Control of translation initiation involves a factor-induced rearrangement of helix 44 of 16S ribosomal RNA

Daoming Qin[1,2] and **Kurt Fredrick**[1,2,3]

[1]Ohio State Biochemistry Program, The Ohio State University, OH 43210, USA.

[2]Center for RNA Biology, The Ohio State University, OH 43210, USA.

[3]Department of Microbiology, The Ohio State University, OH 43210, USA.

Summary

Initiation of translation involves recognition of the start codon by the initiator tRNA in the 30S subunit. To investigate the role of ribosomal RNA (rRNA) in this process, we isolated a number of 16S rRNA mutations that increase translation from the non-canonical start codon AUC. These mutations cluster to distinct regions that overlap remarkably well with previously identified class III protection sites and implicate both IF1 and IF3 in start codon selection. Two mutations map to the 790 loop and presumably act by inhibiting IF3 binding. Another cluster of mutations surrounds the conserved A1413○G1487 base pair of helix 44 in a region known to be distorted by IF1 and IF3. Site-directed mutagenesis in this region confirmed that this factor-induced rearrangement of helix 44 helps regulate initiation fidelity. A third cluster of mutations maps to the neck of the 30S subunit, suggesting that the dynamics of the head domain influences translation initiation. In addition to identifying mutations that decrease fidelity, we found that many P-site mutations increase the stringency of start codon selection. These data provide evidence that the interaction between the initiator tRNA and the 30S P site is tuned to balance efficiency and accuracy during initiation.

Introduction

Translation initiation involves recognition of the start codon by the initiator tRNA in the ribosomal P site. In bacteria, this process is kinetically controlled by three initiation factors (IF1, IF2 and IF3) and occurs in two major phases (Gualerzi and Pon, 1990; Gualerzi *et al.*, 2001; Boelens and Gualerzi, 2002). The first phase is assembly of the 30S initiation complex (30SIC). During this phase, the initiator tRNA (fMet-tRNA^{fMet}) binds the 30S P site and its anti-codon pairs with the start codon of mRNA. The second phase includes the docking of the 50S subunit onto the 30SIC, IF2-dependent GTP hydrolysis, and dissociation of the initiation factors. This generates a 70S initiation complex (70SIC) ready for the first round of elongation.

The efficiency of translation initiation is determined by *cis*-acting elements located in the translation initiation region of the mRNA. One important element is the start codon. In bacteria, AUG is the most common start codon, but GUG and UUG are also used quite often (Eduardo *et al.*, 1999). Another important element is a purine-rich sequence (e.g. AGGA)

*For correspondence. fredrick.5@ osu.edu; Tel. (+1) 614 292 6679; Fax (+1) 614 292 8120..

termed the Shine–Dalgarno (SD) (Shine and Dalgarno, 1974), which lies 6–12 nucleotides upstream from the start codon (Shultzaberger *et al.*, 2001). The SD pairs with the 3′ end of 16S ribosomal RNA (rRNA), termed anti-Shine–Dalgarno sequence (ASD), and this mRNA–rRNA interaction facilitates positioning of the start codon in the 30S P site (Steitz and Jakes, 1975; Kaminishi *et al.*, 2007; Korostelev *et al.*, 2007). The sequence of the SD, sequence of the start codon, number of nucleotides between the SD and start codon, and the secondary structure of the translation initiation region influence the efficiency of initiation (Ringquist *et al.*, 1992; Vellanoweth and Rabinowitz, 1992; de Smit and van Duin, 2003; Studer and Joseph, 2006), indicating the importance of each of these determinants in the initiation process.

Initiation at the correct start codon is critical for establishing the translational reading frame. A number of mutations have been isolated in *Escherichia coli* that increase spurious initiation (i.e. translation from non-canonical start codons such as ACG, AUU, AUC and CUG). These mutations were mapped to *infC*, the gene encoding IF3, strongly implicating the factor in start codon selection (Sacerdot *et al.*, 1996; Sussman *et al.*, 1996; Haggerty and Lovett, 1997). Reducing the concentration of wild-type IF3 in the cell also increases spurious initiation, while overexpression of IF3 confers the opposite phenotype (Olsson *et al.*, 1996; Sacerdot *et al.*, 1996). Indeed, the ability of IF3 to repress translation from non-canonical start codons explains how the factor autoregulates translation of its own gene, which starts with AUU (Brombach and Pon, 1987; Butler *et al.*, 1987; Sacerdot *et al.*, 1996).

IF3 appears to act at both stages of initiation to ensure accuracy. IF3 destabilizes fMet-tRNA$^{\text{fMet}}$ during 30SIC formation, which presumably makes its binding more dependent on cognate codon–anti-codon interaction (La Teana *et al.*, 1993; Antoun *et al.*, 2006a,b). IF3 also inhibits formation of a stable 70SIC when the mRNA contains a non-canonical start codon such as AUU (Grigoriadou *et al.*, 2007; Milon *et al.*, 2008).

Although IF1 is an essential protein in the cell (Cummings and Hershey, 1994), its role in translation initiation remains elusive. IF1 is a small (~8 kDa) β-barrel protein that binds the 30S A site and enhances the activities of the other two factors (Stringer *et al.*, 1977; Pon and Gualerzi, 1984; Moazed *et al.*, 1995; Sette *et al.*, 1997; Carter *et al.*, 2001; Antoun *et al.*, 2006a), which may stem in part from cooperative binding (Weiel and Hershey, 1982; Zucker and Hershey, 1986; Moazed *et al.*, 1995). Recent biochemical studies suggest that IF1 negatively regulates 70SIC formation to ensure high fidelity (Milon *et al.*, 2008). However, *in vivo* evidence that IF1 increases the accuracy of initiation is lacking.

Ribosomal RNA undoubtedly plays a role in initiation, but precisely how 16S rRNA participates in the process remains unclear. Here, we randomly mutagenized 16S rRNA and isolated a number of mutations that increase translation from the non-canonical start codon AUC. These mutations map to distinct regions including the neck, the 790 loop, and helix 44 (h44). The mutations in h44 map near the non-canonical A1413○G1487 pair in a region distorted by either IF1 or IF3. Site-directed mutagenesis in this region supports the idea that this factor-induced rearrangement of h44 helps control translation initiation. We also report that many P-site mutations increase the stringency of start codon selection. These data provide evidence that the affinity of fMet-tRNA$^{\text{fMet}}$ for the 30S P site is tuned to balance accuracy and efficiency during initiation.

Results

Identification of 16S rRNA mutations that increase translation from AUC

In previous work, a specialized ribosome system was used to identify two 16S rRNA mutations, G1338A and A790G, which increase translation from non-canonical start codons

in vivo (Qin *et al.*, 2007). Using the same genetic system, we sought to identify additional mutations with the same phenotype. Plasmid pKF207, encoding specialized 16S rRNA (ASD*: 5′-GGGGU-3′), was mutagenized by propagation in the mutator strain XL1-Red (Stratagene) and used to transform the indicator strain KLF2672. Strain KLF2672 expresses *lacZ* mRNA with the complementary SD sequence (SD*: 5′-AUCCC-3′) and the start codon AUC. Transformants were screened on Xgal plates for those expressing increased levels of β-galactosidase. Nineteen independent isolates carrying one or more mutations in 16S rRNA were identified (Table 1). With one exception (U13G), these mutations were transitions, a bias that appears to be due largely to the mutagenesis with XL1-Red (data not shown). Mutation G1539A mapped to the ASD* and is predicted to change the only wobble base pair in the SD*-ASD* helix to a Watson-Crick base pair. This mutation uniformly increased expression from SD*-AUC-*lacZ* and SD*-AUG-*lacZ* by about threefold (data not shown). The other 16S rRNA mutations cluster to distinct regions that overlap with class III protection sites (Fig. 1). Class III sites were previously implicated in ligand-induced conformational changes based on chemical protection experiments (Moazed and Noller, 1987). Two mutations, U789C and A790G, map to the IF3-binding site. Four mutations cluster in h44, just 'down' from the 30S A site, and surround A1413○G1487 (Fig. 1B and C), a non-canonical pair conserved in bacteria and perturbed by either IF1 or IF3 (Moazed *et al.*, 1995; Dahlquist and Puglisi, 2000; Carter *et al.*, 2001; Cannone *et al.*, 2002). Another group of mutations cluster near the neck of the 30S subunit (Fig. 1D).

To determine which mutations recovered were responsible for the phenotype, each mutation was introduced into pKF207 *de novo* by site-directed mutagenesis. The resulting plasmids were transformed into an indicator strain containing SD*-AUC-*lacZ*. Mutations U13G, U789C, A790G, G886A, U1083C, A1092G, G1094A, C1389U, A1410G, C1411U, U1414C, G1486A were each confirmed to increase expression from SD*-AUC-*lacZ*. To quantify the phenotypes, the plasmids were also moved into strains containing SD*-ACG-*lacZ* or SD*-AUG-*lacZ* and β-galactosidase activity of all resulting strains was compared (Table S1). Translation from AUC and ACG was increased specifically and to various extents depending on the mutation. The level of β-galactosidase from SD*-AUC-*lacZ* or SD*-ACG-*lacZ* relative to SD*-AUG-*lacZ* was used to approximate the frequency of spurious initiation (Fig. 2). Mutations U789C and A790G appeared most defective in initiation fidelity (fivefold to sixfold decrease), similar to effects conferred by various *infC* alleles (Sacerdot *et al.*, 1996; Sussman *et al.*, 1996; Maar *et al.*, 2008). The other mutations had modest but significant effects. We also determined the effects of the 16S rRNA mutations on initiation fidelity in an *infC362* background. This mutation in the IF3 gene increased spurious initiation by about fivefold. The effects of *infC362* and the 16S rRNA mutations were completely additive in some cases (U1083C, A1092G, G1094A, C1389U, C1411U and U1414C) and partially additive in the others (Fig. 2).

Mutant ribosomes can support the growth of E. coli

As a first step towards analysing the mutant 30S subunits *in vitro*, we moved a number of mutations (U13G, U789C, A790G, A790U, A790C, G886A, G1094A, G1338A, G1338U, A1408G, A1410G and U1414C) into a Δ7 prrn strain of *E. coli*, SQZ10. This strain lacks all chromosomal rRNA operons and relies on a single plasmid-borne rRNA operon, and hence a homogeneous population of ribosomes. Each mutation could be readily moved into the Δ7 prrn background, and the resulting strains did not exhibit obvious growth defects (data not shown). All ligand-binding experiments described below were performed using 30S subunits purified from these Δ7 prrn strains as described (Qin *et al.*, 2007).

A790G and U789C confer a defect in IF3 binding

A790G was previously shown to decrease the affinity of IF3 for the 30S subunit by ~10-fold (Qin *et al.*, 2007). We presumed that the adjacent mutation U789C similarly inhibited IF3 binding. To test this, we purified subunits containing U789C or each substitution at position 790 and compared their ability to bind IF3 (Qin *et al.*, 2007). IF3 was labelled with amino-reactive fluorophore (Alexa Fluor 488) and sucrose gradient sedimentation analysis was used to detect the binding of labelled IF3 (IF3-AF) to the mutant 30S subunits (Fig. 3). Little or no IF3-AF co-migrated with 30S subunits containing A790G or U789C, indicating that both mutations conferred a substantial defect in IF3 binding. A790U reduced the level of co-migrating IF3-AF while A790C had no appreciable effect on the level of co-migrating IF3-AF.

A1410G and G886A confer a defect in IF1 binding

A group of mutations mapped to h44 of 16S rRNA (Fig. 1B and C). Structural and biochemical studies have shown that IF1 binds the 30S A site, close to where our h44 mutations cluster (Moazed *et al.*, 1995; Dahlquist and Puglisi, 2000; Carter *et al.*, 2001). Thus, we considered the possibility that some of our mutations affected IF1 binding. 30S subunits containing U13G, G886A, A1408G, A1410G or U1414C were purified and the affinities of [^{35}S]-IF1 for these subunits were measured using membrane filtration devices (Microcon YM100, MWCO 100K; Millipore Corporation) to separate free and bound [^{35}S]-IF1 (Fig. 4). The dissociation constant (K_D) for IF1 binding to control 30S subunits was estimated at ~0.6 μM, consistent with earlier studies using true equilibrium methods (Zucker and Hershey, 1986; Dahlquist and Puglisi, 2000). G886A and A1410G reduced the affinity for IF1 by twofold and threefold respectively, while the other mutations tested did not affect IF1 binding (Fig. 4A, Table 2). 50S subunits failed to bind [^{35}S]-IF1, indicating that interaction between [^{35}S]-IF1 and the 30S subunit was specific. As an additional control, we tested A1408G, a mutation shown previously to confer a defect in IF1 binding (Dahlquist and Puglisi, 2000). A1408G conferred a defect in our hands as well, but of lesser magnitude than that reported previously.

As IF1 and IF3 bind cooperatively to the 30S subunit, we also measured the binding of [^{35}S]-IF1 to mutant 30S subunits in the presence of saturating IF3 (Table 2). IF3 (350 nM) increased the affinity of IF1 for control 30S subunits by ~30-fold, consistent with previous studies (Zucker and Hershey, 1986). G886A or A1410G decreased the affinity for IF1 by twofold to threefold in the presence of IF3, as was observed in its absence (Table 2). The fact that these mutations similarly affected IF1 binding in the absence and presence of IF3 indicates that they do not disrupt the mechanism of cooperativity between the two factors.

A1408G causes spurious initiation in vivo

A1408G was not isolated in the genetic screen but conferred a defect in IF1 binding. Thus, it was of interest to determine whether A1408G also increased spurious initiation. We engineered A1408G into pKF207, transformed the appropriate indicator strains, and found that, indeed, A1408G increased translation from AUC and ACG to the same degree as other h44 mutations (Fig. 2, Table S1). These data further implicate IF1 in the mechanism of initiation fidelity.

Watson-Crick base pairs at 1413–1487 and 1414–1486 decrease initiation fidelity

In contrast to mutation A1410G, U1414C did not affect IF1 binding despite that it conferred a similar decrease in initiation fidelity (Fig. 2). Structural studies have shown that bound IF1 locally displaces one strand of h44 along the helical axis, disrupting several base pairs including A1413○G1487 and U1414•G1486 (Carter *et al.*, 2001). Both U1414C and

G1486A were isolated and are predicted to change the wobble U1414•G1486 base pair to C-G and U-A respectively, suggesting that they may decrease initiation fidelity by stabilizing the paired conformation of h44. With this in mind, we engineered mutations to generate alternative base pairs at positions 1413–1487 and 1414–1486 and tested their effects on initiation fidelity (Fig. 5, Table S2). Substitutions predicted to stabilize base-pairing at either position conferred increased levels of spurious initiation. In contrast, mismatch A1413 × A1487 decreased spurious initiation, while G1413 × A1487 and U1414 × C1486 had no appreciable effect. The only exceptional case was G1414 × G1486, which increased spurious initiation somewhatdespite that it would be predicted to destabilize the paired conformation of h44.

A1410 forms a Watson-Crick base pair with U1490, which remains paired upon IF1 binding (Carter *et al.*, 2001). A1410G was isolated and is predicted to change the Watson-Crick base pair to a wobble G•U pair. We engineered alternative base pairs at this position and found that U1490C increased spurious initiation by about fourfold. A1410G–U1490C double mutation, which generates an alternative Watson-Crick base pair at 1410–1490, restored initiation fidelity (Fig. 5, Table S2). These data suggest that a Watson-Crick base pair at 1410–1490 is important for initiation fidelity, consistent with 95% conservation of a Watson-Crick pair at this position (Cannone *et al.*, 2002).

Most 30S P-site mutations increase the stringency of start codon selection

It has been proposed that IF3 acts to increase fidelity in part by destabilizing fMet-tRNA^{fMet} in the 30S P site (La Teana *et al.*, 1993; Antoun *et al.*, 2006a,b). To explore this possibility, we analysed a number of P-site mutations for their effects on initiation fidelity. Most P-site mutations decreased translation from both SD*-AUC-*lacZ* and SD*-AUG-*lacZ*, but decreased translation from AUC to a larger degree (Table 3). In other words, these P-site mutations effectively increase the stringency of start codon selection.

Effect of 30S mutations on tRNA^{fMet} binding

Most P-site mutations would be expected to destabilize fMet-tRNA^{fMet} in the 30SIC. A notable exception is G1338A, which stabilizes tRNA^{fMet} in the P site (Qin *et al.*, 2007). To determine how other 16S rRNA mutations affected tRNA^{fMet} binding, we estimated the affinity of 3′-[^{32}P]-tRNA^{fMet} for the 30S P site in various mutant 30S subunits using filter binding as described (Qin *et al.*, 2007) (Fig. 4B, Table 2). Of the mutations tested, G1338U, A790C and A790U were the most defective in tRNA^{fMet} binding. A790G, U789C and G1094A conferred a smaller defect, while A1410G, U1414C and G886A showed no apparent defect.

Discussion

In this study, a number of mutations in 16S rRNA that increase translation from AUC were isolated. These mutations cluster to distinct regions that overlap remarkably well with previously identified class III protection sites. Class III sites were defined as those nucleotides protected from chemical probes by multiple ligands (e.g. 50S subunits, tRNA, various antibiotics). Because these ligands can bind simultaneously to the 30S subunit, these class III protections were attributed to ligand-induced conformational changes, rather than to direct protections (Moazed and Noller, 1987). The fact that many of our mutations map near class III sites suggests that they perturb rRNA rearrangements important for initiation.

Nucleotides 783–793 constitute a major portion of the IF3-binding site (Moazed *et al.*, 1995; Dallas and Noller, 2001; Fabbretti *et al.*, 2007). Two mutations (U789C and A790G) lie in this region and confer the strongest phenotypes (Fig. 2). A790G and the adjacent G791A

were shown previously to decrease the affinity of IF3 for the 30S subunit by ~10-fold (Tapprich *et al.*, 1989; Qin *et al.*, 2007), and U789C appears similarly defective in IF3 binding (Fig. 3). It is well known that IF3 regulates initiation complex formation to prevent initiation from non-canonical start codons (Sacerdot *et al.*, 1996; Sussman *et al.*, 1996; Haggerty and Lovett, 1997). Chromosomal mutations that increase spurious initiation events have been mapped almost exclusively to the IF3 gene (*infC*), in line with our observation that 16S rRNA mutations that target the IF3-binding site are the most defective in initiation fidelity. When mutations in IF3 (*infC362*) and its binding site (A790G) are present in the same cell, AUG is discriminated from AUC by a factor of 10 rather than 300 (Fig. 2), underscoring the importance of IF3 in regulating initiation.

A second cluster of mutations surrounds the conserved A1413○G1487 base pair in h44. Structural studies reveal that IF1 binds the 30S A site and induces a distortion of h44, involving displacement of one strand away from the helical axis and consequential disruption of base pairs A1413○G1487 and U1414•G1486 (Carter *et al.*, 2001). This rearrangement of h44 is centred right where our mutations cluster, strongly implicating IF1 in the mechanism of initiation fidelity. While certain mutations (i.e. A1410G, A1408G, G886A) decrease the affinity of IF1 for the subunit, U1414C does not. These data suggest that defects in fidelity might stem from inhibition of IF1 binding, the factor-induced rRNA rearrangement, or both. With this in mind, we tested a number of substitutions predicted to stabilize base pairs 1413–1487 and 1414–1486. In each case, an increase in spurious initiation was observed, consistent with the idea that initiation is regulated by the factor-induced rearrangement of h44. By contrast, engineered mismatches G1413 × G1487, A1413 × A1487 and U1414 × C1486 did not decrease initiation fidelity. The only anomalous result came from the G × G mismatch at 1414–1486, which conferred a slight increase in spurious initiation. It is possible this purine–purine pair generates a unique conformation responsible for its phenotype, which fails to follow the trend.

A recent biochemical study suggests that IF1 helps IF3 negatively regulate 70SIC formation to enhance start codon selection (Milon *et al.*, 2008). Changing the start codon from AUG to AUU slowed both IF3 dissociation and subunit joining, and omission of IF1 from the reaction increased the rates of these events in the presence of AUU. Interestingly, the ability of IF1 to inhibit IF3 release and subunit joining was lost in the presence of streptomycin. It was proposed that IF1 induces an 'initiation-unfavourable' conformation of the 30S subunit, which could be reversed by cognate codon–anti-codon pairing in the 30SIC or by streptomycin. Our data are consistent with this model and further suggest that the structural corollary of the 'initiation-unfavourable' conformation is the distorted state of h44, in which base pairs 1413–1487 and 1414–1486 are unpaired, as observed in the cocrystal structure of 30S-IF1 (Carter *et al.*, 2001). Binding of either IF1 or IF3 to the 30S subunit causes G1487 to become hyper-reactive to kethoxal, suggesting that both factors stabilize the unpaired state of h44 (Moazed *et al.*, 1995; Dahlquist and Puglisi, 2000). Streptomycin, on the other hand, protects G1487 from kethoxal modification, as does tRNA (presumably P-tRNA) (Moazed and Noller, 1987). These ligands do not directly interact with G1487; instead, they most probably stabilize the paired state of h44. Clues of how the unpaired state of h44 might inhibit 70SIC formation come from structural studies. The centre of the IF1-induced distortion lies between intersubunit bridges B2a and B3 (Carter *et al.*, 2000; Korostelev *et al.*, 2006; Selmer *et al.*, 2006), suggesting that docking of the 50S subunit might be inhibitedby displacement of the 16S rRNA nucleotides that contribute to these bridges. The idea that IF1 helps regulate 50S docking is consistent with evidence that IF1 and IF3 work together to facilitate subunit splitting (Noll and Noll, 1972; Godefroy-Colburn *et al.*, 1975; Pavlov *et al.*, 2008).

A previous report showed that several mutations in *inf*A failed to increase translation from GUG and UUG (Croitoru *et al.*, 2005), and those data have been taken by some investigators as evidence that IF1 plays no role in start codon selection. However, mutations in *infC* also fail to increase translation from GUG and UUG (Sacerdot *et al.*, 1996; Sussman *et al.*, 1996), indicating that IF3 does not discriminate against these naturally occurring non-AUG start codons. In our view, there is no reason to suspect that IF1 would discriminate against these start codons either. For technical reasons, Croitoru *et al.* were unable to measure the effects of *infA* alleles on spurious initiation (i.e. translation from Class IIa codons such as AUU, AUC and ACG). Whether these *infA* alleles increase spurious initiation remains an open question worth addressing.

One of our mutations, U13G, maps to the central pseudoknot and has been isolated previously in a selection for streptomycin resistance (Harris *et al.*, 1989). Streptomycin binds adjacent to U13 (Carter *et al.*, 2000), and other base substitutions at this position have been shown to inhibit streptomycin binding (Pinard *et al.*, 1993). Although not yet understood, this connection between streptomycin and initiation fidelity *in vivo* is noteworthy, particularly in light of the effects of streptomycin on the ability of IF1 to regulate initiation *in vitro* (Milon *et al.*, 2008).

A third cluster of mutations localizes to the neck region, and these mutations may act by altering the dynamics of the head domain. Structural and biochemical studies have revealed that the head can assume various positions, and its orientation depends on which ligands (e.g. 50S subunit, translation factors, tRNA, antibiotics) are bound to the 30S subunit (Carter *et al.*, 2001; Ogle *et al.*, 2002; Hickerson *et al.*, 2005; Schuwirth *et al.*, 2005; Berk *et al.*, 2006). Because P-site tRNA influences the orientation of the head domain (Berk *et al.*, 2006), we tested whether G1094A increases the intrinsic stability of the initiator tRNA in the 30S subunit. On the contrary, G1094A decreases the affinity of tRNA^{fMet} for the 30S P site, arguing against the idea that G1094A acts in a manner analogous to G1338A. Upon subunit joining, the head domain of the 30S subunit tilts towards the 50S subunit (Hickerson *et al.*, 2005), which raises the possibility that these neck mutations might promote premature 50S docking. While we have not yet investigated the effects of any of our mutations on subunit association (in the absence or presence of factors), such experiments may prove quite informative.

A number of P-site mutations (e.g. G926A, G926C, G926U, m^{2}G966C, G1338U) increase the stringency of start codon selection (Table 3). These mutations are likely to destabilize fMet-tRNA^{fMet} in the 30SIC, consistent with the fact that G1338U destabilizes tRNA^{fMet} in the 30S P site (Fig. 4B, Table 2). A notable exception is G1338A, which decreases the stringency of start codon selection and stabilizes tRNA^{fMet} in the 30S P site (Qin *et al.*, 2007). These data support to a model in which discrimination against non-canonical start codons relies, at least in part, on the mutual stabilization of mRNA and fMet-tRNA^{fMet} in the 30SIC that comes from codon–anti-codon interaction (La Teana *et al.*, 1993; Antoun *et al.*, 2006a). In G1338U subunits, fMet-tRNA^{fMet} is less stable in the 30SIC, and consequently the probability of productive initiation depends more heavily on cognate codon–anti-codon pairing. In G1338A subunits, the lifetime of 30SIC with fMet-tRNA^{fMet} paired to AUC is lengthened, allowing a higher level of spurious initiation. Indeed, our data are consistent with the proposal that IF3 can repress translation from non-canonical start codons by simply destabilizing fMet-tRNA^{fMet} in the 30SIC (Antoun *et al.*, 2006a).

A790 interacts with both IF3 and fMet-tRNA^{fMet} (Moazed *et al.*, 1995; Dallas and Noller, 2001; Selmer *et al.*, 2006), making it potentially more difficult to predict the effects of mutations at this position on 30SIC stability. However, of the three substitutions, A790G destabilizes IF3 the most and tRNA^{fMet} the least, and it is the only mutation that increases

spurious initiation. A790C has no apparent effect on IF3 binding, destabilizes tRNA[fMet] to the greatest degree, and decreases spurious initiation. A790U has an intermediate effect on IF3 binding, destabilizes tRNA[fMet] to the same degree as A790C, and confers the least effect on spurious initiation. All these results are in line with the model proposed by Ehrenberg in which IF3 and fMet-tRNA[fMet] bind the 30S subunit in an anti-cooperative manner, and their interplay during initiation determines the stringency of start codon selection (Antoun *et al.*, 2006a). Clearly, it will be of interest in the future to directly test the effects of these mutations on fMet-tRNA[fMet] stability in the 30SIC.

Although ribosomes with certain P-site mutations can discriminate AUG from AUC very well, these mutant ribosomes translate from AUG with reduced efficiency (Table 3). Thus, it seems that the affinity of fMet-tRNA[fMet] for the 30S P site is tuned to balance efficiency and accuracy during initiation.

Experimental procedures

Bacterial strains

Strain KLF2511 is CSH142 [F-*ara* Δ(*gpt-lac)5*] containing λ(*P*ant-SD*-AUC-*lacZ*) and was constructed as described previously (Qin *et al.*, 2007). Strain KLF2672 is KLF2511 containing Δ*(recA-srl)306 srl301::Tn10*. Other specialized ribosome strains were described previously (Qin *et al.*, 2007). All *E. coli* Δ7 prrn strains were made using strain SQZ10 as described previously (Qin *et al.*, 2007).

Genetic screen

Mutations were generated in the 16S rRNA gene by propagation of pKF207 in the mutator strain XL1-Red (Stratagene). Twelve transformants were independently grown to saturation iteratively for 7 days. Mutagenized plasmids were then purified from each culture and used to transform the reporter strain KLF2672. The transformed cells were plated on Luria–Bertani plates containing ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}), L-arabinose (5 mM) and Xgal (20 μg ml^{-1}), and the resulting colonies were screened for those that exhibited a bluer colour than their neighbours. Plasmids were purified from the positive isolates, and the 16S rRNA gene was sequenced.

β-Galactosidase assay

Specific β-galactosidase activity was measured as described (Qin *et al.*, 2007), except that cells were grown overnight on plates rather than in liquid culture.

Purification of ^{35}S-labelled IF1

IF1 was overexpressed in strain BL21/DE3 (pLysS) from a pET24b-based construct (kindly provided by A. Dallas and H. Noller). The overproducer cells were grown at 37°C in 250 ml M9 medium supplemented with casamino acids (0.05%), thiamine (10 μg ml^{-1}), proline, tryptophan, leucine (80 μg ml^{-1} each) and kanamycin (30 μg ml^{-1}). At an OD$_{600}$ of 0.5, IPTG (1 mM) and [^{35}S] L-methionine (2 μCi ml^{-1}, MP Biomedical) were added to the culture. The incubation was continued for 2 h, after which the cells were collected by centrifugation at 2800 *g* for 15 min, washed with buffer A [20 mM Tris-HCl (pH 7.6), 20 mM NaCl, 10 mM MgCl$_2$, 6 mM β-ME, 0.5 mM EDTA]. The cells were lysed by freezing and thawing them twice, and the lysate was clarified by centrifugation at 1600 *g* for 10 min. The supernatant was passed through a 0.22 μm filter and loaded onto a Resource S column (GE Corporation). The bound protein was washed with 10 column volumes of buffer A, a NaCl gradient was applied, and IF1 was collected as a single peak (at ~125 mM NaCl). The IF1 preparation was dialysed against buffer B [20 mM Tris-HCl (pH 7.5), 100 mM NH$_4$Cl,

10 mM Mg(OAc)$_2$, 1 mM DTT and 10% glycerol] and stored at −70°C. The purity of IF1 was > 95%, as judged by SDS-PAGE.

Binding assays

Control and mutant 30S subunits were purified from Δ7 prrn strains of *E. coli* as described previously (Qin *et al.*, 2007). To determine the affinity of IF1 for the 30S subunit in the absence of IF3, [^{35}S]-IF1 (250 nM) was incubated with activated 30S subunits (various concentration) at room temperature for 20 min in buffer C [10 mM Tris-HCl (pH 7.5), 100 mM NH$_4$Cl, 5 mM Mg(OAc)$_2$, 6 mM β-ME and 0.03% Nikkol]. Binding reactions were loaded onto Microcon YM100 filter units (Millipore) and spun at 1600 *g* for 5 min to separate the bound and free [^{35}S]-IF1. The flow through was collected and the signal was measured by liquid scintillation assay. A no-subunit control was included to estimate the total free [^{35}S]-IF1. The concentration of bound [^{35}S]-IF1, averaged from at least three independent experiments, was plotted versus the concentration of 30S subunits. To determine K_D, the data were fit to the equation: [30S·IF1] = {[30S]$_{input}$ + [IF1]$_{input}$ + K_D − ((([30S]$_{input}$ + [IF1]$_{input}$ + K_D)^2−4 × [IF1]$_{input}$ × [30S]$_{input}$)$^{1/2}$}/2, using the program KaleidaGraph. The standard error reported in Table 2 derived from the curve-fitting. The same procedure was used to measure [^{35}S]-IF1 binding in the presence of IF3 (350 nM), except that the concentration of [^{35}S]-IF1 was 20 nM.

Assays used to measure binding of 3′-[^{32}P]-tRNA^{fMet} and IF3-AF to the 30S subunit were described previously (Qin *et al.*, 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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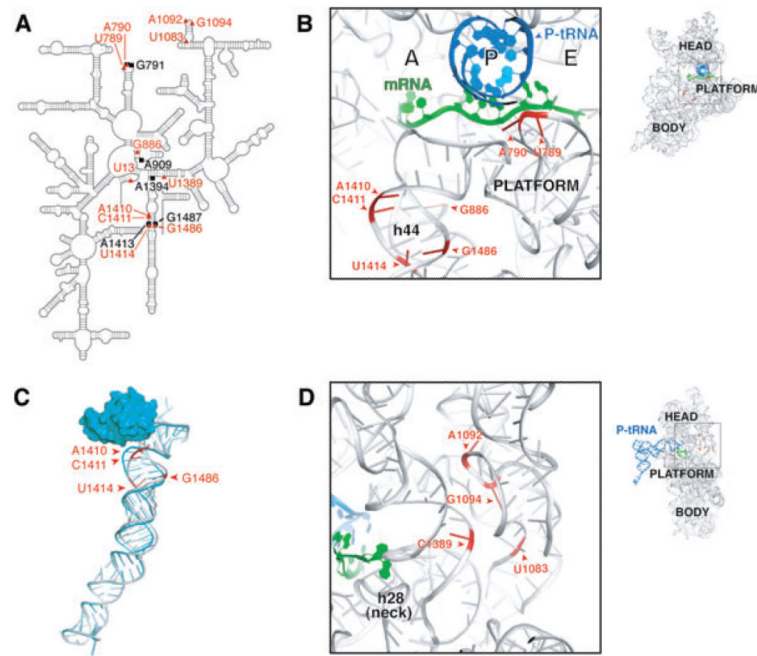
Fig. 1.
Nucleotides identified in this study as important for initiation fidelity cluster to h44, the 790 loop and the neck region. A. Mutation sites overlap with class III protection sites. Positions of mutations identified in this study (red triangles); class III protection sites attributed to ligand-induced conformation changes (black squares; Moazed and Noller, 1987). B. Positions of mutations in h44 and in the 790 loop (image based on Selmer *et al.*, 2006; PDB 2J02). C. Binding of IF1 to the 30S subunit distorts h44 where our mutations map. Blue ribbon, with IF1; grey ribbon, without IF1 (image based on Carter *et al.*, 2001; PDB 1HR0 and 1J5E). D. Positions of mutations in the neck region (image based on Selmer *et al.*, 2006; PDB 2J02).
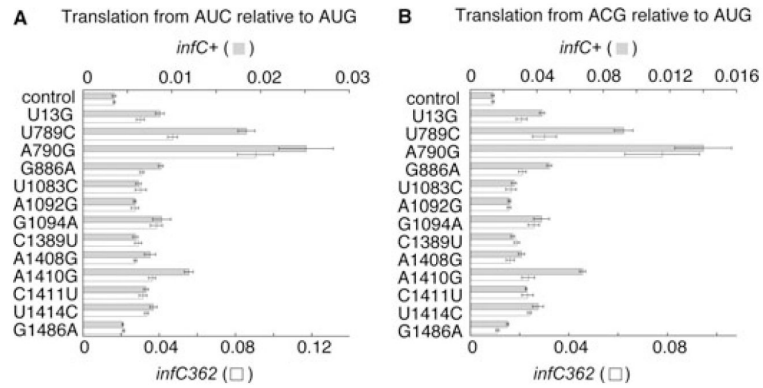
Fig. 2.
Effects of 16S rRNA mutations on the relative level of translation from AUC and ACG. Values correspond to the level of β-galactosidase expressed by specialized ribosomes from either SD*-AUC-*lacZ* (A) or SD*-ACG-*lacZ* (B) relative to SD*-AUG-*lacZ*. Grey bars, wild-type *infC*+ background; white bars, *infC362* background. Values represent the quotient of two means ± standard error.
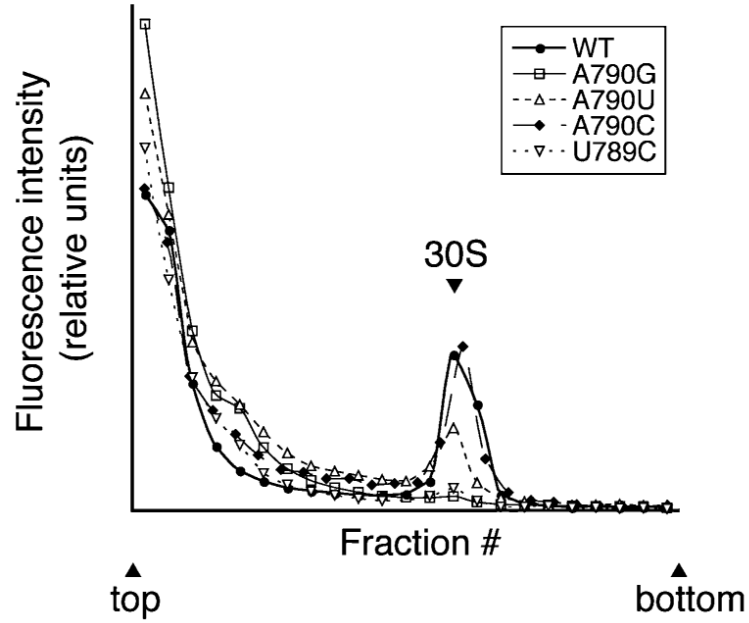
Fig. 3.
Effect of mutations in the 790 loop on the binding of IF3-AF to the 30S subunit. Fluorescently labelled IF3 (IF3-AF; 1 μM) was incubated with each preparation of 30S subunits (1 μM) at 37°C for 20 min and then subjected to sucrose gradient sedimentation analysis. Fractions (0.5 ml) were collected and the fluorescence intensity (excitation 495 nm; emission 520 nm) of each was quantified to determine the distribution of IF3-AF in the gradient. The position of 30S subunits in the gradient, as identified by A260, is indicated.
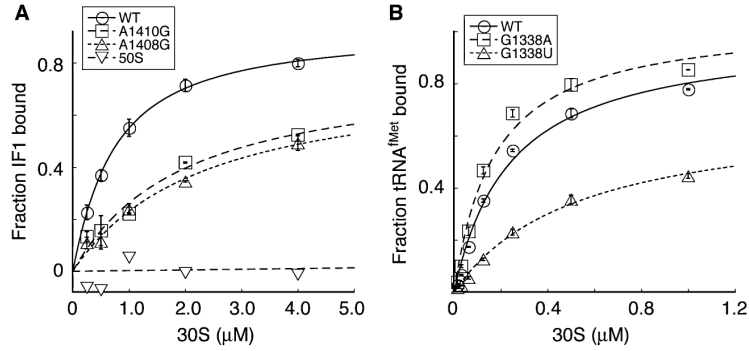
Fig. 4.
Effects of 16S rRNA mutations on the binding of IF1 or tRNA^{fMet} to the 30S subunit. A. Examples of experiments used to estimate the affinity of IF1 for mutant 30S subunits. [^{35}S]-IF1 (0.25 μM) was incubated with 30S subunits (various concentration) in buffer C [10 mM Tris-HCl (pH 7.5), 100 mM NH4Cl, 5 mM Mg(OAc)2, 6 mM β-ME and 0.03% Nikkol] at room temperature for 20 min. Microfiltration devices were used to separate free and bound [^{35}S]-IF1. The fraction of [^{35}S]-IF1 bound, averaged from at least three independent experiments, was plotted as a function of the concentration of 30S subunits, and the data were fit using KaleidaGraph. B. Examples of experiments to estimate the affinity of tRNA^{fMet} for mutant 30S subunits. 3′-[^{32}P] -tRNA^{fMet} (5 nM) was incubated with 30S subunits (various concentration) with a model mRNA containing an AUG start codon (m701; 3 μM) in buffer D [50 mM Tris-HCl (pH 7.5), 100 mM NH4Cl, 20 mM MgCl2, 6 mM β-ME] for 20 min at 37°C. The free and bound 3′-[^{32}P]-tRNA^{fMet} were separated by filtration through a bilayer of nitrocellulose and nylon membranes. The fraction of 3′-[^{32}P]-tRNA^{fMet} bound was plotted as a function of 30S subunit concentration, and the data were fit using KaleidaGraph. Equilibrium binding constants (*K*D) listed in Table 2 represent the mean ± SEM from three independent experiments.
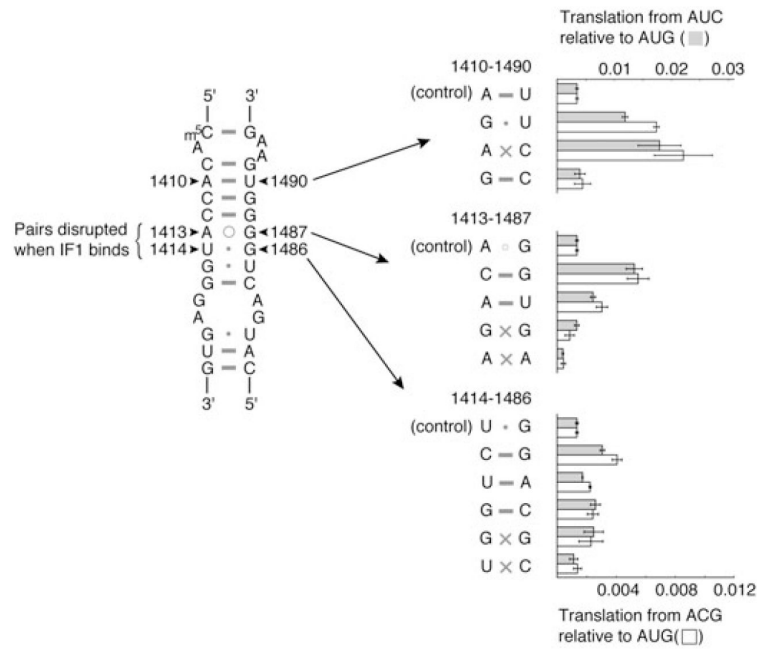
Fig. 5.
Effects of alternative base pairs at 1410–1490, 1413–1487 and 1414–1486 on the relative level of translation from AUC and ACG. Values correspond to the level of β-galactosidase expressed by specialized ribosomes from either SD*-AUC-*lacZ* (grey bars) or SD*-ACG-*lacZ* (white bars) relative to SD*-AUG-*lacZ*. Values represent the quotient of two means ± standard error.

Table 1

Mutations in 16S rRNA obtained in a screen for increased translation from AUC.

16S mutation(s)	Independent isolates[a]	Location
U13G	1	Central pseudoknot
A790G	1	790 loop
G886A	1	h27
U1083C	1	Neck region
A1092G	1	Neck region
G1094A	1	Neck region
A1179G	1	Junction of h38-h39-h40
C1389U	1	Neck helix (h28)
A1410G	4	h44
C1411U	1	h44
U1414C[b]	2	h44
U1414C[b], G616A	1	h44, h21
U789C, U1118C	1	790 loop, junction of h38-h39-h40
G1486A[b], C840U	1	h44, h26
ASD sequence (GGGGU⇒GGGAU)[c]	1	3′ end

[a] Isolates were only considered independent if they originated from separate preparations of mutagenized pKF207.

[b] U1414 and G1486 form a wobble base pair.

[c] This mutation changes the single U-G wobble base pair in the SD*-ASD* helix to U-A.

Table 2

Effects of 16S rRNA mutations on ligand binding.

16S allele[a]	IF1 binding[b]		tRNA binding[c]	IF3 binding[d]
	−IF3	+IF3		
Control	600 ± 80	30 ± 6	240 ± 10	++
U13G	700 ± 100	ND	ND	ND
U789C	ND	ND	340 ± 10	−
A790G	700 ± 100	ND	300 ± 10	−
A790U	ND	ND	510 ± 30	+
A790C	ND	ND	520 ± 10	++
G886A	1100 ± 200	60 ± 9	240 ± 10	++
G1094A	ND	ND	350 ± 30	ND
G1338A	700 ± 200	ND	170 ± 10	++
G1338U	ND	ND	540 ± 50	ND
A1408G	2100 ± 600	ND	ND	ND
A1410G	2200 ± 500	90 ± 20	210 ± 20	++
U1414C	600 ± 100	40 ± 20	240 ± 10	++

ND, not determined.

[a] Mutant 30S subunits were purified from a Δ7 prrn strain of *E. coli* (see text).

[b] Values correspond to the estimated K_D ± standard error (nM) derived from curve fitting. When present, the concentration of IF3 was 350 nM.

[c] Values correspond to K_D (nM; mean ± SEM) obtained from three independent experiments.

[d] Binding of IF3-AF to 30S was assessed by the level of co-migration after sucrose gradient sedimentation assay. ++, extent of co-migration indistinguishable from control; +, extent of co-migration substantially reduced; −, little or no co-migration.

Table 3

Effects of P-site mutations on translation from AUC and AUG.

16S allele[a]	β-Galactosidase activity[b]		Relative translation level[c]
	AUC	AUG	AUC/AUG
Control	8.0 ± 0.4	2300 ± 80	0.0035 ± 0.0002
G926A	< 0.12	490 ± 90	< 0.0002
G926U	< 0.12	470 ± 100	< 0.0003
G926C	< 0.12	330 ± 90	< 0.0004
G1338A	11 ± 0.3	2400 ± 60	0.0046 ± 0.0001
G1338U	0.5 ± 0.1	510 ± 30	0.001 ± 0.0002
G1338C	< 0.12	110 ± 20	< 0.001
C1400A	1.5 ± 0.5	450 ± 20	0.0032 ± 0.001
C1400U	1.9 ± 0.4	1200 ± 300	0.0016 ± 0.0004
C1400G	0.2 ± 0.05	190 ± 30	0.0012 ± 0.0003
A790G	13 ± 0.1	500 ± 60	0.025 ± 0.003
A790C	1.5 ± 0.2	1300 ± 300	0.0012 ± 0.0003
A790U	4.3 ± 0.6	1500 ± 200	0.0027 ± 0.0005
G966A	1.6 ± 0.3	800 ± 100	0.0019 ± 0.0005
G966U	0.5 ± 0.1	700 ± 80	0.0008 ± 0.0002
G966C	0.2 ± 0.01	500 ± 50	0.0004 ± 0.00005

[a]Each 16S allele contains the specialized SD* sequence to allow specific translation of the *lacZ* reporter gene.

[b]Specialized 16S rRNA (without or with mutations as indicated) was expressed in indicator strains KLF2526 and KLF2528 (harbouring SD*-AUC-*lacZ* or SD*-AUG-*lacZ* respectively) and specific β-galactosidase activity (based on cleavage of chlorophenol red-β-D-galactopyranoside) was measured. Values represent the mean ± SEM (arbitrary units) of at least three independent experiments.

[c]Values represent the quotient of two means ± standard error.