The catalytic RNA of RNase P from *Escherichia coli* cleaves *Drosophila* 2S ribosomal RNA in vitro: a new type of naturally occurring substrate for the ribozyme

Yoshiaki Hori, Terumichi Tanaka, Yo Kikuchi*

Division of Bioscience and Biotechnology, Department of Ecological Engineering, Toyohashi University of Technology, Tempaku-cho, Toyohashi, Aichi 441-8580, Japan

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Abstract We have found that the catalytic RNA of RNase P of *Escherichia coli* (M1 RNA) can cleave 2S ribosomal RNA (2S rRNA) of *Drosophila melanogaster* at specific positions in vitro. The cleavage mainly occurred at two sites between nucleotides 11 and 12, and between 16 and 17 of 2S rRNA. Kinetic analyses of the reaction revealed that a dimer caused by intermolecular interaction of 2S rRNA may be the substrate for the cleavage between 11 and 12, while a simple monomer is the substrate for the cleavage between 16 and 17. Substrate recognition by M1 RNA is also discussed.

Key words: RNase P; Ribozyme; rRNA; Hyperprocessing; M1 RNA; *Drosophila*

1. Introduction

Hyperprocessing is defined as a further processing of mature RNA that produces another functional RNA [1–3]. In *Drosophila*, a 5′-half fragment (nucleotides 1–39) produced by hyperprocessing of initiator methionine tRNA is used as the primer for minus-strand reverse transcription of retrovirus-like particles [4]. We have reported that the catalytic RNA subunit of RNase P (M1 RNA) catalyzes this hyperprocessing in vitro [1,2]. *Drosophila* initiator methionine tRNA can be cleaved by M1 RNA in vitro at the site between nucleotides 39 and 40, as well as at other sites [1,2]. Usually, RNase P is a tRNA processing enzyme that cleaves tRNA precursors to make mature 5′-ends of tRNAs [5,6]. This enzyme is a ribonucleoprotein, and the RNA component of eubacterial variants of RNase P has catalytic activity. Hyperprocessing of *Drosophila* initiator methionine tRNA by M1 RNA is dependent of the occurrence of an altered conformation of this tRNA substrate. The tRNA with an altered conformation is the substrate for the catalytic RNA of RNase P in the in vitro hyperprocessing reaction [1,2]. Since the hyperprocessing and the conformational change may be the crucial step to produce a new functional (primer) tRNA fragment, screening of other conformationally changeable tRNAs, if they exist, may be a clue to find out new biological functions of tRNAs. Using the in vitro hyperprocessing reaction by M1 RNA, we have attempted to screen conformationally changeable tRNAs from *Drosophila* [7]. To search new tRNAs that can be hyperprocessed in vitro, we used total mature tRNA preparation from *Drosophila* as substrate for in vitro M1 RNA reaction. During the course of this experiment, we found that 2S ribosomal RNA (2S rRNA) of *Drosophila* can be hyperprocessed by M1 RNA. *Drosophila* 5.8S rRNA has been known to be processed into two pieces. The 2S rRNA is 30 nucleotides long and the 3′-piece of the 5.8S rRNA [8].

Here, we report that M1 RNA can further cleave the 2S rRNA in vitro and that the direct substrate for one cleavage reaction may be a dimer of 2S rRNA while a simple monomer is also a substrate for the other cleavage by M1 RNA.

2. Materials and methods

2.1. Preparation of RNAs and chemicals

The 2S rRNA was prepared from *Drosophila* adult flies. *Drosophila* adult flies were kindly supplied by Drs. R. Ueda and K. Kuroda of Mitsubishi Kasei Institute of Life Sciences. First, total RNA was extracted by the hot phenol method according to Jowett [9]. The 2S rRNA was purified by 10% polyacrylamide gel electrophoresis and labeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase essentially as described [1]. M1 RNA was prepared by in vitro transcription with T7 RNA polymerase (Toyobo) of plasmid pYAY as described previously [1,10]. This preparation was used as catalytic M1 RNA throughout this study. Other enzymes and chemicals were purchased from commercial sources as described [1,2,4,10].

2.2. Cleavage reactions

The standard reaction mixture contained 50 mM Tris–HCl (pH 7.6), 100 mM NH_4Cl, 60 mM $MgCl_2$, 5% (w/v) polyethylene glycol, 1.56 μM M1 RNA and 0.05–6.4 μM 5′-end-labeled 2S rRNA in a total volume of 20 μl. The mixture was incubated at 37°C for 1 h, and the reaction was stopped by addition of 4 μl of 0.5 M EDTA. The products were separated by electrophoresis through 20% polyacrylamide, 8 M urea gels and were detected by autoradiography essentially as described [1].

2.3. Kinetic analyses

To test whether the direct substrate is a dimer of 2S rRNA, as well as to obtain kinetic parameters of the reaction, kinetic analyses of the cleavage reactions were performed. Different concentrations (0.05–6.4 μM) of substrate were added to the standard reaction mixture and the values of reaction rate were determined. The substrate was previously fully denatured by heating to avoid a statically pre-existing dimer molecule presumed. Quantitative analyses of the reactions were performed by counting photo-stimulated luminescence of the product bands in a radiogram of the gel using PhosphorImager (Molecular Dynamics).

3. Results and discussion

Since our 5′-end ^{32}P-labeled total mature tRNA prepara-

*Corresponding author. Fax: (81)-532-44 6929.
E-mail: kikuchi@eco.tut.ac.jp

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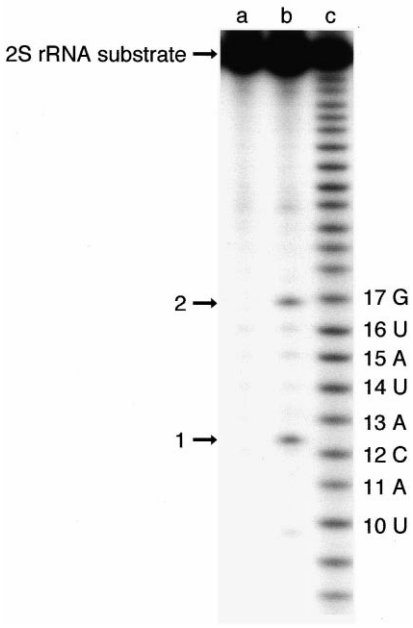

Fig. 1. Cleavage of the 5′-end-labeled 2S rRNA prepared from *Drosophila* adult flies by M1 RNA: electrophoretic analysis of the reaction. Mixtures were incubated for 1 h and analyzed on a 20% polyacrylamide, 8 M urea gel. An autoradiogram of the gel is shown. Lane a, minus M1 RNA control; lane b, the complete reaction mixture; lane c, partial alkaline digest of the substrate. The nucleotide sequence of residues 10–17 is shown on the right. The nucleotide sequence was determined by comparison of the degradation pattern of lane c with those of partial alkaline digests in enzymatic sequencing gel (data not shown). These bands can easily be recognized by the characteristic spacing they produce in the ladders; e.g. removal of a G residue results in a more pronounced increase of electrophoretic mobility as compared with removal of A, U or C [1]. The arrows 1 and 2 on the left indicate the cleavages between 11 and 12 and between 16 and 17.

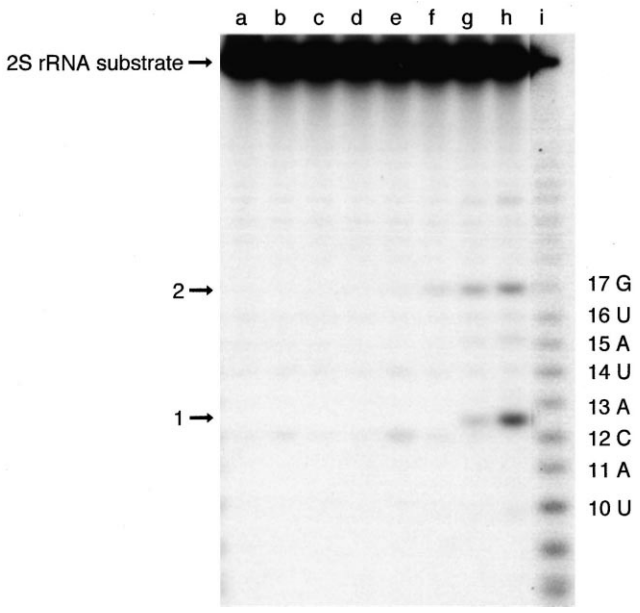

Fig. 2. Effect of Mg^{2+} concentration on the cleavage reaction. The 5′-end-labeled 2S rRNA was incubated in the reaction mixture containing 0–80 mM Mg^{2+}. Electrophoretic analysis was performed and an autoradiogram is shown. Lane a, minus M1 RNA control; lanes b–h, the reactions containing 0, 2, 5, 10, 20, 40 and 80 mM Mg^{2+}, respectively; lane i, partial alkaline digest of the substrate. The nucleotide sequence of residues 10–17 is shown on the right. The nucleotide sequence was determined as described in Fig. 1. The arrows 1 and 2 on the left indicate the cleavages between 11 and 12 and between 16 and 17.

tion always contained the 2S rRNA, we have tested whether this molecule can be a substrate for the hyperprocessing reaction by M1 RNA. The standard mixture containing 0.5 μM substrate was incubated at 37°C for 1 h, and the reaction was stopped by addition of 2 μl of 0.5 M EDTA. The products were separated by electrophoresis through 20% polyacrylamide, 8 M urea gels and were detected by autoradiography. Fig. 1 shows an autoradiogram of this reaction. Product bands are visible in lane b in Fig. 1. The mobilities of these product fragments did not correspond to those of any fragments produced by partial alkaline hydrolysis (Fig. 1, lane c) which would contain 2′- or 3′-phosphate ends. This indicates that the fragments produced in the reaction (Fig. 1, lane b) had 3′-OH ends, as expected for cleavage catalyzed by M1 RNA activity. Furthermore, the appearance of these product bands depended on the presence of both M1 RNA and more than 40 mM Mg^{2+} in the reaction mixture (Figs. 1 and 2). M1 RNA requires a Mg^{2+} concentration of >30 mM for the substrate cleavage [1]. From these experiments, we have concluded that these fragment bands are cleavage products generated by M1 RNA. Two fragments (arrows 1 and 2 of Fig. 1) indicate cleavage between nucleotides 11 and 12, and between 16 and 17, respectively.

From the substrate specificity of M1 RNA reported so far [11], we have presumed that a dimer formation by intermolecular interaction of 2S rRNA may be necessary for these

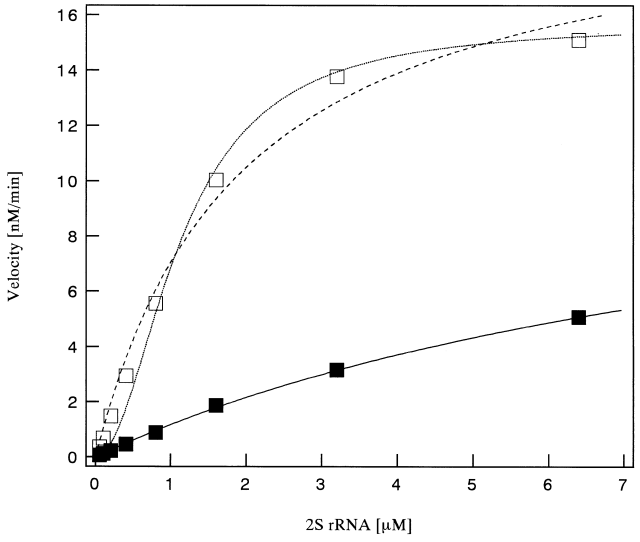

Fig. 3. Kinetic analyses of the cleavage reactions. The standard reaction mixture containing 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 or 6.4 μM 5′-end-labeled 2S rRNA was incubated and each initial reaction rate was determined as described in Section 2. Open squares, cleavages between 11 and 12; closed squares, cleavages between 16 and 17. Solid line and dashed line, theoretical Michaelis–Menten curve for cleavage between 16 and 17, and between 11 and 12, respectively. These lines were obtained by fitting the data to the equation, $v=[S]V_{max}/([S]+K_m)$. Broken line, theoretical curve for dimer substrate. This line was obtained by fitting the data to the equation, $v=[S]^2V_{max}/([S]^2+K_m)$. Plots from the experimental data of cleavage between 11 and 12 (open squares) agree more closely with this sigmoidal curve than with the Michaelis–Menten curve.

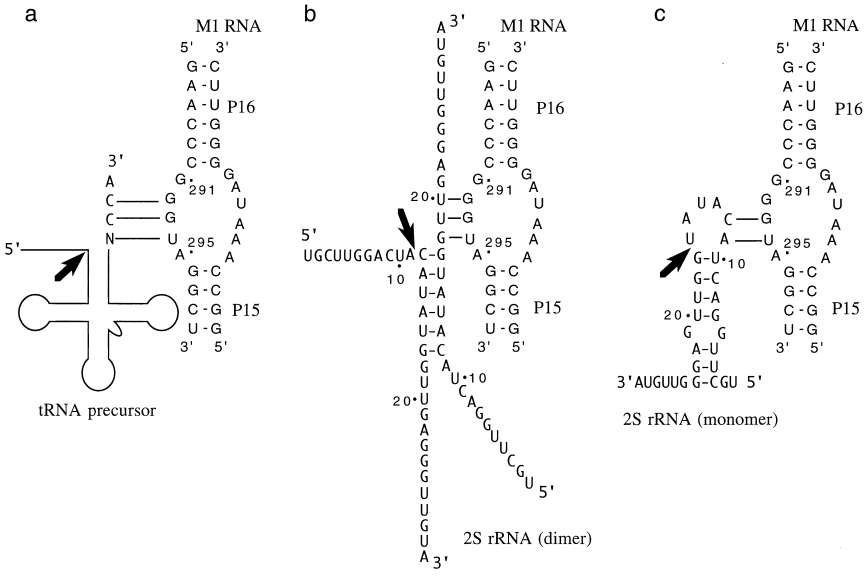

Fig. 4. Models for the cleavage site selection. Arrows indicate cleavage sites. a: The base pairing between 292–294 (GGU) of M1 RNA and NCCA of the 3′-end of tRNA precursor. Only a part around the P15 and P16 is depicted for M1 RNA. P15 and P16 are stems (pairs) in M1 RNA [6]. These base pairs have an important role in the cleavage site selection in M1 RNA reaction [12]. b: Possible dimer model and pairing between 292–294 (GGU) of M1 RNA and 18–20 (GUU) of 2S rRNA. c: Possible monomer model and pairing between 293–294 (GU) of M1 RNA and 11–12 (AC) of 2S rRNA for the cleavage between 16 and 17 of 2S rRNA.

cleavages. To test whether or not the substrate is a dimer of 2S rRNA, the effect of concentration of the 2S rRNA on the reaction rate was examined. As shown in Fig. 3, plots for the cleavage reaction between 11 and 12 were found to fit a theoretical curve for a dimer substrate model, especially in the substrate concentrations of 0.8–6.4 μM, rather than a typical Michaelis–Menten curve. This indicates that the direct substrate for the cleavage between 11 and 12 is not a monomer of 2S rRNA but probably a dimer. Apparent K_m and k_{cat} values for the cleavage between 11 and 12 were calculated to be 1.29 μM and 0.010 min^{-1}, respectively. Fig. 4b shows a dimer model of 2S rRNA. These structures may be recognized by M1 RNA. Interestingly, however, plots for the cleavage reaction between 16 and 17 were found to perfectly fit a typical Michaelis–Menten curve (Fig. 3). This indicates that the substrate for the cleavage between 16 and 17 may be a simple monomer. The K_m and k_{cat} values for the cleavage between 16 and 17 were calculated to be 10.2 μM and 0.0084 min^{-1}, respectively. This K_m value is two orders of magnitude higher than that of the cleavage of the tRNA precursor. Similarly, the k_{cat} value is also two orders of magnitude lower than that of the cleavage of the tRNA precursor so far reported [14]. This cleavage between 16 and 17 may be explained by a model shown in Fig. 4c.

M1 RNA has a bulge between helices P15 and P16 (P15/16 bulge) as shown in Fig. 4a. The GGU sequence in the bulge (292–294) has been shown to be important in binding the NCCA 3′-end of pre-tRNA [12]. This interaction plays an important role for the cleavage site selection (Fig. 4a). The nucleotides 18, 19 and 20 (GUU) of 2S rRNA instead of NCC of pre-tRNA may be recognized by this bulge of M1 RNA for cleavage between 11 and 12 (Fig. 4b). Also, the nucleotides 11 and 12 (AC) may be recognized by the sequence in the bulge to be cleaved between 16 and 17 (Fig. 4c). Kufel and Kirsebom recently reported that if a substrate has different cleavage sites, these sites are aligned differently in the active site of M1 RNA [13]. In the case of our 2S rRNA, these two different cleavage sites may also be aligned differently on M1 RNA. Our kinetic data also revealed that these two cleavage reactions are independent from each other.

Several molecules other than a tRNA precursor or the 4.5S RNA of *Escherichia coli* have previously been reported to be substrates for RNase P. The enzyme or M1 RNA alone cleaves the tRNA-like structure of the 3′-end of turnip yellow mosaic virus RNA [14,15]. It is well known that the model substrate is an efficient substrate for RNase P. The model substrate is the smallest substrate for M1 RNA, retaining only the domain of the aminoacyl stem, the TΨC stem and loop, and the 3′-terminal CCA sequence of a tRNA [11,16]. Also, M1 RNA can cleave RNA substrates that lack the conserved features of natural substrates of RNase P if an external guide sequence (EGS) is present. The EGS contains a sequence complementary to the substrate and a 3′-CCA sequence [17]. It has been reported that a dimer of an artificial self-complementary EGS can also be cleaved by *E. coli* RNase P [17]. Although this was the first reported dimer substrate for RNase P, the 2S rRNA may be the first reported naturally occurring dimer substrate for RNase P.

We have reported here that *E. coli* RNase P cleaves *Drosophila* 2S rRNA. This reaction can never occur in naturally living cells. However, characterization of RNA cleavage reaction by M1 RNA may be important to consider an ancient RNA world [18]. Many RNA enzymes like M1 RNA are thought to have existed in a very early era in molecular evolution [18–20]. The ability of M1 RNA that recognizes higher-order structures of RNA may have had a very important role to determine the shapes of contemporary RNAs during the era of the RNA world.

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