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Note

Functional Study of the Residue C899 in the 900 Tetraloop of *Escherichia coli* Small Subunit Ribosomal RNA

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A mutant ribosome bearing C899G in the 900 tetraloop of *Escherichia coli* 16S rRNA, one implicated in a conformational switch in the dynamic movements of the ribosome, showed defects in subunit association and 30S initiation complex formation. Our results explain the basis of the loss of protein synthesis ability caused by a perturbation of the 900 tetraloop.

Key words: small subunit ribosomal RNA (SSU rRNA); 900 tetraloop; intersubunit bridge; subunit association; specialized ribosome

The ribosome is a ribonucleoprotein complex composed of two unequally sized subunits. Ribosomal RNAs (rRNAs) account for approximately two-thirds of the ribosome and form the core of the subunits, which are stabilized by RNA-RNA interactions in addition to RNA-protein interactions. Elucidation of the crystal structures of the bacterial ribosomes defined intersubunit bridges that involve protein-RNA and protein-protein interactions, in addition to RNA-RNA contacts, which are conserved in ribosomes derived from different microorganisms.[1–4] The intersubunit bridges located in the central areas encompassing the decoding center in the small subunit (SSU) and the reaction center in the large subunit (LSU) contribute more than 80% of individual intersubunit contacts. Nucleotides of rRNA in intersubunit contacts revealed by the crystal structures of the ribosomes have been also identified by chemical footprinting[5,6] and chemical cross-linking.[7]

One of the highly conserved intersubunit bridges of the ribosome, B2c, consists of the 900 tetraloop (positions 900 and 901) and helix 24 (positions 770 and 771) in the SSU rRNA and helix 67 (positions 1832 and 1833) in the LSU rRNA. The 900 tetraloop of SSU rRNA docks into the minor groove of the base-pairs between nucleotides at positions 768–770 and 809–811 of helix 24 in the crystal structure of 30S, which forms a structural motif interacting with helix 67 of LSU rRNA.[8,9] Genetic studies have shown that the 900 tetraloop functionally interacts with helix 1 of SSU rRNA,[10] although no direct physical interaction has been identified in the crystal structure of the 30S. Functional studies have shown that a nucleotide substitution at position 900 (A900G) severely decreases ribosomal activity, and the mutant ribosome exhibits defects in ribosomal subunit association and translational fidelity.[9] These results indicate that the 900 tetraloop plays important roles in several aspects of the translation process.

In the present study, we investigated a functional role of residue C899 of the 900 tetraloop in protein synthesis. We chose C899 from among four nucleotides in the loop, because no functional studies have been carried out with a complete set of single mutants at this position.[11] To do this, we constructed site-directed mutations at position 899 of the SSU rRNA gene in a specialized ribosome system[12–14] and assayed for their protein synthesis ability. In the specialized ribosome system we utilized, the chloramphenicol acetyltransferase (CAT) reporter mRNA is translated exclusively by plasmid (pRNA122)-derived ribosomes. In addition, because of alterations present in the ribosome binding site of the CAT mRNA and mRNA binding site of SSU rRNA expressed from pRNA 122, chromosome-derived ribosomes cannot translate CAT mRNA, while the specialized ribosomes cannot translate normal cellular mRNA. Thus the level of function of plasmid-derived mutant ribosomes could be assayed *in vivo* by determining the degree of resistance of the cells to chloramphenicol (Cm) or the amount of CAT protein produced in the cells.

A base substitution from C (the wild type) to U or G at position 899 (C899U or C899G) resulted in deleterious effect (MIC = 200 and 150 respectively), whereas the C899A mutation resulted in a moderately deleterious effect (MIC = 300) on ribosome function, indicating that C899 is important to ribosome function (Fig. 1). These results are consistent with a previous study that showed 75% and 86% loss of GFP protein synthesis ability of mutant ribosomes containing C899A and C899G respectively.[11] To confirm the correlation between the degree of resistance to Cm of the cells that expressed mutant ribosomes and the amount of CAT protein synthesized by mutant ribosomes in the cells, Western blot analysis was performed using polyclonal

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Abbreviations: Cm, chloramphenicol; MIC, minimum inhibitory concentration; RT, reverse transcriptase; SSU rRNA, small subunit ribosomal RNA; LSU rRNA, large subunit ribosomal RNA

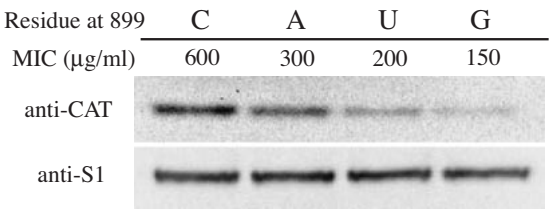

Fig. 1. Functional Analysis of Mutations Constructed at Position 899 of SSU rRNA in pRNA122.

Mutations were introduced at position 899 by cloning PCR products containing A, C, or T at position 899 into pRNA122 plasmid using *Bgl*II and *Dra*III sites. The overlap extension PCR method was used to create mutations, and the primers used were 16S-537F (5′-GGAGGGTGCAAGCGTTAATCGGAA-3′), 16S-899D (5′-ACCCTCATGCCGGCDTTCCAATTTTGAGTTTACTTAACT-3′, D = A, G, and T), ASD-B (5′-GGCGACTTTCACTCACAAAC-3′), and 16S-905F (5′-TAAAACTCAAATGAATTGACGG-3′). DH5α cells harboring pRNA122 or derivatives were grown to $OD_{600} = 0.1$, and 1 mM IPTG was added to induce synthesis of pRNA122-ribosomes. Culture samples were harvested 2 h after induction to obtain total protein. Western blot analysis of CAT protein has been described.[16] The same membrane probed with anti-CAT polyclonal antibody was stripped and reprobed with anti-ribosomal protein S1 polyclonal antibody. MICs were determined as previously reported.[12]

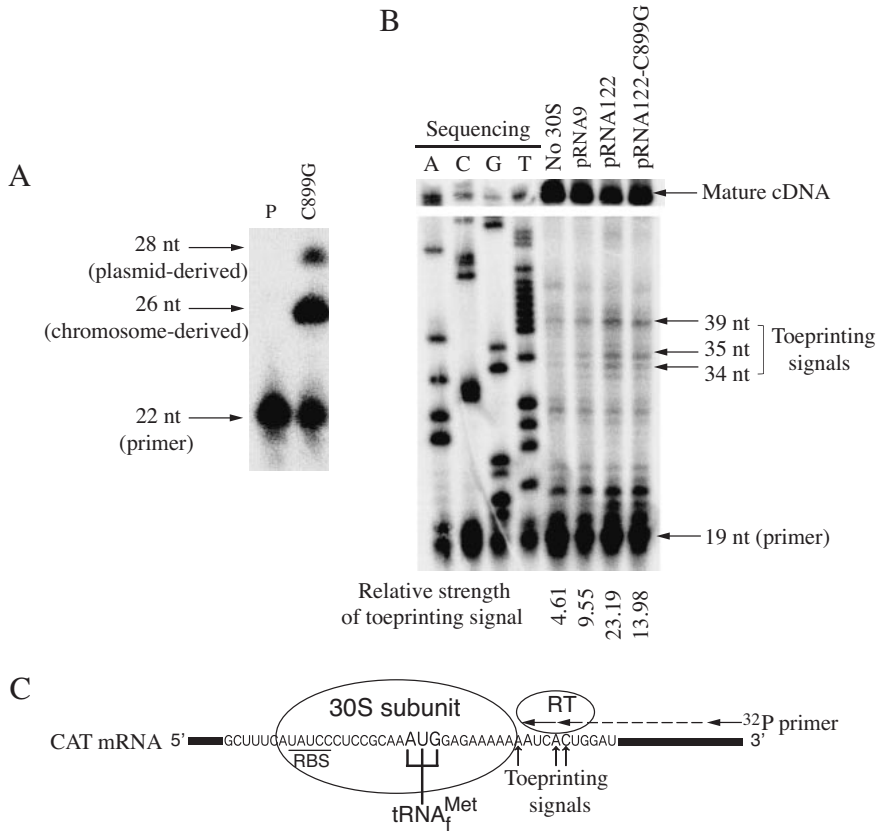

Fig. 2. Effects of a Base Substitution at Position 899 of SSU rRNA on Ribosomal Subunit Association and 30S Initiation Complex Formation.

A, Distribution of plasmid-derived mutant SSU rRNA. The 28-nucleotide-long DNA fragments synthesized from plasmid-derived mutant rRNA to the 26-nucleotide-long DNA fragments synthesized from chromosome-derived wild-type rRNA are indicated. 30S and 70S ribosomes were isolated from cells grown in the way described in the Fig. 1 legend by the method of Powers and Noller.[17] The ratio of plasmid to chromosome-derived rRNA in the rRNA prepared from 70S ribosome was determined by modified primer extension[12,15] by annealing the end-labeled 16S-924R (5′-GTCAATTCATTTGAGTTTTAAC-3′) complementary to the 899 mutation site and extending it through the mutation site using AMV reverse transcriptase. The extension reaction contained a mixture of dATP, dCTP, dTTP, and ddGTP. The synthesized cDNAs were resolved by PAGE, and the ratios of mutant to non-mutant rRNA were determined by comparing the amounts of radioactivity in the two bands. In lane P, a sample from the extension reaction carried out without RNA was loaded. B, Effects of C899G on the rate of 30S initiation complex formation. *In vitro* synthesized CAT mRNA was used in toeprinting assays with 30S subunits purified from cells expressing pRNA9, pRNA122, or pRNA122-C899G ribosomes. Ratios were calculated as percentages of toeprinting bands relative to the sum of the mature product and the toeprinting bands. Preparation of 30S ribosomal subunits and toeprinting assay were performed as described by Lee *et al.*[18] Ten-microliter reactions were prepared in standard buffer (10 mM Tris-acetate, pH 7.4, 60 mM NH_4Cl, 6 mM β-mercaptoethanol, and 10 mM magnesium acetate), and contained 10 nM of *in vitro* transcribed CAT mRNA, 0.5 μM 30S subunits, and 5.0 μM $tRNA_f^{Met}$ (Sigma-Aldrich, St. Louis, MO, the United States of America). For *in vitro* synthesis of CAT mRNA of pRNA122, PCR-generated DNA was prepared using primers T7-cat-TSS (5′-taatac gactcactatagggATGTGTGGAAGCGGCCGCTTTCA-3′) and CAT-NcoRI (5′-CCTGAATCGCCAGCGGCA-3′), with pRNA122 as template. CAT mRNA was synthesized using the MEGAscript T7™ kit (Ambion, Austin, TX) and PCR DNA as template according to the manufacturer's instructions. The primer cat + 50 (5′-TGCCATTGGGATATATCAA-3′) was 5′-end-labelled with [Υ-^{32}P]ATP and T4 polynucleotide kinase, and was used in the toeprinting assay. C, Schematic representation of the toeprinting assay. Reverse transcriptase (RT) terminates cDNA synthesis when it encounters 30S initiation complex.

antibodies to CAT. Mutant ribosomes lost their ability to synthesize CAT protein at different degrees, resulting in different levels of resistance of the cells to Cm (Fig. 1). The results were also consistent with previous functional studies of the 900 tetraloop.[11)]

Based on the structural and functional analyses of others, we speculated that mutant ribosomes bearing a base substitution at position 899 have a defect in ribosomal subunit association and consequently lose their protein-synthesis ability. To test this hypothesis, we purified mutant 70S ribosomes bearing C899G using a sucrose gradient and analyzed the distribution of mutant 30S subunits in the 70S peak to measure their ability to form 70S ribosome. The C899G mutation was chosen for this experiment because ribosomes bearing this mutation showed the lowest protein synthesis function among the mutant ribosomes with a point mutation at this position (Fig. 1). Sucrose gradient profiles of the mutant ribosomes showed an increased abundance of 30S subunits as compared to those from cells expressing wild-type ribosomes (data not shown). Primer extension analysis showed that the mutant SSU rRNA was notably under-represented in the peaks of the 70S ribosomes (about 4%), indicating that perturbed ribosomal subunit association is the primary cause of inhibition of translation (Fig. 2A). It has been found that wild-type rRNA synthesized from pRNA122 constitutes approximately 35% of 70S ribosomes.[13,15)]

Since mutant ribosomes showed a defect in ribosomal subunit association, we tested their ability to form 30S initiation complex, which is a step prior to 70S formation in the translation process. We carried out toeprinting assays, which measure the efficiency of formation of translation initiation complexes between *in vitro*-synthesized CAT mRNA and mutant 30S ribosomal subunits. 30S subunits purified from cells harboring pRNA122-C899G exhibited a 40% decrease in the toeprinting signal as compared to those purified from cells harboring pRNA122 (Fig. 2B). The reaction mix without 30S subunits resulted in premature cDNA whose size was similar to the cDNA for the toeprinting signal, but the strength of the signal was approximately 5 times lower than that generated by wild-type 30S subunits purified from cells harboring pRNA122. In addition, the reaction carried out with 30S subunits purified from cells harboring pRNA122-C899G resulted in a toeprinting signal slightly higher than that of the reaction mix with 30S subunits purified from cells harboring pRNA9[11)] that expressed SSU rRNA with an unaltered mRNA binding site, which was essentially same as chromosome-derived SSU rRNA. Considering that approximately 35% of the 30S subunits purified from the cells harboring pRNA122 or pRNA122-C899G were derived from the plasmid, the ability of mutant 30S subunits bearing C899G to form the 30S initiation complex was estimated to be reduced by about 60% as compared to the wild-type 30S. These results indicate that *in vitro* synthesized CAT mRNA derived from pRNA122 was recognized mainly by 30S subunits expressed from pRNA122, and that the 30S subunits bearing C899G have a significant defect in forming 30S initiation complex. Based on these results, we conclude that a perturbation of the 900 tetraloop impairs subunit association as a result of a defect in 30S initiation complex formation. Considering that the 900 tetraloop caps helix 27, which interacts with the decoding center of helix 44 as well as helix 24, and that these interactions induce large-scale rearrangements in both ribosomal subunits,[19)] a perturbation of the 900 tetraloop might result in defects in several aspects of ribosome-mediated reactions, such as mRNA selection and tRNA binding, that are required for 30S initiation complex formation. Structural studies of the mutant ribosome should clarify the basis of its defect in 30S initiation complex formation.

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