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Functional investigation of residue G791 of *Escherichia coli* 16S rRNA: implication of initiation factor 1 in the restoration of P-site function

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Abstract

Using a specialized ribosome system, previous studies have identified G791 in *Escherichia coli* 16S rRNA as an invariant and essential residue for ribosome function. To investigate the functional role of G791, we searched for multicopy suppressors that partially restored the protein synthesis ability of mutant ribosomes bearing a G to U substitution at position 791 (U791 ribosomes). Analyses of isolated multicopy suppressors showed that overexpression of initiation factor 1 (IF1) enhanced the protein synthesis ability of U791 ribosomes. In contrast, overexpression of initiation factor 2 (IF2) or IF3 did not enhance the protein synthesis ability of wild-type or U791 ribosomes, and overexpression of IF1 did not affect the function of wild-type or mutant ribosomes bearing nucleotide substitutions in other regions of 16S rRNA. Analyses of sucrose gradient profiles of ribosomes showed that overexpression of IF1 marginally enhanced the subunit association of U791 ribosomes and indicated lower binding affinity of U791 ribosomes to IF1. Our findings suggest the involvement of IF1 in the restoration of the P-site function that was impaired by a nucleotide substitution at residue G791.

Introduction

rRNA occupies more than 60% of the ribosome mass and is responsible for most, if not all, catalytic reactions in protein synthesis (for a recent review, see Ramakrishnan, 2002). Segments of highly conserved rRNA sequences are implicated in various catalytic reactions, and the 790 loop (positions 787–795) of the small subunit rRNA is an example of a highly conserved segment. The 790 loop is positioned in the front half of the platform in the crystal structure of the 30S subunit and forms bridges of electron density that extend toward the 50S subunit in the 70S ribosome crystal structure (Cate *et al.*, 1999; Clemons *et al.*, 1999; Wimberly *et al.*, 2000; Yusupov *et al.*, 2001). Consistent with the structural data, nucleotide substitutions in this loop have been shown to result in defects in ribosomal subunit association (Tapprich *et al.*, 1989; Lee *et al.*, 1997; Song *et al.*, 2007). Residues in the 790 loop are protected from chemical probes through binding of initiation factor 3 (Muralikrishna & Wickstrom, 1989; Moazed *et al.*, 1995), 50S subunits (Moazed & Noller, 1986), P-site bound $tRNA^{phe}$ (Moazed & Noller, 1986; Dinos *et al.*, 2004), as well as the P-site specific antibiotics edeine, kasugamycin, and pactamycin (Moazed & Noller, 1987; Mankin, 1997; Dinos *et al.*, 2004), indicating that this loop interacts with various ligands at different stages of translational initiation.

During translation initiation, mRNA competes for binding to available 30S subunits, the initiator tRNA is selected over other tRNAs, and the start codon is decoded at the P-site. This process requires three initiation factors, and the interactions of these initiation factors with 16S rRNA have been characterized by chemical protection studies. The sites affected by IF1 overlap with those affected by A-site-bound tRNA (Moazed & Noller, 1986, 1990; Moazed

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et al., 1995), and IF1 also enhances the reactivity of a subset of class III sites (A1413, G1487, A908, and A909) that are protected by tRNA, 50S subunits, and certain antibiotics (Moazed & Noller, 1987; Dahlquist & Puglisi, 2000). It has also been demonstrated that initiation factor 3 (IF3) protects another class III site, G791, while no IF2 footprint has been observed for this site (Moazed *et al.*, 1995). IF3 is known to promote subunit dissociation and to discriminate other tRNAs from the initiator tRNA; mutations at positions 787, 791, 792, and 795 result in a decrease in subunit association (Tapprich *et al.*, 1989; Santer *et al.*, 1990; Lee *et al.*, 1997). The decreased binding affinity of IF3 to 30S when residues at 791 and 792 are altered (Tapprich *et al.*, 1989; Santer *et al.*, 1990) indicates that this is not due to a lack of antiassociation activity, but rather a loss of ability of the initiator tRNA to select or bind to the P-site, resulting in a decrease in subunit association. The footprinting and structural data suggest that these residues are heavily involved in tRNA selection in the P-site and it is likely that these sites may form a structural motif that interacts with IF3 and recruits the initiator tRNA to the P-site. The P-site-specific antibiotics edeine, pactamycin, and kasugamycin, which stabilize the P-site-bound tRNA, show a footprint in the 790 loop; this also supports the involvement of the 790 loop in the recruitment of initiator tRNA to the P-site.

Here, we describe how we functionally analyzed the role of G791 in protein synthesis. This residue has been shown to be an invariant and essential residue for ribosome function (Lee *et al.*, 1997; Song *et al.*, 2007). To investigate the functional role played by G791 during the process of protein synthesis, we adopted a novel genetic approach (Lee *et al.*, 1997, 2001; Kim *et al.*, 2009) by introducing a base substitution at position 791 and then selecting multicopy suppressors that partially restored the protein synthesis ability of the mutant ribosomes. We identified IF1 as a multicopy suppressor of the mutant ribosome bearing a G to U substitution at position 791. Based on functional analyses of the effects of IF1 on the mutant ribosome, we suggest the involvement of IF1 in the restoration of the P-site that was perturbed by a nucleotide substitution at position 791.

Materials and methods

Strains and plasmids

All plasmids were maintained and expressed in *E. coli* DH5α (Hanahan, 1983). Cultures were grown in Luria–Bertani (LB) medium (Luria & Burrous, 1957) or LB medium containing 100 μg mL^{-1} ampicillin (LB–Amp100). To induce the synthesis of plasmid-derived rRNA from the *lac*UV5 promoter, IPTG was added to a final concentration of 1 mM and 0.1% of L-arabinose was used to induce the synthesis of initiation factors from the BAD promoter.

Plasmids pRNA122 and pRNA16 ST were described previously (Lee *et al.*, 1997, 2001). The construction of pKAN3, pKAN4, and pKAN6 was described previously (Tamura *et al.*, 2006). To construct pRNA122-U791U1192 and pRNA122-G770U1192, the XbaI and SexAI fragment from pRNA122-U1192 (Lee *et al.*, 1996) was subcloned into the same sites in pRNA122-U791 (Song *et al.*, 2007) and pRNA122-G770 (Kim *et al.*, 2007).

To clone the IF1 gene into pKAN3, the coding sequence of IF1 along with its intact promoter P1 was amplified from the DH5α chromosome using two primers, IF1-1 (5′-GAAGCTTGGATCCCCGAACCTGTTTGTTGCGAT) and IF1-2 (5′-CTCTAGAATTCACTTCCAAAGTACTTCATAC), and ligated into the BamHI and EcoRI sites of pKAN3. To clone all three initiation factors under control of the BAD promoter, coding regions were amplified from the DH5α chromosome and cloned into the NotI and XbaI sites of pKAN6. The 5′- primers contained the same ribosome-binding site and spacer to ensure the same level of protein expression. The primer sequence is as follows: 5′-GGCATGCGCGGCCGCAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATG plus 17 nucleotides of the gene-specific sequence (the start codon is underlined). The 3′-primers had the same sequence, except for the sequence corresponding to the coding region, namely 5′-GGATCCTCTAGATTA plus 17 nucleotides of the gene-specific sequence (the stop codon is underlined).

Minimum inhibitory concentration (MIC) and Western blot analysis

MICs were determined as described previously (Lee *et al.*, 1996). Western blot analysis of the CAT protein and IF1 was performed as described previously (Cummings & Hershey, 1994; Kim *et al.*, 2009).

Quantification of the distribution of plasmid-derived rRNA

Ribosome purification and primer extension analysis were performed as described previously (Lee *et al.*, 1996; Kim *et al.*, 2009).

Results and discussion

Isolation of intergenic multicopy suppressors of the pRNA122-U791 ribosome

To investigate the functional role played by G791 during the process of protein synthesis, we adopted a novel genetic approach using the specialized ribosome system (Lee *et al.*, 1997, 2001). In the specialized ribosome system used in this study, the chloramphenicol acetyltransferase (CAT) reporter message is translated exclusively by plasmid

(pRNA122)-derived ribosomes (pRNA122 ribosomes), which cannot translate normal cellular messages. Thus, it is possible to measure the function of the plasmid-derived mutant ribosomes *in vivo* by determining the amount of CAT protein synthesized in cells that express the mutant ribosomes. This specialized ribosome system offers a genetic method to select for mutants that restore CAT protein synthesis ability to mutant ribosomes, because the degree of resistance to chloramphenicol (Cm) of cells is proportional to the CAT activity or the amount of CAT protein produced in the cells by the mutant ribosomes (Lee *et al.*, 1997, 2001; Song *et al.*, 2007; Kim *et al.*, 2009).

We suspected the involvement of the 790 loop in interaction with ligands involved in translation because of the accessibility of the loop to solvents and the structural features of bases at positions 789–791. Consequently, we considered the possibility that a base substitution at position 791 may cause a structural perturbation in the 790 loop that prevents the 30S ribosome from interacting with ligands. To examine this possibility, we used a genetic complementation approach to identify such ligands. A genomic library was constructed in pKAN3 using *Escherichia coli* genomic DNA from the DH5α strain partially digested with EcoRI. This plasmid contains a replication origin from pACYC177 (Chang & Cohen, 1978) and a deletion of *bla*. Constructs of this vector are compatible with the pRNA122 plasmid, which is a pBR322 derivative. The genomic library was transformed into DH5α cells containing pRNA122 plasmid with the U791 mutation (pRNA122-U791). An empty vector (pKAN3) was transformed as a negative control. The U791 mutation was chosen, because this mutation was shown to be the most detrimental to ribosome function compared with other possible mutations at this position without affecting the assembly of the 30S ribosomal subunit (Song *et al.*, 2007). To select for genomic library clones containing genes that restored protein synthesis ability in U791 ribosomes when overproduced, transformants were plated on LB-agar medium containing 50 μg of chloramphenicol per milliliter of LB (Cm50); at this concentration, cells expressing pRNA122-U791 ribosomes could not grow. The survival ratio for the transformants was about 3×10^{-4}, which was about $400\times$ higher than the background (7×10^{-6} when pKAN3 was transformed).

Plasmids were prepared separately from 50 of the surviving geneomic clones and cotransformed with pRNA122-U791 into *E. coli* cells. The resulting transformants were tested for their degree of resistance to chloramphenicol in the presence and absence of the inducer IPTG. All clones derived from the genomic library were resistant to Cm100 (MIC = 150) only in the presence of IPTG. These results indicated that the CAT mRNA translation in these cells was dependent on both pRNA122-A789 ribosomes and genomic clones. However, all clones showed a MIC of 50 regardless of the presence of an inducer when 10 of the clones derived from cells harboring an empty vector were subjected to the same procedure as described above. These negative control clones may have survived initially because they managed to grow on selective media due to heavy plating of cells or chromosomal mutations.

Identification of intergenic multicopy suppressors of the pRNA122-U791 ribosome

Restriction enzyme sites analyses were performed with plasmids purified from the 50 clones using the initial EcoRI cloning site. All the clones exhibited a common 6 kbp EcoRI fragment. The results of sequencing analysis of the chromosomal DNA in five clones that contained only the 6.0 kb EcoRI fragment showed that the fragment was located at ~20 min of the *E. coli* chromosome. It contained the coding regions of *aat*, *cydC*, *cydD*, two unknown ORFs, and *infA*. We chose to test whether overexpression of *infA* was responsible for the partial restoration of the protein synthesis function of the pRNA122-U791 ribosome because this gene encodes a known translational factor, IF1. The coding region for the *inf*A gene was subcloned into pKAN6B, a derivative of pKAN3, and expressed under the arabinose-inducible promoter (pKAN6-IF1). Plasmid pKAN6-IF1 was cotransformed with pRNA122-U791 into DH5α cells and the resulting transformants were tested for their degree of resistance to chloramphenicol in the presence and absence of the inducer IPTG. The cells that expressed the pRNA122-U791 ribosomes and IF1 protein from pKAN6-IF1 showed resistance to chloramphenicol at the same levels (MIC = 150 μg mL^{-1}) as cells that expressed the pRNA122-U791 ribosomes and the IF1 protein from the EcoRI genomic clones (Fig. 1a). We therefore concluded that multiple copies of the wild-type IF1 gene, probably due to overexpression of IF1, enhanced the protein synthesis ability of pRNA122-U791 ribosomes. Overexpression of IF1 also allowed cells that expressed pRNA122-A791 or pRNA122-C791 ribosomes to exhibit resistance to higher concentrations of chloramphenicol (MIC = 300, 200 μg mL^{-1}, respectively), whereas the degree of chloramphenicol resistance of cells expressing the wild-type pRNA122 ribosomes was not affected by IF1 overexpression (Fig. 1a).

Next, the amount of CAT and IF1 proteins in cells was quantified using Western blot analysis to examine whether increased CAT protein synthesis by the mutant ribosomes was responsible for the enhanced resistance to chloramphenicol of cells coexpressing the pRNA122-U791 ribosomes and IF1. Cells expressing both pRNA122-U791 ribosomes and IF1 showed an ~1.5-fold increase in the amount of CAT protein when compared with cells that expressed only the pRNA122-U791 ribosomes (Fig. 1b). Analogous results were obtained when the amount of CAT protein was

quantified in cells expressing pRNA122-C791 ribosomes in the presence and absence of IF1 overexpression. The amount of CAT protein was moderately increased in cells expressing pRNA122-A791 when IF1 was coexpressed compared with cells that expressed only the pRNA122-A791 ribosomes. These results indicate that the degree of complementation by IF1 overexpression is somewhat dependent on the nucleotide identity at position 791. Overexpression of IF1 had no significant effect on the amount of CAT protein synthesized by the wild-type pRNA122 ribosomes. These results demonstrated a good correlation between the degree of cellular resistance to chloramphenicol and the quantity of CAT synthesized in these cells. The amount of IF1 protein in cells harboring pKAN6-IF1 was increased by approximately 20-fold compared with cells harboring pKAN6 (Fig. 1b). This indicates that IF1 was overexpressed from pKAN6-IF1 and was responsible for the increase in protein synthesis from the mutant ribosomes.

Effects of IF2 or IF3 overexpression on CAT protein synthesis by pRNA122-U791 ribosomes

It has been shown that the 790 loop interacts with IF3 and initiation factors are known to interact functionally with one another during translational initiation. We therefore tested whether two other initiation factors, IF2 and IF3, could complement the pRNA122-U791 ribosomes. The coding regions of IF2 and IF3 were cloned into pKAN6 under the control of an arabinose-inducible promoter (pKAN6-IF2 and pKAN6-IF3), and these proteins were expressed in cells harboring pRNA122-U791. Neither the overexpression of IF2 nor IF3 complemented pRNA122-U791 ribosomes (MIC = 50) (data not shown here).

Specificity of IF1 overexpression on ribosome function

To test the effect of IF1 overexpression on wild-type ribosomes, we measured the amount of CAT protein produced by cells expressing CAT mRNA with a natural *E. coli* consensus ribosome-binding sequence (5′-GGAGG-3′) and 16S rRNA with an mRNA-binding sequence (5′-CCUCC-3′) present in chromosomally derived rRNA (pRNA9) (Lee *et al.*, 1996) in the presence and absence of IF1 overexpression. Overexpression of IF1 did not enhance the level of CAT protein synthesis by wild-type ribosomes (Table 1).

To test whether the effects of increased IF1 as a multicopy suppressor were specific to U791 ribosomes, DH5α cells expressing pRNA122 ribosomes bearing a nucleotide substitution (A516 or G770) were transformed with pKAN6 or pKAN6-IF1, and the resulting transformants were tested for their degree of resistance to chloramphenicol. These mutations were chosen because they have been shown to exhibit a protein synthesis ability as poor as that of pRNA122-U791 ribosomes (Lee *et al.*, 2001; Kim *et al.*, 2007). IF1 overexpression had no effect on mutant ribosomes bearing a

Table 1. Effects of IF1 overexpression on mutant ribosomes

Ribosomes expressed from	IF1 overexpression	MIC (μg mL^{-1})
pRNA9	–	600
	+	600
pRNA122-U791	–	50
	+	150
pRNA122-A516	–	50
	+	50
pRNA122-G770	–	150
	+	150

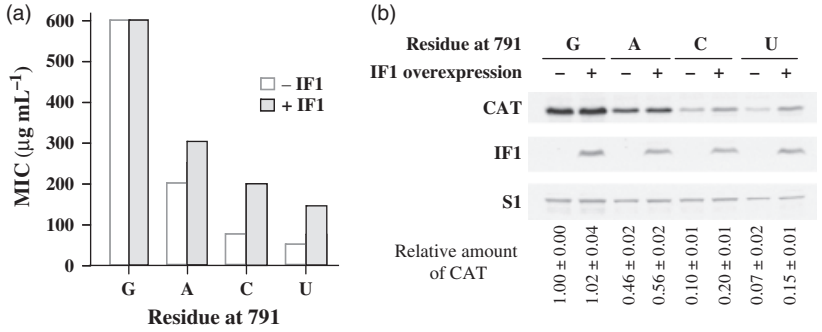

Fig. 1. Effects of IF1 overexpression on the function of pRNA122 ribosomes containing a mutation at position 791. (a) Degree of resistance to chloramphenicol. (b) Western blot analysis of the CAT protein. Cultures were grown to $OD_{600\,nm}$ = 0.1 and 1 mM IPTG and 0.2% arabinose were added to induce the synthesis of pRNA122 ribosomes and IF1. Culture samples were either tested for MIC or harvested 2 h after induction to obtain total protein. The same membrane was probed with anti-CAT monoclonal antibody, anti-IF1 monoclonal antibody, and anti-S1 polyclonal antibody. The relative abundance of the CAT and IF1 protein bands was quantified by setting the amount of protein produced by wild-type pRNA122 ribosomes in the absence of IF1 overexpression equal to 1. The experiments were repeated three times and averaged. The SE of the mean (± numbers) was used to indicate the range of assay results.

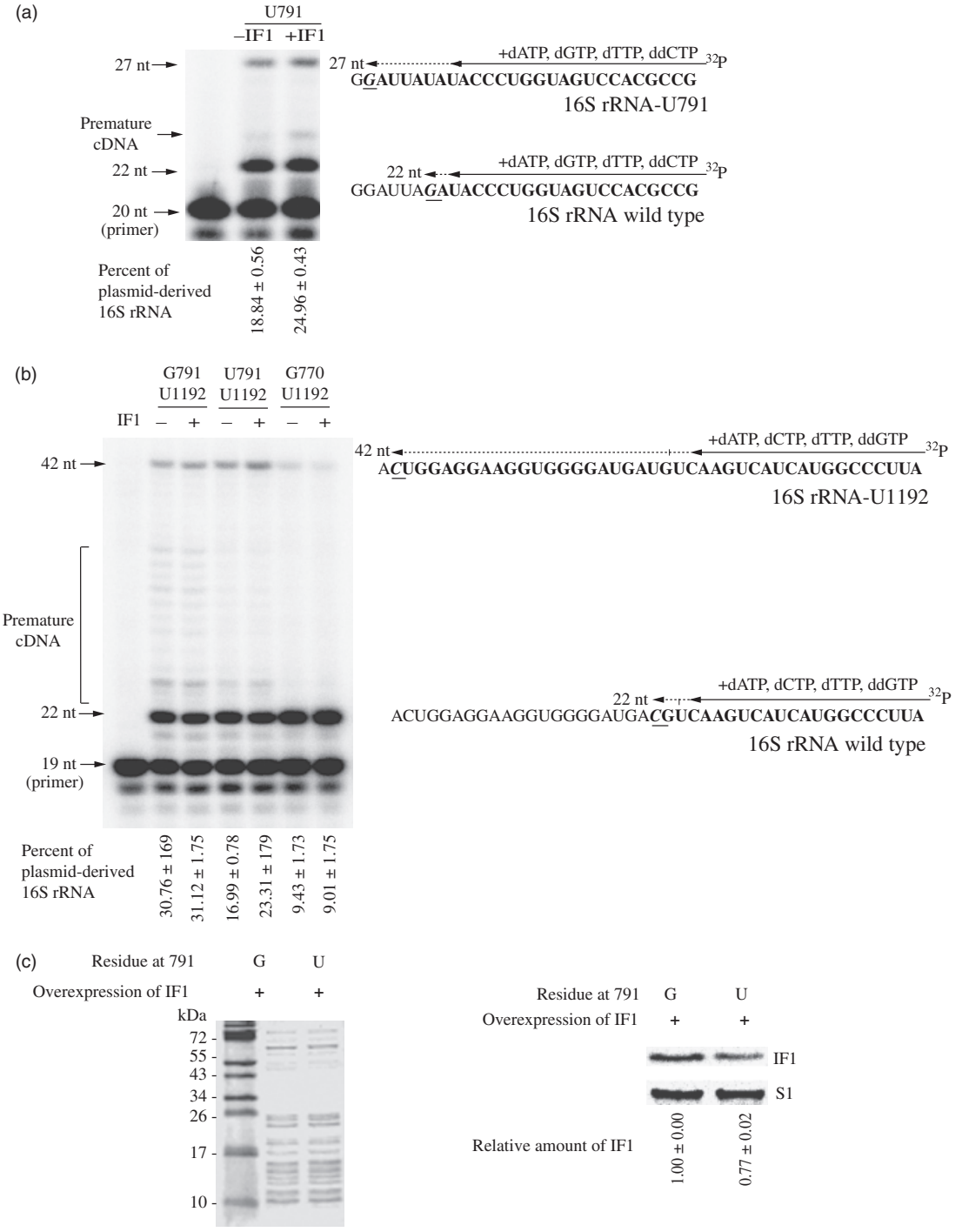

Fig. 2. Effects of IF1 overexpression on the ribosomal subunit association of pRNA122-U791 ribosomes. The distribution of plasmid-derived mutant 16S rRNA was measured by annealing with a 5′-end-labeled primer complementary to the sequence downstream of the 791 mutation site (a) or the 1192 mutation site (b) and extending through the mutation sites using AMV reverse transcriptase. The synthesized cDNAs were resolved by polyacrylamide gel electrophoresis and the ratios of mutant to nonmutant rRNA were determined via comparisons of the amount of radioactivity in each of the two bands. The amount of radioactivity in premature cDNA bands, which were indicated, was also taken into account to determine the ratios of mutant to nonmutant rRNA where they were found. (c) Western blot analysis of the IF1 protein in the 30S peak. 30S fractions purified from *Escherichia coli* cells coexpressing IF1 and pRNA122-G791 or pRNA122-U791 ribosomes via sucrose gradient were separated in 13% tricine-sodium dodecyl sulfate gels, and either stained with coommassie blue (left panel) or transferred to a nitrocellulose membrane for Western blotting (right panel) as described in the legend of Fig. 1. The experiments were repeated three times and averaged. The SE of the mean (± numbers) is used to indicate the range of the assay results.

nonfunctional mutation in other regions of 16S rRNA, thus indicating that the effect of IF1 on ribosome function is not a general phenomenon (Table 1).

Effects of IF1 overexpression on the ribosomal subunit association of pRNA122-U791 ribosomes

A previous study demonstrated that pRNA122-U791 ribosomes have ribosomal subunit association defects (Song *et al.*, 2007). For this reason, we measured the effects of IF1 overexpression on pRNA122-U791 ribosomes in terms of the formation of 70S ribosomes. Total ribosomes were purified from cells that expressed pRNA122-U791 ribosomes in the presence and absence of IF1 overexpression using a sucrose gradient, and we analyzed the ability of pRNA122-U791 ribosomes to form 70S ribosomes. Primer extension analysis revealed that 16S rRNA containing U791 was notably under-represented in the 70S ribosome peaks (~19%) of the total ribosomes purified from cells harboring pRNA122-U791 and pKAN6A, as has been shown previously (Song *et al.*, 2007), while the distribution of 16S rRNA containing U791 was increased up to ~25% in the 70S ribosome peaks purified from cells harboring pRNA122-U791 and pKAN6-IF1 (Fig. 2a).

To test whether the effect of IF1 overexpression on the formation of 70S ribosomes is specific to pRNA122-U791 ribosomes, we measured the effects of IF1 overexpression on wild-type and U770 mutant 30S ribosomes in terms of their ability to form 70S ribosomes. To do this, we subcloned a C to T mutation at position 1192 in the 16S rRNA coding region of pRNA122, pRNA122-U791, and pRNA122-U770. This mutation (U1192) has been shown to have no effect on ribosome function and has therefore been used to assess the distribution of plasmid-derived ribosomes in the cell (Sigmund *et al.*, 1984; Makosky & Dahlberg, 1987). Total ribosomes were purified and analyzed using primer extension analysis. IF1 overexpression had no significant effect on pRNA122 wild-type and pRNA122-U770 ribosomes, while we found that the subunit association increased only by pRNA122-U791U1192 ribosomes, suggesting that the IF1 effect is specific to pRNA122-U791 ribosomes (Fig. 2b).

We further tested whether the increased subunit association of pRNA122-U791 ribosomes by overexpression of IF1 stems from the lower binding affinity of pRNA122-U791 ribosomes to IF1 by measuring the amount of IF1 that was associated with 30S subunits. The results indicated that amounts of IF1 are lower by ~23% in the 30S fraction from *E. coli* cells coexpressing U791 ribosomes and IF1 than those expressing G791 ribosomes and IF1 (Fig. 2c). The composition of ribosomal proteins in both 30S fractions was similar (Fig. 2c), indicating that the U791 mutation does not affect assembly of ribosomal proteins to 16S rRNA. Considering that the proportion of mutant 30S subunits in the 30S peak from the sucrose gradient analysis is ~40% (data not shown here), we conclude that the U791 mutation severely inhibits IF1 binding to the 30S ribosomal subunit.

Overexpression of IF1 resulted in increased ribosomal subunit association, probably by stabilizing P-site-bound initiator tRNA, which is mediated by its cooperation with IF2 and its interaction with the initiation codon of the mRNA (Hartz *et al.*, 1990; Wu & RajBhandary, 1997; Meinnel *et al.*, 1999). Although no clear function has been assigned to initiation factor 1, considering that IF1 is known to aid IF2 and IF3 in translational initiation and increase the rate of both subunit association and dissociation (Grunberg-Manago *et al.*, 1975), and that IF1 footprinting mimics A-site-bound tRNA, a local change in the A-site due to an increase in IF1 binding to the A-site may be transmitted to the P-site (790 loop), thus restoring the functional conformation of the P-site for initiator tRNA binding and consequent ribosomal subunit association. The crystal structures of ribosomes also support this hypothesis. The 790 loop interacts with the 900 region and the 900 region docks somewhere in the vicinity of residues at positions 1413–1418 and 1483–1487 (Cate *et al.*, 1999; Clemons *et al.*, 1999), which interact with IF1 (Carter *et al.*, 2001).

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