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Functional Analysis of the Invariant Residue G791 of *Escherichia coli* 16S rRNA

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The nucleotide at position 791(G791) of *E. coli* 16S rRNA was previously identified as an invariant residue for ribosomal function. In order to characterize the functional role of G791, base substitutions were introduced at this position, and mutant ribosomes were analyzed with regard to their protein synthesis ability, via the use of a specialized ribosome system. These ribosomal RNA mutations attenuated the ability of ribosomes to conduct protein synthesis by more than 65%. A transition mutation (G to A) exerted a moderate effect on ribosomal function, whereas a transversion mutation (G to C or U) resulted in a loss of protein synthesis ability of more than 90%. The sucrose gradient profiles of ribosomes and primer extension analysis showed that the loss of protein-synthesis ability of mutant ribosomes harboring a base substitution from G to U at position 791 stems partially from its inability to form 70S ribosomes. These findings show the involvement of the nucleotide at position 791 in the association of ribosomal subunits and protein synthesis steps after 70S formation, as well as the possibility of using 16S rRNA mutated at position 791 for the selection of second-site revertants in order to identify ligands that interact with G791 in protein synthesis.

Keywords: specialized ribosome, rRNA, subunit association, G791, protein synthesis

Ribosomal RNA occupies more than 60% of ribosomal mass, and is involved directly in the catalytic process in protein synthesis. Segments of most highly conserved sequences exist in the rRNAs and the 790 loop (positions 787 to 795) in small subunit rRNA is one of them (Gutell *et al.*, 1994; Cannone *et al.*, 2002) (Fig. 1A). Residues within the 790 loop are protected from chemical probes by the initiation of factor 3 binding (G791 and U793) (Muralikrishna and Wickstrom, 1989; Moazed *et al.*, 1995), 50S subunits (A790 and G791) (Moazed and Noller, 1986), P-site bound $tRNA^{phe}$ (A794 and C795) (Mankin, 1997; Dinos *et al.*, 2004), and antibiotics, kasugamycin, and edeine (residue A794) or by pactamycin and edeine (residue C795) (Moazed and Noller, 1987; Mankin, 1997; Dinos *et al.*, 2004). The crystal structure of the 30S subunit positions the 790 loop in the front half of the platform and the 790 loop forms bridges of electron density which extend toward the 50S subunit in the crystal structure of the 70S ribosome (Cate *et al.*, 1999; Clemons *et al.*, 1999; Wimberly *et al.*, 2000; Yusupov *et al.*, 2001).

Via the simultaneous mutation of all of the nucleotides in the 790 loop and the selection of alternative functional sequences, invariant nucleotides which may perform a direct role in ribosome function via interaction with ligands have been identified in previous studies (Lee *et al.*, 1997). One of these residues (G791) is very well conserved in all small subunit rRNAs (Cannone *et al.*, 2002) (Table 1). In this report, we have determined the functional role of this residue and have demonstrated the involvement of G791 in the association of ribosomal subunits using a specialized ribosome system (Lee *et al.*, 1996; Lee *et al.*, 1997; Lee *et al.*, 2001).

Materials and Methods

Strains and plasmids

All plasmids were maintained and expressed in *Escherichia coli* DH5α [F⁻, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(r_k^-, m_k^+), *phoA*, *supE44*, λ⁻, *thi-1*, *gyrA96*, *relA1?*]. The cultures were maintained in LB and antibiotics were added at 100 μg /ml for ampicillin (Ap100). In order to induce the synthesis of plasmid-derived rRNA from the *lacUV5* promoter, IPTG was added to a final concentration of 1 mM. Unless indicated otherwise, the transformants were grown in SOC medium.

Mutations were introduced at position 791 via the cloning of PCR products containing H (A, C, or T) at position 791 into the pRNA122 plasmid (Lee *et al.*, 1996; Lee *et al.*, 1997) using the *Bgl*II and *Dra*III sites. A recombinant PCR technique (Higuchi, 1989) was utilized in order to create the mutations, using the following primers: 16S-537F; 5'-GGAG GGTGCAAGCGTTAATCGGAA, 16S-786R; 5'-CCTGTTT GCTCCCCACGCTTTCGCACCTGAGCG, 16S-791H; 5'-C

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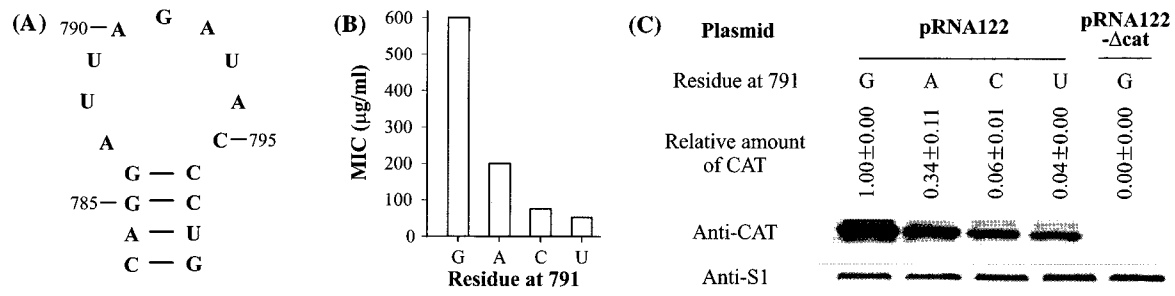

Fig. 1. Effects on ribosome function of mutations at position 791. (A) The secondary structure of the 790 loop. (B) Degree of resistance to Cm by *E. coli* cells expressing pRNA122 ribosomes harboring a base substitution at position 791. (C) Western blot analysis of CAT protein in *E. coli* cells expressing pRNA122 ribosomes harboring a base substitution at position 791. The cultures were grown to an $O.D._{600}$=0.1 and 1 mM IPTG was added in order to induce the synthesis of pRNA122-ribosomes. Culture samples were harvested two hours after induction in order to obtain the total proteins. The same membranes probed with anti-CAT monoclonal antibody were stripped and reprobed with anti-S1 polyclonal antibody. The relative abundance of protein bands was quantified by establishing the amount of CAT protein generated by wild-type pRNA122 ribosomes as one, using the Versa Doc imaging system (Bio-Rad) and Quantity One (ver. 4.5.1; Bio-Rad). The experiments were repeated three times and averaged. The standard error of the mean (± numbers) was used to indicate the range of the assay results.

Table 1. Nucleotide frequency at position 791 in SSU RNA

Origin of rRNA	Nucleotide at position 791					Number of sequences
	A	C	G	U	gap	
Total	0.07	0.04	99.56	0.04	0.29	7244
Three domains	0.02	0.05	99.92	0.00	0.02	6140
Eubacteria	0.02	0.06	99.88	0.00	0.04	4867
Archaea	0.00	0.00	100.00	0.00	0.00	231
Chloroplast	0.60	0.00	99.40	0.00	0.00	168
Eucarya (Nuclear)	0.00	0.00	100.00	0.00	0.00	1042
Mitochondria	0.32	0.00	97.44	0.32	1.92	936

TCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAH ATACCCTGGTAGTCCACGCCGTAA and ASD-B; 5'-GGC GACTTTCACTCACAAAC. The pRNA122Δcat plasmid was constructed via the ligation of the *Bam*HI fragment containing the *rrnB* operon from pRNA122 with *Bam*HI and *Bgl*II-digested PCR DNA harboring the replication origin and *bla* from pBR322. The primers utilized were as follows: pBR-F; 5'-ATGGATCCATCGATGCACCATTATGTTC and pBR-R; 5'-AGATCTCTAGATTCTTGAAGACGAAA.

Minimum Inhibitory Concentration (MIC) and Western blot analysis

MICs were determined as reported previously (Lee *et al.*, 1996). In brief, cultures grown overnight 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+1 mM IPTG and chloramphenicol at increasing concentrations. Cultures were grown for 24 h, and the lowest concentration of chloramphenicol required for the complete inhibition of growth was designated as the MIC. The Western blot analysis of the CAT protein has been described previously (Kim *et al.*, 2007).

Quantitation of the distribution of plasmid-derived rRNA

Cultures were grown to an $O.D._{600}$=0.1, and 1 mM IPTG was added in order to induce the synthesis of pRNA122-ribosomes, and were then harvested two hours after induction in order to obtain the crude ribosomes. 30S and 70S ribosomes were isolated from 400 ml of cells via the previously described technique (Kim *et al.*, 2007). The distribution of plasmid- derived rRNA containing U791 in total rRNA was determined via a modified primer extension technique (Lee *et al.*, 1997; Kim *et al.*, 2007) by annealing the end-labeled primer 16S-812R; 5'-CGGCGTGGACTACCAGGGTA complementary to the 791 mutation site and extending through the mutation site, using AMV reverse transcriptase. The extension reaction contained a mixture of three kinds of deoxynucleotides and one dideoxynucleotide (Fig. 2B). The synthesized cDNAs were resolved by PAGE and the ratios of mutant to non-mutant rRNA were determined via comparisons of the amount of radioactivity in each of the two bands.

Results and Discussion

Genetic analysis of site-directed mutations constructed at the position 791 of the loop

In order to verify the importance of the invariant residue G791 of the 16S rRNA in protein synthesis (Lee *et al.*, 1997), site-directed mutations were constructed at position 791 in a specialized ribosome system, in order to assess the protein-synthesis ability of the mutant ribosomes. In this specialized *in vivo* system, the chloramphenicol acetyltransferase (CAT) reporter message was exclusively translated by plasmid-derived ribosomes that cannot translate normal cellular mes-

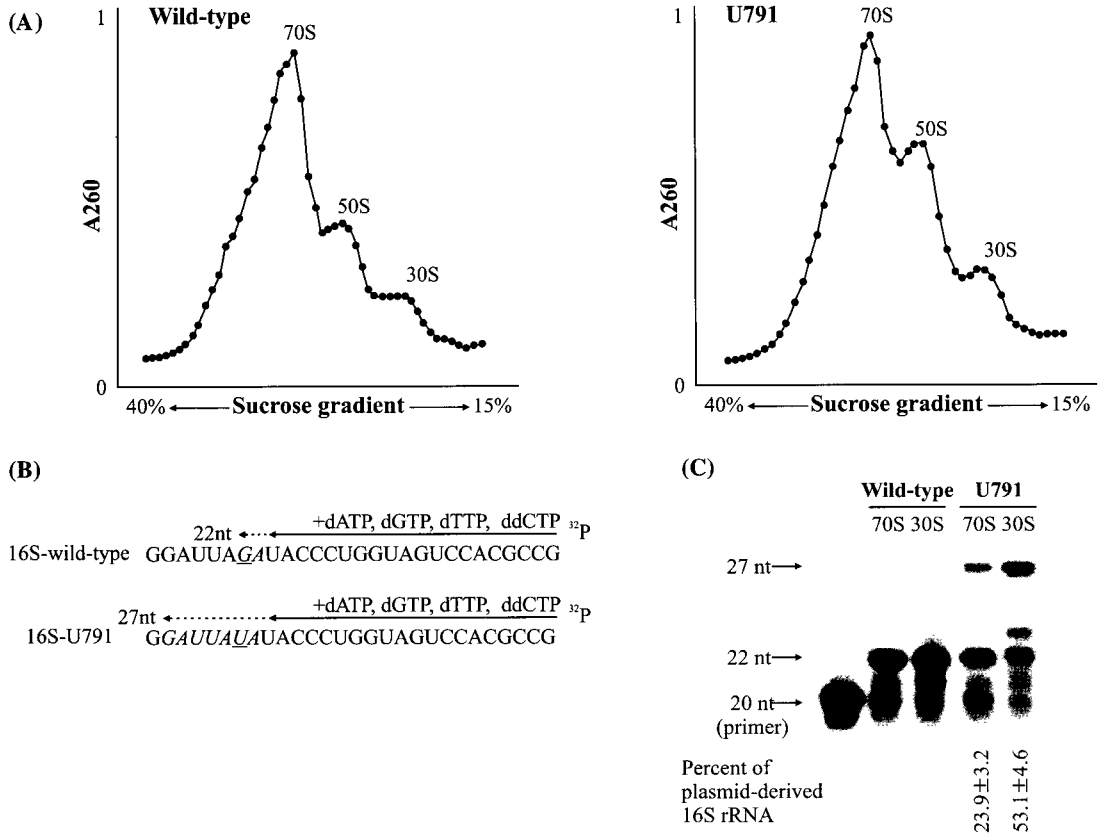

Fig. 2. Effects of a base substitution at the position 791 of 16S rRNA on ribosomal subunit association. (A) Sucrose gradient profiles of ribosomes prepared from cells expressing pRNA122 or pRNA122-U791. Positions of 30S subunits, 50S subunits, and 70S subunits are indicated. (B) Schematic representation of the modified primer extension method. (C) Distribution of plasmid-derived mutant 16S rRNA. The DNA fragments synthesized from plasmid-derived mutant rRNA to the DNA fragments synthesized from chromosome-derived wild-type rRNA are shown. In lane P, the sample from extension reaction conducted without RNA was loaded.

sages (Lee *et al.*, 1996; Lee *et al.*, 1997; Lee *et al.*, 2001). Thus, the level of function of the plasmid-derived mutant ribosomes could be assayed *in vivo* by growing clones in growth media containing different levels of chloramphenicol, and subsequently determining the minimal inhibitory concentration (MIC).

Consistent with the results of previous analyses of functional 790 loop mutant sequences (Lee *et al.*, 1997), ribosomes harboring point mutations at position 791 evidenced significant reductions in their ability to synthesize the CAT protein (Fig. 1B). A transition mutation (G to A) at position 791 resulted in the highest MIC (200 μg/ml) and therefore the most functional mutant ribosomes, whereas transversion mutations resulted in MICs lower than 100 μg/ml.

Analysis of protein synthesis ability of mutant ribosomes

In an effort to confirm that the degree of resistance to Cm is correlated with the quantity of CAT protein synthesized by the mutant ribosomes, Western blot analyses were conducted using polyclonal antibodies to CAT. As is shown in Fig. 1C, there was a good agreement between them, indicating that mutant ribosomes had lost their ability to synthesize the CAT protein at different degrees, resulting in different levels of resistance of the cells expressing them to Cm. The mutant ribosomes harboring A791 retained approximately 34% of the protein synthesis ability of wild-type ribosomes, while those harboring a transversion mutation (C791 or U791) lost more than 90% of their wild-type ribosomal function.

The most functional mutant (A791) is found second most frequently in the 7,244 rRNA sequences derived from all three domains (Eubacteria, Archaea, Eucarya), including chloroplasts and mitochondria (Table 1), which is consistent with the notion, deduced from previous mutational analyses of 16S rRNA using the specialized ribosome system (Lee *et al.*, 1997; Morosyuk *et al.*, 2000; Lee *et al.*, 2001; Kim *et al.*, 2007), that the protein synthesis ability of a specific rRNA mutant is correlated with the frequency of its occurrence in nature. This phenomenon implies that a species-specific variation in rRNA sequence that fits the physiological requirements of each species harboring specific variations is not completely independent of the minimum structure and function of ribosome required to carry out protein synthesis in the cell.

Effects of a base substitution at the position 791 on ribosomal subunit association

Based on the structural analyses conducted by others, as well as our own results, we speculated that mutant ribosomes

harboring a base substitution at position 791 evidence defects in ribosomal subunit association, coupled with a consequent loss in protein-synthesis ability. In order to evaluate this hypothesis, the total ribosomes were purified from cells that expressed mutant ribosomes bearing a base substitution at position 791 using a sucrose gradient, and analyzed for the ability of mutant ribosomes to form 70S ribosomes. U791 was selected for use in this experiment, as ribosomes bearing this mutation evidenced the lowest protein synthesis function. Sucrose gradient profiles of mutant ribosomes prepared from cells expressing 16S rRNA harboring U791 (16S-U791) evidenced an increased abundance of 30S subunits as compared to those from cells expressing wild-type 16S rRNA (Fig. 2A). Primer extension analysis of the U791 mutant revealed that mutant 16S rRNA was prevalent in the 30S peak (~53%) and was notably underrepresented in the 70S ribosome peaks (~24%), thereby indicating that a G to U mutation at position 791 resulted in a disruption of ribosomal subunit association. It has been demonstrated previously that wild-type rRNA synthesized from pRNA122 constitutes approximately 40% of both free subunits and 70S ribosomes (Lee *et al.*, 1997). It remains unclear, however, as to whether the inhibition of subunit association induced by U791 is the result of direct involvement of the wild-type residue with some portion of the 50S subunit, or is indirectly due to a change in structure or interruption of the initiation pathway resulting in the formation of the 70S ribosome. Furthermore, considering that mutant ribosomes harboring 16S-U791 retained only 4% of the protein synthesis ability evidenced by the wild-type ribosome (Fig. 1C), a relatively high distribution of 16S-U791 in the 70S peak (~24%) implies that these mutant ribosomes harbor additional defects in protein synthesis steps occurring after the formation of 70S. In support of this view, mutations in 16S rRNA in the 790 stem-loop that have been shown to affect ribosomal subunit association, including G770 (Kim *et al.*, 2007) and U787A795 (Lee *et al.*, 1997) resulted in partially functional ribosomes with 24% and 37% of the protein synthesis ability evidenced by the wild-type ribosomes, respectively, while the distribution of 16S-G770 and 16S-U787A795 in the 70S peak was ~6% and ~10%, respectively, which was much lower than that of 16S-U791 in the 70S peak.

Nevertheless, the results of the present study demonstrate that the G791 residue is involved in ribosomal subunit association, and 16S rRNA mutated at this residue can be employed as a primary mutant for the selection of second-site revertants that would help identify ligands that interact with G791 and their role in protein synthesis, including mRNA selection, the binding of the initiator tRNA and initiation factors, and ribosomal subunit association. These studies will provide us with the detailed molecular mechanisms of G791 function in protein synthesis.

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