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Interaction of 10Sa RNA with ribosomes in *Escherichia coli*

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Abstract 10Sa RNA is a bacterial small stable RNA, in which the 5′- and 3′-end sequences are folded into a tRNA-like structure. The RNA accepts alanine in vitro, and interacts with 70S ribosomes in the cells. In this study, we examined the ribosome-binding properties of *Escherichia coli* 10Sa RNA in vivo, and found that the aminoacylation ability of 10Sa RNA with alanine is necessary for the binding to 70S ribosomes. 10Sa RNA was also found to bind only to 70S monosomes and not to polysomes. Recently, *E. coli* 10Sa RNA was suggested to be used as mRNA for tag peptides, which were found to attach to the C-termini of truncated peptides synthesized in vivo. The present results are consistent with the 'trans-translation' model, which has been proposed for tag-peptide synthesis.

Key words: *Escherichia coli*; 10Sa RNA; Ribosome; Polysome; Aminoacylation; Translation

1. Introduction

10Sa RNA is one of the small stable RNAs found in *Escherichia coli* [1]. So far, 10Sa RNA homologs or its genes have been found in seven other bacterial species [2–6]. We previously reported the existence of tRNA-like structures in the 5′- and 3′-end regions in all 10Sa RNAs [4]. Furthermore, 10Sa RNA is aminoacylatable with alanine in vitro, and interacts with 70S ribosomes in vivo [4,7,8]. Recently, *E. coli* 10Sa RNA has been shown to encode the sequence for the tag peptide, which is found to attach to the C-terminus of truncated polypeptide synthesized in vivo [9]. Keiler et al. [10] have shown that attachment of tag peptide occurs when polypeptides are translated from mRNAs lacking a stop codon, and proposed a 'trans-translation' model for tag synthesis. According to the model, alanine-charged 10Sa RNA enters the ribosome when translation is stopped at the 3′-end of the truncated mRNA, and translation of the tag-coding sequence occurs by switching the template from mRNA to 10Sa RNA. We have also shown that 10Sa RNA itself directly serves as an mRNA for tag peptides using an in vitro translation system, and that the alanylation of 10Sa RNA is required for template activity for tag synthesis (Himeno et al., submitted for publication). In this reaction, 10Sa RNA plays a dual function both as an alanine tRNA and as an mRNA for the tag peptide.

In the present study, we analyzed the ribosome-binding properties of *E. coli* 10Sa RNA in vivo to assess the validity of the above model. We have constructed a mutant 10Sa RNA which cannot accept alanine, and examined its ribosome binding ability. We also investigated the distribution of 10Sa RNA in the cell lysate to ascertain whether it binds to polysomes.

2. Materials and methods

2.1. Strains and culture conditions

E. coli strain W3110 (wild-type) and the 10Sa RNA gene-deleted mutant (W3110Δ*ssrA*), a derivative of W3110 [7], were kindly provided by Dr. H. Inokuchi of Kyoto University; *E. coli* strain A19 (RNase I$^-$) was used for polysome preparation. Cells were cultured in LB medium [4] at 37°C with vigorous aeration, and harvested at the mid-log phase by centrifugation (6000 rpm, 5 min). The cells were washed with ice-cold TMK-1 buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 60 mM KCl, 6 mM 2-mercaptoethanol, pH 7.8), and kept at −80°C.

2.2. Construction of recombinant plasmids containing ssrA and its derivative

The wild-type 10Sa RNA gene, *ssrA*, was amplified from *E. coli* W3110 DNA by polymerase chain reaction (PCR) with the first primer (5′)-ACGATAACGCTCTAGAGGCTGGTCATGGCGCTCA-(3′) including the sense strand sequence of the 5′-upstream region of the promoter of the RNA gene and *Xba*I site (underlined), and the second primer (5′)-AGTTCTCTTCAAGCTTCGCGGGA-CAAATTGAGG-(3′), including the antisense strand of the 3′-downstream sequence of the transcription terminator and *Hin*dIII site (underlined). The mutant 10Sa RNA gene *ssrA(A3)*, which has a G to A mutation at the third base from the 5′-end of the RNA, was amplified using the first primer (5′)-ATAACGCTCTAGAGGCTGGTCATGG-CGCTCATAAATCTGGTATACTTACCTTTACACATTGGAGC-(3′), including the G to A mutation (bold letter) (Fig. 1A). The sequences of primers were designed according to the reported *ssrA* and its flanking sequences [7,11]. The amplified DNA fragments were digested with *Xba*I and *Hin*dIII, ligated to an *Xba*I-*Hin*dIII fragment of the plasmid pGEMEX-2 DNA, and transformed to W3110Δ*ssrA* cells. The transformant colonies containing the recombinant plasmids were selected on LB plates containing 50 μg/ml ampicillin. The resulting recombinant plasmids were named p10Sa(W) and p10Sa(A3), respectively. The RNA products of the transformant cells were isolated from the transformant cells, and the sequences of the 3′- or 5′-end regions were determined by enzymatic methods as described in a previous paper [4].

2.3. Alanylation of 10Sa RNA

The reaction mixture (50 μl) contained 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 2.5 mM ATP, 26.2 mM L-[U-^{14}C]alanine (5.62 GBq/mmol), 0–0.4 μg 10Sa RNA and 0.9 units of alanyl-tRNA synthetase (1 unit was defined as the amount of enzyme which catalyzes the incorporation of 1 nmole of alanine into $tRNA^{Ala}$ in 10 min). Alanyl-tRNA synthetase was partially purified from *E. coli* W3110 cells as described [12]. The mixture was incubated at 37°C for 15 min, and trichloroacetic acid insoluble radioactivity was measured by a liquid scintillation counter.

2.4. Sucrose gradient centrifugation

Frozen cells (about 0.5 g) were ground with an equal amount of alumina powder, extracted with 1 ml TMK-2 buffer (10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 300 mM KCl, 6 mM 2-mercaptoethanol) containing 50 units of DNase I (FPLC pure, Pharmacia-LKB). The extract was centrifuged at 12000 rpm for 5 min to remove cell debris and alumina powder, and a 0.5 ml portion of the supernatant fraction was loaded on a 5–20% linear sucrose gradient (25 ml) made in TMK-2 buffer. Centrifugation was carried out at 22000 rpm for 6 h at 4°C

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in a Hitachi RPS-25 rotor. Fractions (1 ml) were collected from the bottom of the tube. A 0.2 ml portion of each fraction was taken and used to measure the absorbance at 260 nm.

2.5. Northern hybridization

RNA was extracted by the phenol extraction method from each tube, and dissolved in 10 μl distilled water. A 1 μl portion from each preparation was fractionated by 1.5% agarose gel containing 5% formaldehyde and transferred to a Nylon membrane (Hybond-N^+, Amersham). Northern hybridization was performed using DIG-labeled synthetic DNA probes as described in a previous paper [4]. The sequences of the probes are (5′)-TGCCAGCTGCGGACGGA-CACGCCACTAACA-(3′) for 10Sa RNA (complementary to the nucleotide position 251–280), and (5′)-GCAGGCGCTCTCCCAGCT-GAGCTAT-(3′) for $tRNA^{Ala}$ (position 7–31).

2.6. Polysome preparation and fractionation

E. coli lysates were prepared according to the method of Phillips et al. [13]. *E. coli* strain A19 cells (about 0.5 g) were harvested at the mid-log phase and washed with 10 ml TS buffer (0.1 M Tris-HCl (pH 7.8), 20% (w/v) sucrose, 0.1 mg/ml chloramphenicol). The cells were suspended in 1.6 ml TS buffer, and spheroplasts were formed by mixing the suspension with 160 μl of 1 mg/ml lysozyme and 3.5 μl of 0.5 M EDTA for 3 min at 0°C, followed by the addition of 1/10 volume of 0.1 M $MgSO_4$. The spheroplasts were collected by centrifugation at 4000 rpm for 5 min, resuspended in 0.7 ml TMK-1 buffer containing 3 U/ml DNase I, and lysed by the addition of 190 μl 3% Brij 58. The lysate was clarified by centrifugation at 12000 rpm for 10 min and loaded on a 10–40% linear sucrose gradient (25 ml) made in TMK-1 buffer. Centrifugation was carried out at 22000 rpm for 4 h at 4°C.

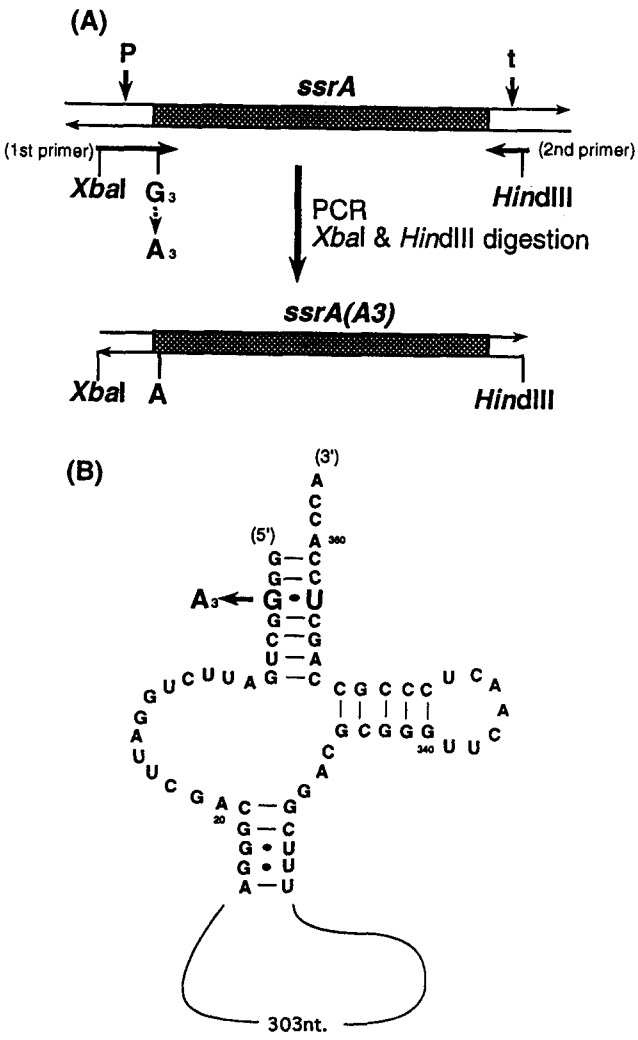

Fig. 1. Construction of recombinant plasmid containing a mutant 10Sa RNA gene. The DNA fragment including the mutant *ssrA*, having a G to A exchange at the third base position from the 5′-end of the RNA coding sequence, was amplified from *E. coli* genomic DNA by PCR using the first primer including the G to A substitution and the *Xba*I site, and the second primer containing the *Hin*dIII site (A). P, promoter; t, terminator. The fragment was digested with *Xba*I and *Hin*dIII, and the product was ligated into the *Xba*I-*Hin*dIII sites of plasmid pGEMEX-2. The in vivo product of the mutant *ssrA(A3)* has an A-U pair instead of the G-U wobble pair at the third base-pair position in the amino acid-acceptor stem of the tRNA-like structure in 10Sa RNA (B).

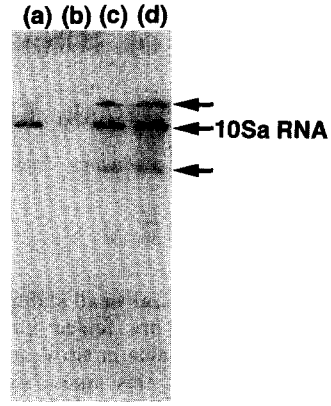

Fig. 2. Accumulation of 10Sa RNA in the transformant cells. The total RNAs were prepared from cells of *E. coli* strain W3110 (a), W3110Δ*ssrA* (b), W3110 transformed with plasmid p10Sa(W) (c), and that with p10Sa(A3) (d), and separated by 1.5% agarose gel electrophoresis. The 10Sa RNA sequence was detected by Northern hybridization using (3′)DIG-labeled oligonucleotides complementary to 10Sa RNA as a probe. The extra bands (arrows) detected in lanes (c,d) are a precursor and a degradation product of 10Sa RNAs.

3. Results

3.1. Construction and expression of a mutant 10Sa RNA gene

A mutant of the 10Sa RNA gene (*ssrA*), whose product has an A residue instead of G at the third base position of the RNA (10Sa(A3)), was constructed by site-directed mutagenesis with PCR (Fig. 1A), and was inserted into multicopy plasmid pGEMEX-2. The mutant RNA product contains an A-U pair instead of a G-U wobble pair at the third base-pair position of the amino acid-acceptor stem in the tRNA-like structure (Fig. 1B). The recombinant plasmid (p10Sa(A3)) was transformed to *E. coli* strain W3110Δ*ssrA*, a deletion mutant of *ssrA* [7]. The recombinant plasmid containing the wild-type *ssrA* was also constructed (p10Sa(W)) and used for control experiments. As shown in Fig. 2, the plasmid-encoded *ssrA(W)* and *ssrA(A3)* were expressed in the transformant cells. At least three RNA bands were detected by Northern hybridization; the major band corresponded to the control 10Sa RNA band. The partial sequencing including the 5′- and 3′-end regions of the RNA extracted from major band revealed that it contains the end sequences identical to those of the mature-type 10Sa RNA [7]. The longer and shorter bands may be a precursor [7] and a degradation product of 10Sa RNA, respectively. Northern hybridization (Fig. 2) showed that the amount of 10Sa RNA in the transformant cells was 3–5-times greater than that in the wild-type parental cells (W3110). While the growth rates of the transformant cells in LB medium at 37°C were lower (about half) than

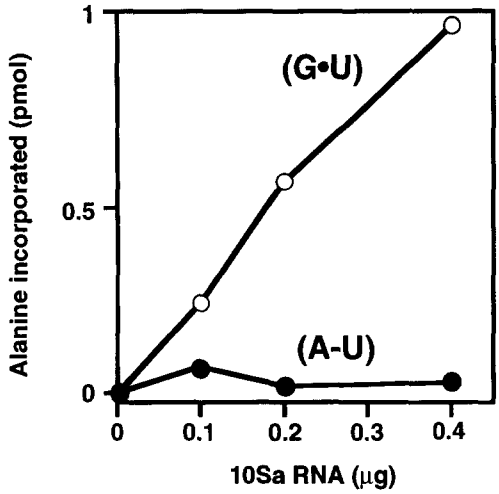

Fig. 3. Alanylation of 10Sa RNA and its mutant possessing A-U at the third base-pair of the acceptor stem. Alanylation of wild-type (○) or mutant (●) 10Sa RNA. The reaction mixture (50 μl) was incubated at 37°C for 15 min, and cold trichloroacetic acid insoluble radioactivity was measured in a liquid scintillation counter.

that of the strain W3110Δ*ssrA* without plasmids, no significant difference of the growth rates between the transformant with p10Sa(W) and that with p10Sa(A3) was observed.

The wild-type and mutant RNAs were isolated from each transformant cell, and their aminoacylation abilities with alanine were examined in vitro. As expected, the wild-type 10Sa(W) RNA could be aminoacylated with alanine, but the mutant 10Sa(A3) RNA could not (Fig. 3).

3.2. Ribosome binding

The extracts of the transformant cells were separated by sucrose density gradient centrifugation, and the distribution of 10Sa RNA was examined by Northern hybridization. A considerable amount of the wild-type 10Sa(W) RNA cosedimented with 70S ribosomes (Fig. 4A), confirming the earlier results [4,8]. However, the mutant 10Sa(A3) RNA did not bind to 70S ribosomes, showing that the alanylation ability of 10Sa RNA is necessary for the 70S ribosome binding.

3.3. Polysome binding

Next, we prepared the lysates of *E. coli* cells to determine whether 10Sa RNA binds to polysomes. Sucrose density gradient centrifugation of the lysate and Northern hybridization analysis showed that most of the 10Sa RNAs were located at 70S ribosome and soluble fractions; no significant hybridization signal was observed in the polysome fraction (Fig. 5A). For further confirmation, the polysome and 70S monosome fractions were respectively pooled, and RNA was extracted from each fraction. The RNAs from these two fractions separated by agarose gel electrophoresis were probed for 10Sa RNA, and for $tRNA^{Ala}$ as a control. As seen in Fig. 5B, 10Sa RNA was detected only in the 70S ribosome fraction, while $tRNA^{Ala}$ was detected in both the polysome and 70S fractions. It is thus clear that 10Sa RNA interacts with 70S ribosomes but not with polysomes.

4. Discussion

Our previous study showed that 10Sa RNA of *Bacillus subtilis* interacts predominantly with 70S ribosomes, but not with dissociated ribosomal subunits [4]. Essentially, the same results were obtained in *E. coli* [8]. The trans-translation model [10] predicts that alanine-charged 10Sa RNA binds to the A-site of the stalled 70S ribosome, and thus alanylation of RNA is essential for tag-peptide synthesis on the ribosome. In the present study, we constructed a mutant 10Sa RNA having an A-U pair instead of a G-U pair at the third position to test this trans-translation hypothesis. As we have already reported, all 10Sa RNAs have a G-U wobble at the third base-pair position of the amino acid acceptor stem in the tRNA-like structure [4,7] (see Fig. 1), that is a major identity determinant of $tRNA^{Ala}$ [14,15]. In fact, 10Sa RNAs of *E. coli* and *B. subtilis* have been shown to accept alanine in vitro [4,7]. Here, we confirmed that the mutant 10Sa RNA does

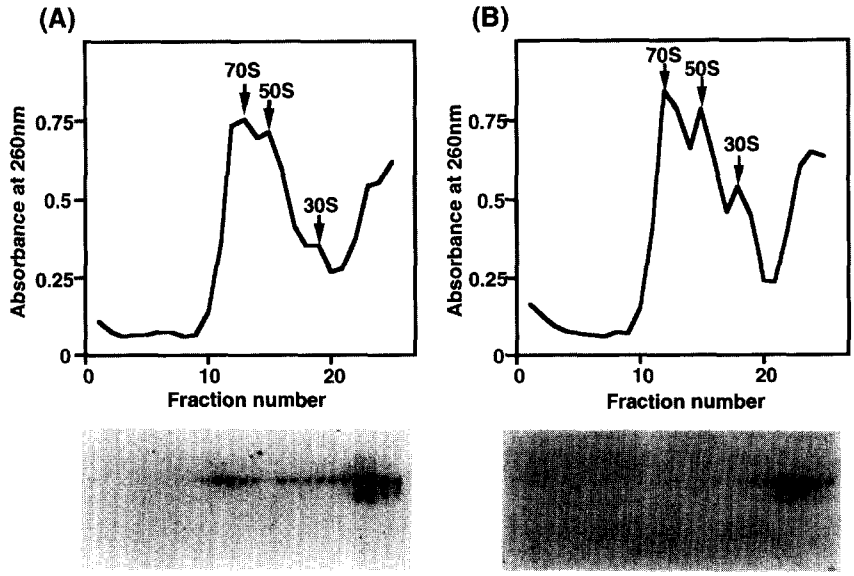

Fig. 4. Binding of 10Sa RNA to ribosomes. Crude extracts of *E. coli* W3110Δ*ssrA* transformed with plasmid p10Sa(W) (A) or plasmid p10Sa(A3) (B) were fractionated by sucrose density gradient centrifugation (upper panels). RNA from each fraction was separated by 1.5% agarose gel electrophoresis, and 10Sa RNAs were detected by Northern hybridization (lower panels).

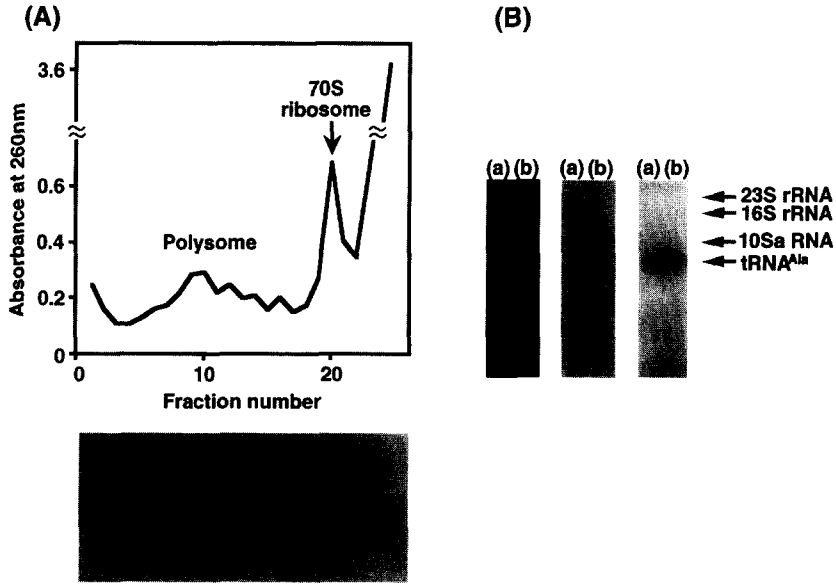

Fig. 5. Distribution of 10Sa RNA in cell lysate. (A) The cell lysate of *E. coli* A19 was separated by sucrose density gradient centrifugation (upper panel). RNA extracted from each tube was separated by agarose gel electrophoresis, and 10Sa RNA was detected by Northern hybridization (lower figure). (B) The polysome fraction (tube nos. 4–10 of (A)) and the 70S fraction (tube nos. 12–15) were respectively pooled, and equal amounts of RNAs extracted from the fractions were separated by agarose gel electrophoresis and stained by ethidium bromide (left). 10Sa RNA (center) or $tRNA^{Ala}$ (right) was detected by Northern hybridization. (a) RNA from polysomes; (b) RNA from 70S ribosomes.

not accept alanine in vitro, while the wild-type *E. coli* 10Sa RNA is aminoacylatable (Fig. 3). The observation that only aminoacylatable 10Sa RNA can bind to 70S ribosomes (Fig. 4) is thus consistent with the trans-translation model. 10Sa RNA contains a complete amino acid acceptor stem including the CCA-(3′) sequence and a TΨC-arm [4,7], which are the minimum requirements for the formation of the ternary complex of aminoacyl-tRNA, EF-Tu and GTP [16]. It is conceivable that alanyl-10Sa RNA also enters the A-site of ribosome as a ternary complex with EF-Tu and GTP, as in the case of aminoacyl-tRNA binding to the ribosome.

According to the trans-translation model [10], when translation is stopped at the 3′-end of the truncated mRNA lacking a stop codon, alanyl-10Sa RNA enters the ribosome A-site and translation of the tag-coding sequence in 10Sa RNA begins. At this moment, the pre-existing truncated mRNA, which consists of the polysome, is released from the 10Sa RNA-bound ribosome. As a result, tag-peptide translation occurs solely on the 10Sa RNA-bound 70S monosome. The observation that 10Sa RNA binds only to 70S ribosomes but not to polysomes (Fig. 5) thus supports the above model.

10Sa RNA has two functions in the trans-translation reaction: the first as an alanine tRNA and the second as an mRNA for the tag peptide. The ribosome binding properties described in this paper demonstrate that the function as a tRNA is a prerequisite for that as an mRNA of 10Sa RNA.

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