-tRNA takes also a productive conformation at 0−4 mM Mg^{2+}. Therefore, 6 mM may be the minimal concentration of Mg^{2+} for catalytic cleavage. At 40 or 80 mM Mg^{2+}, the cleavage of M1 RNA-pre-tRNA is a partial intermolecular trans-cleavage (Figure 5). This indicates that these high concentrations of Mg^{2+} may act as counterions to facilitate intermolecular enzyme-substrate complex formation.

Fortunately, the M1 RNA-pre-tRNA originally constructed is the most active for cleavage among variants having various spacer lengths. Self-cleavage activity is rather strongly affected by the spacer length of the molecule (Figure 6). The original M1 RNA-

pre-tRNA has 29 nucleotides as the spacer (Figure 1A). This spacer length may be suitable to take a productive conformation of this molecule for self-cleavage. It is possible that, also in the configuration of an intermolecular enzyme-substrate complex, the 3'-end of M1 RNA may be located 29-nucleotide distant from the cleavage site of the substrate. However, since we tested optimal spacer length only using the variants derived from the original molecule (Figure 1A), it cannot be at present concluded that this length (29 nucleotides) of any sequence is optimal spacer length for self-cleavage. To confirm optimal spacer length, experiments using spacers of different sequences may be required.

Here we would propose that M1 RNA-pre-tRNA can be used for *in vitro* evolution experiments of M1 RNA. Joyce *et al.* developed an *in vitro* evolution system of the *Tetrahymena* group I intron (19−21). In this case, it was relatively easy to select an active variant from a population of randomly mutated variants, because active group I intron molecules are joined to a part of substrate during the reaction (3). Using an oligonucleotide complementary to the substrate sequence joined, active group I intron sequences can be amplified by the reverse transcription and polymerase chain reaction (20, 21). In the case of M1 RNA, however, selection of an active M1 RNA variant from a population of variants was difficult, because there was no way to differentiate active M1 RNA variants from a population of mutants. However, active M1 RNA sequences are now selectable using the self-cleavage reaction of M1 RNA-pre-tRNA. As described above, under the condition of 10 mM Mg^{2+}, self-cleavage reaction of M1 RNA-pre-tRNA is an intramolecular reaction. Therefore, only active M1 RNA sequences can be separated as a product band from inactive or uncleaved M1 RNA-pre-tRNA molecules by gel electrophoresis, as shown in Figure 2. As is the case of group I intron (20, 21), populations of M1 RNA sequences may be pushed to evolve using this selection method. Although this system is restricted to this self-cleavage reaction, it may be expected to obtain new M1 RNAs of novel properties.

ACKNOWLEDGEMENTS

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