J. Microbiol. Biotechnol. (2007), **17**(7), 1204–1207

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Functional Analysis of the Residues C770 and G771 of *E. coli* 16S rRNA Implicated in Forming the Intersubunit Bridge B2c of the Ribosome

KIM, HONG-MAN, JI-HYUN YEOM, HYE-JUNG HA, JONG-MYUNG KIM, AND KANGSEOK LEE*

Department of Life Science, Chung-Ang University, Seoul 156-756, Korea

Received: April 11, 2007 Accepted: May 24, 2007

Abstract Structural analyses have shown that nucleotides at the positions 770 and 771 of *Escherichia coli* 16S rRNA are implicated in forming one of highly conserved intersubunit bridges of the ribosome, B2c. To examine a functional role of these residues, base substitutions were introduced at these positions and mutant ribosomes were analyzed for their protein synthesis ability using a specialized ribosome system. The results showed requirement of a pyrimidine at the position 770 for ribosome function regardless of the nucleotide identity at the position 771. Sucrose gradient profiles of ribosomes revealed that the loss of protein-synthesis ability of mutant ribosome bearing a base substitution from C to G at the position 770 stems from its inability to form 70S ribosomes. These findings indicate involvement of nucleotide at the position 770, not 771, in ribosomal subunit association and provide a useful rRNA mutation that can be used as a target to investigate the physical interaction between 16S and 23S rRNA.

Keywords: Intersubunit bridge, subunit association, 16S rRNA, specialized ribosome

The ribosome is a ribonucleoprotein complex composed of two unequally sized subunits. Ribosomal RNAs (rRNAs) account for two-thirds of the ribosome and form the core of subunits, which are stabilized by RNA-RNA interactions in addition to RNA-protein interactions [1, 18, 19, 27]. They are responsible for most, if not all, catalytic reactions in protein synthesis that include mRNA selection, decoding the genetic information, formation of peptide bonds, nascent peptide release, GTP hydrolysis, etc. (reviewed in [15]).

Nucleotides of rRNA in intersubunit contacts have been detected by chemical footprinting [2, 6, 12] and chemical cross-linking [13]. Structural studies further defined intersubunit bridges that involve protein-RNA and protein-protein interactions, in addition to RNA-RNA contacts in ribosomes derived from different microorganisms [3, 4, 20, 22, 23]. Some of these bridges made of RNA-protein and protein-protein contact are often broken and rearranged during specific steps in protein synthesis [5, 16, 24, 26]. These dynamic movements of the intersubunit bridges have been proposed to play a role in translocation of tRNAs during elongation step and dissociation of subunits in termination step. The model of Yusupov *et al.* [28] presents 12 intersubunit bridges that seem to be largely conserved in all living organisms [4, 22]. The intersubunits 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 the individual intersubunit contacts [4]. Most of the identified intersubunit contacts based on structural analyses involve RNA and one of highly conserved intersubunit bridges of the ribosome, B2c, involves residues C770, G771, C899, G1514, and G1515 of 16S rRNA, C1832 and C1833 of 23S rRNA, and Mg ion [20, 21]. However, involvement of these residues in ribosomal subunit association was indicated only based on the structural analyses and their function in protein synthesis has not been characterized. In the present study we investigated a functional role of residues C770 and G771 of 16S rRNA in protein synthesis using a specialized ribosome system [9–11].

MATERIALS AND METHODS

Strains and Plasmids

All plasmids were maintained and expressed in *Escherichia coli* DH5α. Cultures were maintained in LB containing 100 μg of ampicillin ml^{-1} (LB-Ap100). To induce the synthesis of plasmid derived rRNA from the *lacUV5* promoter, IPTG was added to a final concentration of 1 mM.

Mutations were introduced at the positions 770 and 771 by cloning PCR products containing N (A, C, G, or T) at the positions 770 and 771 into pRNA122 plasmid [9–11] using BglII and DraIII sites. A recombinant PCR method

*Corresponding author
Phone: 82-2-820-5241; Fax: 82-2-822-5241;
E-mail: kangseok@cau.ac.kr

[7] was used to create mutations and the primers used are 16S-537F (5'-GGAGGGTGCAAGCGTTAATCGGAA), N770N771 (5'-ATCTAATCCTGTTTGCTCCCCANNCT-TTCGCACC), 777F (5'-AGCAAACAGGATTAGATACC) and ASD-B (5'-GGCGACTTTCACTCACAAAC).

Minimum Inhibitory Concentration (MIC)

MICs were determined as previously reported [9]. Briefly, overnight grown cultures in LB-Ap100 were diluted and induced in the same medium containing 1 mM IPTG for 2–3 h. Approximately 10^4 of the induced cells were then added to wells containing LB-Ap100+ and 1 mM IPTG and chloramphenicol at increasing concentrations. Cultures were grown for 24 h, and the lowest concentration of chloramphenicol that completely inhibited growth was determined as the MIC [8].

Protein and RNA Work

Cultures were grown to OD_{600} of 0.1 and 1 mM IPTG was added to induce the synthesis of pRNA122-ribosomes. Culture samples were harvested two hours after induction to obtain total protein or RNA. The relative abundance of protein bands was quantified using Versa Doc imaging system (Bio-Rad) and Quantity One (ver. 4.5.1; Bio-Rad). 30S and 70S ribosomes were isolated from 400 ml of cells grown in the same way described above by the method of Powers and Noller [14]. The ratio of plasmid to chromosome-derived rRNA in total RNA was determined by a modified primer extension [9, 17] by annealing the end-labeled primer 16S 793R (5'-ATCTAATCCTGTTTG-CTCCC) complementary to the 770 mutation site and extending 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 amount of radioactivity in each of the two bands.

Results and Discussion

Genetic Analysis of Site-directed Mutations Constructed at the Positions 770 and 771 of 16S rRNA

To verify the RNA-RNA interactions that constitute intersubunit bridges identified by structural analyses, site-directed mutations were constructed at the positions 770 and 771 of 16S rRNA gene in a specialized ribosome system and the protein-synthesis ability of mutant ribosomes was measured. These sites were chosen because functional analyses of the residues have not been previously done and the intersubunit bridge B2c that they are involved to form is mainly composed of RNA-RNA interactions. In the specialized ribosome system we utilized, the chloramphenicol acetyltransferase (CAT) reporter message was translated exclusively by plasmid-derived ribosomes that cannot translate

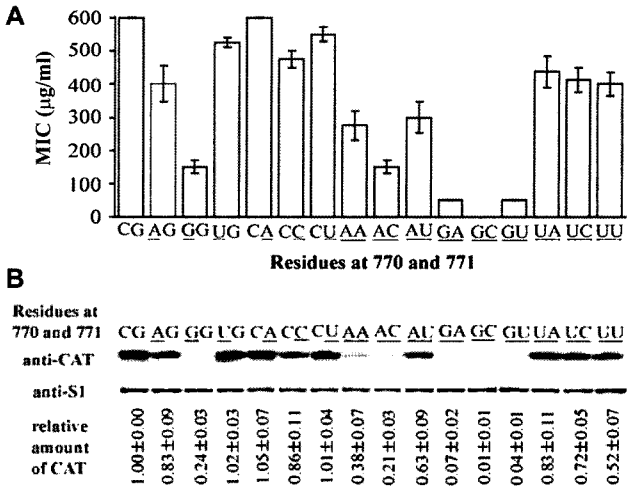

Fig. 1. Functional analysis of mutations constructed at the positions 770 and 771 of 16S rRNA in pRNA122.
A. Nucleotide identities are indicated in the order of 770 and 771, and mutations are underlined. **B.** Western blot analysis of CAT protein synthesized in *E. coli* cells expressing pRNA122 ribosomes bearing mutations at the positions 770 and 771 of 16S rRNA. Cultures were grown to OD_{600} of 0.1 and 1 mM IPTG was added to induce the synthesis of pRNA122-ribosomes. Culture samples were harvested at OD_{600} of 0.6 to obtain total protein. The same membrane probed with anti-CAT polyclonal antibody was stripped and reprobed with anti-S1 polyclonal antibody. The relative abundance of protein bands was quantified by setting the amount of CAT protein produced by wild-type pRNA122 ribosomes as one. The experiments were repeated three times and averaged. Standard error of the mean (±numbers) is used to indicate the range of the assay results.

normal cellular messages [9–11, 25]. Thus, the level of function of plasmid-derived mutant ribosomes could be assayed *in vivo* by growing clones in the growth media containing different levels of chloramphenicol and determining the minimal inhibitory concentration (MIC).

The results showed that a base substitution from C (wild-type) to U at the position 770 (C770U) resulted in mildly deleterious effect (MIC=500) whereas a change to a purine resulted in moderately (C770A, MIC=400) or strongly deleterious effect (C770G, MIC=150) on ribosome function, indicating that a pyrimidine (C or U) at the position 770 is required for ribosome function (Fig. 1). A base substitution at the position 771 had no effect (MIC of G771A=600) or mildly deleterious effect (MIC of G771C and G771U≈500 and 550, respectively) on the protein synthesis ability of the ribosome. A single mutation at each of the positions showed a negative effect to each other on ribosome function since cells expressing mutant ribosomes bearing a double mutation at these positions exhibited a lower MIC compared to those expressing mutant ribosomes bearing a corresponding single mutation.

Analysis of Protein Synthesis Ability of Mutant Ribosomes

To confirm that the degree of resistance to chloramphenicol (Cm) correlates with the amount of CAT protein synthesized by mutant ribosomes, Western blot analysis was performed

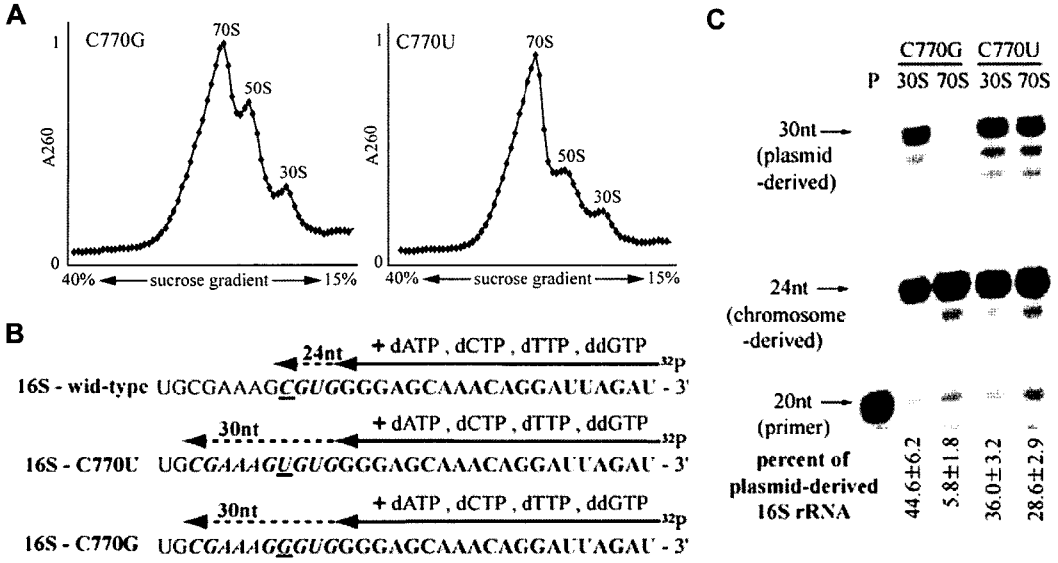

Fig. 2. Effects of a base substitution at the position 770 of 16S rRNA on ribosomal subunit association.
A. Sucrose gradient profiles of ribosomes prepared from cells expressing pRNA122-C770G or -C770U. Positions of 30S subunits, 50S subunits, and 70S subunits are indicated by arrowheads. **B.** Schematic representation of modified primer extension method. **C.** Distribution of plasmid-derived mutant 16S rRNA. The 30 nucleotide-long DNA fragments synthesized from plasmid-derived mutant rRNA and the 24 nucleotide-long DNA fragments synthesized from chromosome-derived wild-type rRNA are indicated. In lane P, sample from extension reaction carried out without RNA was loaded.

using polyclonal antibodies to CAT. As shown in Fig. 2B, there was a good agreement between them showing that mutant ribosomes lost their ability to synthesize CAT protein at different degrees resulting in different levels of resistance of cells expressing mutant ribosomes to Cm.

Mutant ribosomes bearing a substitution at the position 771 still retained more than 85% of protein synthesis ability of wild-type ribosomes, indicating that, inconsistent with a conclusion drawn from the crystal structure of the ribosome [20], the residue C771 is unlikely to be involved in the intersubunit bridge, B2c, for ribosomal subunit association.

Effects of a Base Substitution at the Position 770 on Ribosomal Subunit Association

Based on structural analyses of others and our results, we speculated that mutant ribosomes bearing a base substitution to a purine residue at the position 770 have a defect in ribosomal subunit association and consequently lost their protein-synthesis ability. To test this hypothesis, mutant ribosomes bearing a base substitution at the position 770 were purified using a sucrose gradient and analyzed for their ability to form 70S ribosome. C770U and C770G mutations were chosen for this experiment because ribosomes bearing C770U and C770G showed the highest and lowest protein synthesis function, respectively among mutant ribosomes with a point mutation at this position. Sucrose gradient profiles of mutant ribosomes prepared from cells expressing 16S rRNA bearing C770G (16S-C770G) showed an increased abundance of 30S subunits compared to those from cells expressing 16S-C770U. Primer extension analysis of the C770G mutant showed that the mutant 16S rRNA was prevalent in the 30S peak (~45%) and was notably underrepresented in peaks of 70S ribosomes (~6%), indicating that perturbed ribosomal subunit association is the primary cause of inhibition of translation. A moderately deleterious mutant (C770U) showed a moderate decrease in the amounts of 70S ribosomes (~29%) compared to free 30S subunits (~36%), whereas it has been previously shown that wild-type rRNA synthesized from pRNA122 constitutes approximately 40% of both free subunits and 70S ribosomes [10].

Taken together, the present study demonstrates that the residue C770, not G771, is involved in ribosomal subunit association and a mutated residue at 770 can be used as a target to investigate the physical interaction between 16S and 23S rRNA in ribosomal subunit association.

Acknowledgments

This work was supported by the grant from Seoul R&BD program and Korea Research Foundation Grant (KRF-2004-015-C00502) to K. Lee.

References

1. Ban, N., P. Nissen, J. Hansen, P. B. Moore, and T. A. Steitz. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289:** 905–920.

2. Chapman, N. M. and H. F. Noller. 1977. Protection of specific sites in 16S RNA from chemical modification by association of 30S and 50S ribosomes. *J. Mol. Biol.* **109:** 131–149.
3. Gabashvili, I. S., R. K. Agrawal, C. M. Spahn, R. A. Grassucci, D. I. Svergun, J. Frank, and P. Penczek. 2000. Solution structure of the *E. coli* 70S ribosome at 11.5 Å resolution. *Cell* **100:** 537–549.
4. Gao, H., J. Sengupta, and M. Valle *et al.* 2003. Study of the structural dynamics of the *E. coli* 70S ribosome using real-space refinement. *Cell* **113:** 789–801.
5. Hennelly, S. P., A. Antoun, M. Ehrenberg, C. O. Gualerzi, W. Knight, J. S. Lodmell, and W. E. Hill. 2005. A time-resolved investigation of ribosomal subunit association. *J. Mol. Biol.* **346:** 1243–1258.
6. Herr, W. and H. F. Noller. 1979. Protection of specific sites in 23S and 5S RNA from chemical modification by association of 30S and 50S ribosomes. *J. Mol. Biol.* **130:** 421–432.
7. Higuchi, R. 1989. Using PCR to engineer DNA, pp. 61–70. *In* H. A. Erlich (ed.), *PCR Technology.* Stockton Press, New York, NY, U.S.A.
8. Hwang, B. and S. W. Lee. 2005. Analysis of *in vivo* interaction of HCV NS3 protein and specific RNA aptamer with yeast three-hybrid system. *J. Microbiol. Biotechnol.* **15:** 660–664.
9. Lee, K., C. A. Holland-Staley, and P. R. Cunningham. 1996. Genetic analysis of the Shine-Dalgarno interaction: selection of alternative functional mRNA-rRNA combinations. *RNA* **2:** 1270–1285.
10. Lee, K., S. Varma, J. Santalucia Jr., and P. R. Cunningham. 1997. *In vivo* determination of RNA structure-function relationships: Analysis of the 790 loop in ribosomal RNA. *J. Mol. Biol.* **269:** 732–743.
11. Lee, K., C. A. Holland-Staley, and P. R. Cunningham. 2001. Genetic approaches to studying protein synthesis: Effects of mutations at Ψ516 and A535 in *Escherichia coli* 16S rRNA. *J. Nutr.* **131:** 2994S–3004S.
12. Merryman, C., D. Moazed, G. Daubresse, and H. F. Noller. 1999. Nucleotides in 23S rRNA protected by the association of 30S and 50S ribosomal subunits. *J. Mol. Biol.* **285:** 107–113.
13. Mitchell, P., M. Osswald, and R. Brimacombe. 1992 Identification of intermolecular RNA cross-links at the subunit interface of the *Escherichia coli* ribosome. *Biochemistry* **31:** 3004–3011.
14. Powers, T. and H. F. Noller. 1991. A functional pseudoknot in 16S ribosomal RNA. *EMBO J.* **10:** 2203–2214.
15. Ramakrishnan, V. 2002. Ribosome structure and the mechanism of translation. *Cell* **108:** 557–572.
16. Rawat, U., H. Gao, A. Zavialov, R. Gursky, M. Ehrenberg, and J. Frank. 2006. Interactions of the release factor RF1 with the ribosome as revealed by cryo-EM. *J. Mol. Biol.* **357:** 1144–1153.
17. Ryou, S. M., J. M. Kim, J. H. Yeom, H. L. Kim, H. Y. Go, E. K. Shin, and K. Lee. 2005. Species-specific cleavage by RNase E-like enzymes in 5S rRNA maturation. *J. Microbiol. Biotechnol.* **15:** 1100–1105.
18. Schluenzen, F., A. Tocilj, and R. Zarivach *et al.* 2000. Structure of functionally activated small ribosomal subunit at 3.3 angstroms resolution. *Cell* **102:** 615–623.
19. Shuang, J. L., C. H. Lui, S. Q. An, Y. Xing, G. Q. Zheng, and Y. F. Shen. 2006. Some universal characteristics of intertidal bacterial diversity as revealed by 16S rRNA gene-based PCR clone analysis. *J. Microbiol. Biotechnol.* **16:** 1882–1889.
20. Schuwirth, B. S., M. A. Borovinskaya, C. W. Hau, W. Zhang, A. Vila-Sanjurjo, J. M. Holton, and J. H. Cate. 2005. Structures of the bacterial ribosome at 3.5 Å resolution. *Science* **310:** 827–834.
21. Selmer, M., C. M. Dunham, F. V. Murphy 4th, A. Weixlbaumer, S. Petry, A. C. Kelley, J. R. Weir, and V. Ramakrishnan. 2006. Structure of the 70S ribosome complexed with mRNA and tRNA. *Science* **313:** 1935–1942.
22. Spahn, C. M., R. Beckmann, N. Eswar, P. A. Penczek, A. Sali, G. Blobel, and J. Frank. 2001. Structure of the 80S ribosome from *Saccharomyces cerevisiae*: tRNA-ribosome and subunit-subunit interactions. *Cell* **107:** 373–386.
23. Spahn, C. M., E. Jan, A. Mulder, R. A. Grassucci, P. Sarnow, and J. Frank. 2004. Cryo-EM visualization of a viral internal ribosome entry site bound to human ribosomes: The IRES functions as an RNA-based translation factor. *Cell* **118:** 465–475.
24. Spahn, C. M., M. G. Gomez-Lorenzo, R. A. Grassucci, R. Jorgensen, G. R. Andersen, R. Beckmann, P. A. Penczek, J. P. Ballesta, and J. Frank. 2004. Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation. *EMBO J.* **23:**1008–1019.
25. Szatkiewicz, J. P., H. Cho, S. M. Ryou, J. M. Kim, P. R. Cunningham, and K. Lee. 2006. Genetic analysis of a structural motif within the conserved 530 stem-loop of *Escherichia coli* 16S rRNA. *J. Microbiol. Biotechnol.* **16:** 569–575.
26. Wilson, D. N., F. Schluenzen, J. M. Harms, T. Yoshida, T. Ohkubo, R. Albrecht, J. Buerger, Y. Kobayashi, and P. Fucini. 2005. X-ray crystallography study on ribosome recycling: The mechanism of binding and action of RRF on the 50S ribosomal subunit. *EMBO J.* **24:** 251–260.
27. Wimberly, B. T., D. E. Brodersen, W. M. Clemons Jr., R. J. Morgan-Warren, A. P. Carter, C. Vonrhein, T. Hartsch, and V. Ramakrishnan. 2000 Structure of the 30S ribosomal subunit. *Nature* **407:** 327–339.
28. Yusupov, M. M., G. Z. Yusupova, A. Baucom, K. Lieberman, T. N. Earnest, J. H. Cate, and H. F. Noller. 2001. Crystal structure of the ribosome at 5.5 Å resolution. *Science* **292:** 883–896.