Ribosomal selection of mRNAs with degenerate initiation triplets

He Chengguang1,2,†, Paola Sabatini2,†, Letizia Brandi2, Anna M. Giuliodori2,*, Cynthia L. Pon2 and Claudio O. Gualerzi2,*

1College of Life Sciences, Engineering Research Centre of the Chinese Ministry of Education for Bioreactor and Pharmaceutical Development, Jilin Agricultural University, Changchun 130118, Jilin, China and 2Laboratory of Genetics, University of Camerino 62032 Camerino, Italy

Received March 18, 2017; Revised April 20, 2017; Editorial Decision May 03, 2017; Accepted May 12, 2017

ABSTRACT

To assess the influence of degenerate initiation triplets on mRNA recruitment by ribosomes, five mRNAs identical but for their start codon (AUG, GUG, UUG, AUU and AUU) were offered to a limiting amount of ribosomes, alone or in competition with an identical AUGmRNA bearing a mutation conferring different electrophoretic mobility to the product. Translational efficiency and competitiveness of test mRNAs toward this AUGmRNA were determined quantifying the relative amounts of the electrophoretically separated wt and mutated products synthesized in vitro and found to be influenced to different extents by the nature of their initiation triplet and by parameters such as temperature and nutrient availability in the medium. The behaviors of AUAmRNA, UUGmRNA and AUGmRNA were the same between 20 and 40°C whereas the GUG and AUUmRNAs were less active and competed poorly with the AUGmRNA, especially at low temperature. Nutrient limitation and preferential inhibition by ppGpp severely affected activity and competitiveness of all mRNAs bearing non-AUG starts, the UUGmRNA being the least affected. Overall, our data indicate that beyond these effects exclusively due to the degenerate start codons within an optimized translational initiation region, an important role is played by the context in which the rare start codons are present.

INTRODUCTION

It has been known since the late 60’s that translation initiation begins with the 30S ribosomal subunit which forms a complex with mRNA and initiator tRNA (e.g. ref. 1,2). The possibility of initiating protein synthesis with undisso-
present within the translational initiation region (TIR) of the mRNA represents one of the discriminants targeted by IF3; both in vitro (13,14,16) and in vivo (17,18) analyses have shown that only three start codons (AUG, GUG and UUG) are accepted by IF3 as ‘canonical’ whereas all the others, regarded as ‘non-canonical’, are rejected by the factor, albeit to different levels during 30SIC formation. Thus, the mRNAs using non-canonical start codons are subject to translation repression by this factor and occasionally expressed at low levels. A typical example is represented by the infC gene which uses either AUU or UAC in all bacterial species and is auto-regulated by its own gene product IF3 (19–22).

Genomic analysis of over 600 bacterial species revealed that the canonical triplets AUG (80.1%), GUG (11.6%) and UUG (7.8%) are the most frequent start codons (23) whereas non-canonical degenerate initiation triplets are extremely rare. Nevertheless, other triplets like AUA, AUC and AUU are also found; although the latter is present in only two Escherichia coli genes (infC and pcnB) (24,25), AUU and AUC are found quite frequently in other species such as Mycoplasma gallisepticum (23), the causative agent of avian chronic respiratory disease. Non-canonical triplets such as CUG, AUU, AUC and AUU are found in low percentage (between 0.004 and 0.024%) among all the start codons annotated from 79 bacterial genome and plasmid sequences (26). However, these figures might well represent an underestimation of the occurrence of these initiation triplets because initiation codon identification in bacteria is far from being precise, mainly in light of the generally very low-level of expression of genes containing these uncommon start codons. It is noteworthy in this connection that when translation initiation from all 64 triplet codons was systematically quantified in E. coli, at least 46 codons were found to direct protein synthesis at a level ranging from 10 to 100% relative to AUG for the canonical codons (GUG and UUG), from 1 to 2% for the near-cognates codons (AUA, AUC, AUU and CUG) and between 0.1 and 1% for the others (26).

However, origin and possible functional significance of initiation codon degeneracy remain open questions. Obviously, in some cases randomly occurring mutations of an existing AUG triplet could produce alternative triplets which may cause little or no phenotype or could even have an adaptive value. The conserved presence of non-AUG triplets at the beginning of some genes both isolated or within operons (see ‘Discussion’ section) as well as several other findings or clues suggest that non-AUG start codons may play important regulatory roles and might be at the root of intricate regulatory mechanisms in both bacteria (19–22,27–29) and eukarya (e.g. see 30–32). Furthermore, P-site decoding of the initiation triplet by the initiator tRNA determines start site and reading frame selection and contributes to overall translational efficiency (8,33,34).

In light of the above considerations in this study we sought to analyse the influence that degenerate initiation triplets may have on the mRNA selection by ribosomes and on the overall translational efficiency.

**MATERIALS AND METHODS**

**Buffers**

Buffer A: 20 mM Tris–HCl (pH 7.7); 60 mM NH4Cl; 10 mM MgAcetate; 1 mM dithiothreitol (DTT). Buffer B (gel buffer): 57 mM Bis–Tris/Acetic acid (pH 5.0); Buffer C (upper buffer): 10 mM Bis–Tris/Acetic acid (pH 4.0); Buffer D (lower buffer): 180 mM K acetate/acetic acid (pH 5).

**Genetic constructs**

A 250 bp DNA fragment encoding a synthetic infA* gene (initiation factor IF1) was excised from pXR101 (35) by EcoR1 and HindIII digestion, purified by 1.2% agarose gel electrophoresis, electroelution followed by ethanol precipitation (36) and cloned into the pSelect*-1 (Promega) phagemid to yield pSelectAUGinfAWT. This plasmid was then subjected to site-directed mutagenesis using appropriate oligonucleotides whereby the AUG initiation triplet was changed to GUG, UUG, AUU and AUU to yield pSelectGUGinfAWT, pSelectUUGinfAWT, pSelectAUUinfAWT and pSelectAUAinfAWT, respectively. Another manipulation yielded pSelectAUGinfAD34H in which a single base substitution was introduced into the coding sequence of pSelectAUGinfAWT resulting in the substitution of a His residue for the Asp residue at position 34 of IF1. This substitution produced a mutated IF1 (i.e. IF1 H34D) which is still active in promoting 30S IC formation and mRNA translation, although the mutation yields a factor with a somewhat reduced affinity for the 30S subunit so that a somewhat higher amount of the mutant factor is necessary to attain the same level of translation obtained with the wt protein (37). All the above-mentioned pSelect constructs were then subjected to EcoR1–HindIII digestion and the resulting DNA fragments cloned into pTZ18 (Pharmacia) downstream of the phage T7 RNA polymerase promoter to yield pTZ18::AUGinfAWT, pTZ18::AUGinfAD34H, pTZ18::GUGinfAWT, pTZ18::UUGinfAWT, pTZ18::UUUinfAWT and pTZ18::AUAinfAWT.

**Preparation of mRNAs**

The six different mRNAs encoded by the aforementioned pTZ18 constructs were prepared by in vitro transcription with phage T7 RNA polymerase and subsequently purified as described (38).

**Preparation of cell-free (S30) extracts**

*Escherichia coli* MRE600 cells were grown at 37°C in either Terrific Broth (TB) or in M9 broth until they reached the cell densities indicated in the figures. The cells were harvested by centrifugation, washed three times with Buffer A containing 10% Glycerol and disrupted by grinding with precooled alumina (Sigma) (1.5 x the cell weight) in a prechilled mortar. During grinding RNase-free DNase (2.5 mg/g cells) was added to the cell slurry which was resuspended in an equal volume of Buffer A containing 10% glycerol, 0.5 g/l benzotri, 0.2 mM Benzamidine and 0.2 mM PMSF (added just before use). After gentle stirring, the extract was centrifuged.
for 60 min at 4°C at 12K rpm (SA600 Sorvall rotor) to remove alumina and cell debris to obtain a clear ‘S30 extract’ which was used without dialysis to prevent the loss of potentially important low molecular weight molecules. To ensure that all reaction tubes contained the same amount of ribosomes, before use aliquots of the extracts were subjected to sucrose density gradient centrifugation to determine the amount of ribosomes contained in each S30 preparation.

mRNA translational tests

Depending upon the experimental need, translation tests were performed according to four different experimental protocols as specified in the appropriate figure legends. In some experiments (non-competed mRNA), only one type of mRNA was offered whereas in other cases (competed mRNA) two different mRNAs were given in competition with each other. In some experiments (reconstituted system) mRNA translation was carried out in reaction tubes containing purified ribosomes, post-ribosomal supernatant and purified initiation factors. In other cases (non-reconstituted or crude system) unfractionated extracts (S30 extracts) obtained from cells grown for different times in different media were used.

For translation of non-competed mRNA in the reconstituted system high-salt washed 70S ribosomes, initiation factors and post ribosomal extracts of E. coli MRE600 cells were prepared as described (38,39) and programmed with individual mRNAs beginning with the various start codons as indicated in the appropriate figures. Each reaction tube contained in 50 µl of buffer A: 5 mM DTT; 2 mM adenosine 5’-triphosphate (ATP); 0.4 mM Guanosine 5’-triphosphate GT; 10 mM phosphoenolpyruvate; 1.25 µg pyruvate kinase; 0.12 mM folic acid; 50 mM adenosine 5’-triphosphate (ATP); 0.4 mM Guanosine 5’-triphosphate GT; 10 mM phosphoenolpyruvate; 1.25 µg pyruvate kinase; 0.12 mM folic acid; 50 µM of total tRNA; 0.2 mM of each non-radioactive amino acid but methionine that was present as a mixture of [35S] methionine (0.4 µM, 1 Ci/Mole) and non-radioactive methionine (39.6 µM); 16 pmols 70S ribosomes; 8 pmoles each of IF1, IF2 and IF3; post-ribosomal supernatant (60 µg protein) and 40 pmols mRNA.

For translation of competed mRNA in the reconstituted system the reaction mixtures were prepared as described above but programmed with different combinations of mRNAs. All reaction mixtures contained decreasing amounts (from 40 to 0 pmols) of AUGinf/AD34H mRNA and increasing amounts (from 0 to 40 pmols) of one of the other mRNAs (i.e. AUGinf/AWT; GUAGinf/AWT; UUGinf/AWT; AUUinf/AWT or AUUinf/AWT). In some experiments ppGpp, synthesized and purified as described (40), was added to the reaction mixtures at the concentrations indicated in the appropriate figures.

Translation of both non-competed and competed mRNAs in non-reconstituted (crude) systems was carried out as described above but for the fact that the incubation mixture consisted of an S30 extract supplemented with 2 mM ATP; 0.4 mM GTP; 10 mM phosphoenolpyruvate; 1.25 µg pyruvate kinase; 0.12 mM folic acid; 50 µg of total tRNA; 0.2 mM of each non-radioactive amino acid but methionine that was present as a mixture of [35S] methionine (0.4 µM, 1 Ci/Mole) and non-radioactive methionine (39.6 µM). Translation was programmed with either a single mRNA or with different combinations of mRNAs as described above. All reaction tubes were incubated for 30 min at 35°C or at the temperatures indicated in the appropriate figures. Before electrophoretic analysis of the products, the samples were incubated with RNase A and 0.2 mM DTT.

Electrophoretic method

The translation products were separated on 15% polyacrylamide in Buffer B containing 8M corresponding to the first dimension of the electrophoretic method developed to separate ribosomal proteins (41), using buffers C and D as upper and lower buffers, respectively; the radioactive products were detected and quantified with a Molecular Imager GS-250 (Biorad). An example of this separation can be seen in Supplementary Figure S1.

RESULTS

Proof of principle—competition between two mRNAs both having AUG as start codon

In E. coli, translation initiation factor IF1 (71 amino acids) contains only two His residues at positions 29 and 34 (42). A single base transversion (i.e. CAT to GAT) in the triplet corresponding to His34 causes the substitution of this residue with Asp, generating a mutant molecule which maintains at least part of the activity in translation of the wt factor (37) and can be separated from wt IF1 by electrophoresis as a result of the large pK difference between the side chains of these two amino acids (Supplementary Figure S1). Thus, two mRNAs encoding IF1 and having an AUG start codon and identical sequences but for the aforementioned base transversion were tested in competition with one another for a limiting amount of ribosomes as a function of temperature. For these experiments different proportions of the two mRNAs were added to a translational system and after incubation at 20, 30 or 40°C the amount of the products (i.e. wt IF1 or IF1 H34D) synthesized was determined from the amount of radioactivity incorporated into the two electrophoretically separated proteins. Thus, a series of incubation mixtures were prepared containing a fixed total amount of mRNA with the amount of wt infA mRNA increasing from 0 to 40 pmols and the amount of infA H34D correspondingly decreasing from 40 to 0 pmols. The level of the products synthesized in each sample is indicated by the two curves present in the panels of Figure 1. As seen from this figure, the amount of wt IF1 produced increases with increasing amounts of wt infA mRNA while there is a corresponding decrease of the amount of IF1 H34D with decreasing amounts of infA H34D mRNA. The same amounts of wt IF1 and IF1 H34D are made at the point where the two curves intersect which corresponds to the condition in which equal amounts of the two mRNAs (i.e. 20 pmols each) are present in the incubation mixture (Figure 1). Temperature variations between 20 and 40°C did not affect the competition between the two mRNAs, but the total level of the products formed increases with increasing temperatures from 20°C (Figure 1A) to 30°C (Figure 1B) and to 40°C (Figure 1C).

These results indicate that the single base substitution introduced in the infA mRNA does not affect the transla-
on the ribosomal selection of mRNAs having different initiation triplets.

Ribosomal recruitment of mRNAs with different initiation codons as a function of temperature

Translational activity of non-competed mRNAs. When the translational systems were programmed with individual mRNAs, the levels of product synthesized depended upon both nature of the initiation triplet and temperature (Figure 2). As expected, translational efficiency increases with increasing temperature with all mRNAs. However, the mRNAs beginning with AUG, AUU and UUG are less sensitive to temperature variations and express comparable levels of product which are substantially higher than those obtained with the GUG and AUU mRNAs. Indeed, whereas the amount of IF1 synthesized at 20°C with the AUG, UUG and AUU mRNAs is >70% of that expressed at 40°C, the GUG mRNA is translated with ca. 60% of the efficiency displayed at 40°C. The mRNA beginning with AUU is the most sensitive to temperature variations, its activity at 20°C being <50% of that observed at 40°C (Figure 2A).

Competition between mRNAs with non-AUG and AUG initiation codons. The trend observed in the experiments carried out with non-competed mRNAs is fully confirmed by the results of the experiments in which the individual mRNAs bearing a non-AUG start codon were competed in translation with the mRNA beginning with AUG.

Between 20 and 40°C the reaction mixtures produced different amounts of wtIF1 and IF1 H34D as a function of the amount of wt infA AUG mRNA and infA H34D mRNA offered to the translational systems. The amounts of the two forms of IF1 produced are the same when same amounts (20 pmole each) of the two mRNAs were present in the reaction mixture (Figure 3). This finding indicates that the mRNAs beginning with AUU and AUG are recruited by the ribosomes and compete with each other with the same efficiency. Unlike the case of the AUAmRNA, an excess of the mRNAs beginning with GUG (Figure 4) and AUU (Figure 5) is necessary to synthesize the same amounts of wt IF1 and IF1 H34D, indicating that these mRNAs compete less efficiently with the AUGmRNA. Quite the opposite occurs with the UUGmRNA which wins the competition with the AUGmRNA, at least between 20 and 30°C (Figure 6).

The efficiency of competition between the non-AUG mRNAs and the AUGmRNA as a function of temperature was quantified from the data of the experiments shown above (Figures 3–6) and expressed as percentage of the competition between the two mRNAs starting with AUG (i.e. wt infA and infA H34D mRNA) (Figure 2B). As seen from this figure, the efficiency of GUG and AUU mRNAs to compete with AUGmRNA is only ~25–30% at 20°C and does not exceed 80% (GUG mRNA) and 60% (AUU mRNA) at 40°C. On the other hand, both AUU and UUG mRNAs are good competitors of the AUGmRNA; actually, these mRNAs are slightly favored over the AUGmRNA at lower temperatures (20–30°C) in the case of the UUGmRNA and at higher temperatures (30–40°C) in the case of the AUAmRNA (Figure 2B).
Figure 2. Influence of mRNA initiation triplet on (A) level of IF1 synthesis by non-competed mRNAs and (B) on mRNAs competition for ribosomal recruitment as a function of temperature. (A) The reactions were carried out at 20, 25, 30, 35 and 40 °C, as indicated in the abscissa under conditions identical to those of Figure 1, but for the fact that each tube contained 40 pmol of only one type of mRNA: (green) wt AUG infA mRNA; (red) AUG infA H34D mRNA; (blue) AUA infA mRNA; (black) UUG infA mRNA; (magenta) GUG infA mRNA and (orange) AUU infA mRNA. The amounts (pmoles) of IF1 synthesized in each reaction mixture were quantified by the hot-TCA method (33) and are reported in the ordinate. The results shown in the graph represent the average of four independent experimental points. Error bars are drawn to represent the upper and lower values of each point obtained in the four replicates. (B) Competition for recruitment by a limiting amount or ribosomes between pairs of mRNA consisting of AUG infA H34D mRNA and of another wt infA mRNA having a non-AUG start codon as a function of temperature. The plotted data are taken from the results of Figures 3–6. The competition between AUG infA H34D mRNA and the mRNAs beginning with ▼ AUA; ▲ UUG; ● GUG; and ♦ AUU is expressed in the ordinate as percentage of the competition (taken as 100%) between the two mRNAs beginning with AUG.

Ribosomal recruitment of mRNAs with different initiation codons as a function of the metabolic state of the cells

Whether the availability of nutrients in the growth medium might influence competition between the mRNAs beginning with non-AUG start codons and the AUGmRNA was assessed using extracts of cells grown in rich (i.e. Terrific Broth = TB) and poor (i.e. M9) media. The amounts of wt IF1 and IF1 H34D synthesized by these extracts, normalized for their ribosome content and programmed with different amounts of mRNAs, were determined as indicated above.

Translational activity of non-competed mRNAs. Under conditions of non-competition in the extracts of cells grown in TB the mRNAs beginning with AUA, UUG and GUG codons synthesized wt IF1 with an efficiency comparable to that displayed by the two AUGmRNAs (i.e. wt infA and infA H34D mRNAs) whereas the activity of the AUUmRNA was ~25% lower (Figure 7A). Furthermore, all mRNAs, including the AUUmRNA, had essentially the same activity in extracts of cells harvested during early and mid-exponential growth but their activity was reduced by 25–30% in extracts of cells harvested in stationary phase (Figure 7A).

In sharp contrast to the results obtained with extracts of TB cells, the translational activity of the various mRNAs displayed large differences when tested in the extracts of cells grown in M9; indeed, with the exception of the UUGmRNA, whose activity was only marginally affected (i.e. ~20%), all the other non-AUG mRNAs translated substantially less IF1 than the two AUGmRNAs; as seen from the results of Figure 7B, the amount of IF1 synthesized with AUUmRNA, GUGmRNA and AUAmRNA was reduced respectively by ~50, 40 and 30% compared to that synthesized with the AUGmRNAs. Furthermore, unlike the case of extracts of cells growing in TB the amount of IF1 synthesized is progressively reduced with the aging of the cultures (Figure 7B) indicating that in poor medium the growth phase does influence the translational activity of the mRNAs. The observed activity reductions are of small entity regardless of the mRNA initiation codon, with the notable exception of the AUUmRNA whose translational activity is drastically reduced in extracts of stationary cells (~50% less than in early exponential growth) (Figure 7B). As a result, the amount of product made with AUUmRNA drops from ~42% of that displayed by the AUGmRNA in the extracts of cells in early exponential growth to ~28% in stationary phase extracts (Figure 7B). Thus, aside from the case of the AUUmRNA, the above results indicate that overall the stage of growth has only a modest influence on the translational activity of the mRNAs, especially in the extracts of cells growing in rich medium. Although these results might appear surprising, it should be recalled that the amount of cell extract used was normalized so that each incuba-
Figure 3. Competition between mRNAs beginning with AUG and AUA for translation by a limiting amount of ribosomes as a function of temperature. The translation experiments were carried out as described in the legend of Figure 1 at the temperatures indicated in each panel (from A to E: 20, 25, 30, 35 and 40 °C) with reaction mixtures containing a total amount of 40 pmoles mRNA constituted by a mixture of (▼) wt infA AUG mRNA (0–40 pmoles) and (□) infA H₃₄D AUG mRNA from (40–0 pmoles) as indicated in the scheme presented at the bottom of the figure.

Figure 4. Competition for translation by a limiting amount of ribosomes between mRNAs beginning with AUG and GUG as a function of temperature. The translation experiments were carried out as described in the legend of Figure 1 at the temperatures indicated in each panel (from A to E: 20, 25, 30, 35 and 40 °C) with reaction mixtures containing a total amount of 40 pmoles mRNA constituted by a mixture of (●) wt infA GUG mRNA (0–40 pmoles) and (□) infA H₃₄D AUG mRNA from (40–0 pmoles) as indicated in the scheme presented at the bottom of the figure.
Figure 5. Competition for translation by a limiting amount of ribosomes between mRNAs beginning with AUG and AUU as a function of temperature. The translation experiments were carried out as described in the legend of Figure 1 at the temperatures indicated in each panel (from A to E: 20, 25, 30, 35 and 40°C) with reaction mixtures containing a total amount of 40 pmoles mRNA constituted by a mixture of (♦) wt infA AUU mRNA (0–40 pmoles) and (□) infA H34D AUG mRNA from (40–0 pmoles) as indicated in the scheme presented at the bottom of the figure.

Figure 6. Competition for translation by a limiting amount of ribosomes between mRNAs beginning with AUG and UUG as a function of temperature. The translation experiments were carried out as described in the legend of Figure 1 at the temperatures indicated in each panel (from A to E: 20, 25, 30, 35 and 40°C) with reaction mixtures containing a total amount of 40 pmoles mRNA constituted by a mixture of (▲) wt infA UUG mRNA (0–40 pmoles) and (□) infA H34D AUG mRNA from (40–0 pmoles) as indicated in the scheme presented at the bottom of the figure.
Ribosomes and post-ribosomal fraction are responsible for low translational activity of non-AUG mRNAs in extracts of cells growing in minimal medium

To determine the reason for the reduced translational activity of non-AUG mRNAs in extracts of M9 cells, mRNA competition experiments were carried out in 'hybrid' translational systems reconstituted by mixing components obtained from cells grown in TB and M9 media. A reduced capacity of the non-AUG mRNAs to compete with AUGmRNA was observed when ribosomes from rich media cells were combined with the post-ribosomal supernatant of M9 cells (D panels of Supplementary Figures S12–14). The effect was particularly strong in the case of GUGmRNA and AUAmRNA (Supplementary Figure S13). Competition between AUGmRNA and non-AUG mRNAs tested in competition with AUGmRNA (Supplementary Figures S2 through S11) reflects to a large extent their behavior under conditions of non-competition. In fact, in extracts of cells grown in TB the mRNAs beginning with AUG (Supplementary Figure S3) UUG (Supplementary Figure S4) and GUG (Supplementary Figure S5) competed for translation with the AUG infA H34D mRNA with an efficiency close to 100% of that displayed by AUG wt infA mRNA (Supplementary Figure S2); under these conditions the only exception was the AUAmRNA, whose competition capacity was reduced by ∼30% (Supplementary Figure S6).

In the extracts of cells grown in minimal medium the competition between the two AUG mRNAs was not affected (Supplementary Figure S7) whereas the competitiveness displayed by the other mRNAs was strongly reduced, namely by 30, 45 and 60% for GUG, AUAmRNA, respectively (Supplementary Figures S8, 10 and 11). In contrast, the capacity of the UUGmRNA to compete with the AUGmRNA was hardly affected (∼12% reduction) (Supplementary Figure S9); this property makes the mRNAs beginning with UUG like the cyaAmRNA suitable for translation under conditions of nutritional stress (see ‘Discussion’ section).

A quantification of the results of the mRNA competition experiments shown in Supplementary Data (Supplementary Figures S2 through 11) is presented in the schemes of Figure 8A and B. In these schemes the length of each bar illustrates the different amounts of AUG infA H34D mRNA and of a non-AUG mRNA which must be present in the reaction mixtures to obtain the synthesis of equal amounts of the two products (IF1 H34D and wt IF1, respectively); this allows an immediate visualization of the competition efficiency of the various non-AUG mRNAs for translation in extracts of cells grown in TB (Figure 8A) and M9 (Figure 8B) broths and harvested at different stages. Bars of identical or different lengths indicate that the two mRNAs are recruited by the ribosomes with either identical or different efficiency. It is interesting to note in these figures that the stage of growth (i.e. A600 = 0.8, 3.2 and 14 in TB and A600 = 0.4, 1.0 and 3.0 in M9 medium) at which the cells used to prepare the extracts were harvested, unlike the type of growth medium (i.e. rich/poor), has hardly any influence on the competitiveness of the various non-AUG mRNAs (Figure 8A and B).
Figure 8. Influence of mRNA initiation triplet on mRNAs competition for ribosomal recruitment as a function of growth stage in rich and poor medium. Cell extracts, prepared from cultures growing in (A) rich (i.e. TB) medium and (B) minimal (i.e. M9) medium and harvested at the optical density (A600) indicated on the left side of each panel were programmed with a mixture of two mRNAs (40 pmoles total) constituted in all cases by infA H34D mRNA (white bar) and by a second wt infA mRNA with initiation triplets AUG, AUA, UUG, GUG or AUU, as indicated inside each black bar. The complete results of these experiments are presented in Supplementary Figures S2 through S11 whereas the scheme presented here shows the amounts of the two mRNAs which are present in the reaction mixtures which yield equal amounts of the two products (i.e. IF1 H34D and wt IF1).

UUUGmRNA was only marginally affected (Supplementary Figure S14).

Unlike the above case, in the reaction mixtures prepared with the alternative combination consisting of ribosomes from M9 cells and post-ribosomal supernatant of TB cells the competition capacity of non-AUG mRNAs toward the AUGmRNA was hardly reduced (C panels in Supplementary Figures S12–14). The results of these experiments are summarized in the scheme (Figure 9) in which each bar illustrates graphically the different amounts of AUG infA H34D mRNA and of mRNAs beginning with AUA (top), GUG (middle) and UUG (bottom) which must be present in the reaction mixtures to produce equal amounts of IF1 H34D and wt IF1, respectively.

Taken together, these results indicate that the origin of the ribosomes plays only a minor role in the discrimination between different mRNAs, whereas the presence or absence of some key component(s) in the post-ribosomal supernatant of cells grown in minimal medium is responsible for a reduced ribosomal selection of mRNAs beginning with non-AUG triplets.

Effect of increasing concentrations of IF3 on mRNA selection

It is known that IF3, with the support of IF1, is responsible for discriminating against at least some non-AUG initiation codons (13,14) and that the level of initiation factors with respect to ribosomes is normally kept constant in the cells (43). However, there are special conditions, such as cold stress, which cause the level of the factors to increase while synthesis and assembly of the ribosomes slow down. This determines a substantial increase of the IFs/ribosome ratio (44).

In light of these facts, we sought to determine whether different levels of the IFs might influence the ribosomal selection of non-AUG mRNAs. For these experiments, increasing amounts of IF3 or of all three factors (IFs) were added to translation reactions prepared with extracts of cells grown in either TB (Supplementary Figure S15A) or M9 (Supplementary Figure S15B) broth. These reaction mixtures were supplemented with a single, non-competed mRNAs such as wt infA mRNA or one of the non-AUG mRNAs. Preliminary analyses carried out by semi-quantitative western blotting (44) allowed us to estimate that the extracts of both TB and M9 cells contained an amount of IF3 corresponding to the physiological ~0.15 stoichiometric ratio with respect to the ribosomes present in the same extracts. Under these basal conditions the relative levels of synthesis obtained in the extracts of the TB cells were 1.0 for the AUGmRNA, 0.95 for AUAmRNA, 0.91 for GUGmRNA, 1.05 for UUGmRNA and 0.74 for AUUmRNA (Supplementary Figure S15A). Additions of increasing amounts of IF3, up to a 10-fold excess over the ribosomes, did not significantly alter these results, but for the case of the AU-
UmRNA whose activity was almost halved (Supplementary Figure S15A). Unlike the previous case, in the extracts of M9 cells the activity of all non-AUG mRNAs was substantially reduced compared to that of the AUGmRNA; the relative levels of synthesis were 0.82 for UUGmRNA, 0.71 for GUGmRNA, 0.56 for AUUmRNA and 0.48 for AUAmRNA. Also in this case the addition of increasing amounts of IF3 had only a marginal effect on the activity of the UUGmRNA but caused a clear albeit small reduction (i.e. 20–25% at an IF3/ribosome ratio = 10) of the activity of the AUG and GUG mRNAs. Under the same conditions the activity of the AUUmRNA was strongly decreased (i.e. >60%) (Supplementary Figure S15B).

Effect of additions of increasing amounts of IFs and fMet-tRNA/IF2

Experiments similar to those described above were carried out after addition to the extracts of M9 cells of increasing amounts of either the three initiation factors (Supplementary Figure S15C) or of just IF2 and fMet-tRNA (Supplementary Figure S15D). As seen in the figures, following these additions the performance of all non-AUG mRNAs was only moderately improved without ever reaching the levels of activity observed in the TB extracts. Results qualitatively similar to those shown in Supplementary Figure S15A–D were obtained in mRNAs competition experiments (not shown).

In summary, the above data indicate that the efficiency by which mRNAs beginning with AUG, GUG and UUG are translated is not altered by increasing amounts of IF3 in the reaction mixtures prepared with extracts of TB cells and is only marginally reduced in the M9 cell extracts. The activity of the AUUmRNA, on the other hand, is more affected by increases of the IF3 levels, in full agreement with the known discrimination displayed by this factor against mRNAs beginning with this non-canonical triplet (15–17). In the M9 extracts increasing amounts of the three IFs or of only IF2 and fMet-tRNA improves only marginally the translational activity of all the non-AUG mRNAs whose level remains much lower than that obtained in the TB extracts.

Thus, the reduced activity of the non-AUG mRNAs in the extracts of M9 cells is not due to either the presence of an increased level of IF3/ribosomes ratio or to a deficiency of the other IFs or of the initiator tRNA. An increased level of the ‘alarmone’ guanosine tetraphosphate (ppGpp) in the extracts of cells subjected to nutritional limitations was therefore regarded as a possible reason for the reduced selection of non-AUG mRNAs.

Effect of ppGpp on the ribosomal recruitment of non-AUG mRNAs

To test the above hypothesis, two types of experiments were carried out. In the first experiment, increasing amounts of ppGpp were added to reaction mixtures prepared with extracts of TB cells and programmed with individual mRNAs beginning with AUG, UUG, GUG and AUU. The translational levels obtained with each mRNA and at each alarmone concentration were compared to the translational levels obtained under the same experimental conditions with the AUGmRNA which served as control. Different levels of translation were observed for each non-AUG mRNA as a function of increasing ppGpp concentrations; the strongest inhibitions (relative to the AUGmRNA) were observed with the AUU and AUAmRNAs whereas translation of the GUG and UUG mRNAs were much less affected (Figure 10A).

The second type of experiment was designed to test the capacity of the AUU, AUG and GUG mRNAs to compete with AUGmRNA for a limiting amount of ribosomes in reaction mixtures prepared with extracts of TB cells containing different concentrations of ppGpp. The results of these competitions are presented in the scheme shown in Figure 10B in which the bars represent the amounts of the individual wt infA mRNAs beginning with the aforementioned

Figure 9. Influence of source of ribosomes and post-ribosomal supernatant on the competition between mRNAs with different initiation codons. Ribosomes (R) and post-ribosomal supernatant (S) were prepared from cells grown in TB up to A600 = 14 [R(TB) and S(TB)] or in M9 up to A600 = 3 [R(M9) and S(M9)] and mixed in the combinations indicated on the left side of the figure to prepare reaction mixtures which were programmed with a mixture of two mRNAs (40 pmoles total) constituted in all cases by infA H34D AUG mRNA and by a second wt infA mRNA with initiation triplets AUA, GUG or UUG. The primary data obtained in these translation experiments are presented in Supplementary Figures S12 through S14 where as in the scheme presented here the black and white bars represent the amounts of the two mRNAs which are present in the reaction mixtures which yield equal amounts of the two products (i.e. IF1 H34D and wt IF1).
start codons which must be present in the reaction mixtures together with infA H34D AUG mRNA to allow the synthesis of the same amounts of wt IF1 and IF1 H34D. As seen from the figure, the amount of the non-AUG mRNAs necessary to obtain this result increases with increasing ppGpp concentrations present in the reaction mixtures. The effect is moderate in the case of the GUGmRNA, but particularly strong with the AUAmRNA and, even more so, with the AUUUmRNA. Overall, these results are in full agreement with the differential level of inhibition caused by ppGpp on the translation of the same mRNAs as seen above (Figure 10A).

**General considerations on the occurrence of non-AUG initiation triplets in bacterial mRNAs**

In all kingdoms of life the degeneracy of the genetic code is not restricted to the triplets decoded during elongation but concerns also the translation initiation triplet. In addition to the most common and ambiguous (insofar as it encodes also internal Met residues) AUG triplet, other rarer triplets can be decoded in the P-site of the small ribosomal subunit to initiate protein synthesis. In bacteria, in addition to AUG, GUG and UUG, which are recognized as canonical by IF3 (18), non-canonical start codons such as AUU, AUC, AUA are occasionally found. In eukaryotic and mitochondrial mRNAs, the presence of a large and increasing number of non-AUG start codons have been found not only in the mRNA 5’ termini, where they are selected by the conventional ribosome-scanning mechanism, but also within IRES structures (30–32); ribosomal footprinting studies with yeast and mammalian cells have suggested that non-canonical translation initiation may be much more frequent than previously thought (45–48). Several genes using non-AUG initiation triplets encode growth factors, cation transport channels and proteins involved in transcription and translation regulation, a circumstance which suggests that these triplets are involved in cellular regulations. There are indeed several documented cases of regulatory mechanisms based on the use of non-AUG initiation triplets (30–32,49). When present within a single mRNA these start codons allow the expression of several proteins from a single gene.
generating protein isoforms with distinct functions thereby contributing to protein diversity. For instance, in several mammalian cells CUG is used as an inefficient start codon to allow translation from a downstream initiation point generating two isoforms of thioredoxin/glutathione reductase (30).

As to the reasons for initiation codon degeneracy in bacteria, genomic base composition can play a role in determining the abundance of the individual initiation triplets. ATG represents the almost exclusive initiation triplet in bacterial species whose DNA contains an extremely low percentage of GC whereas GUG is relatively more abundant than ATG and TTG in bacteria whose DNA has a high GC content.

However, the overall reasons for start codon degeneracy has so far remained to a large extent unclear. In some cases, the rare triplets may result from random neutral or near-neutral base mutations of AUG codons whereas in other cases they may have been selected to set at low level the expression of some genes (27–29,50). Indeed, an AUG to AUU mutation caused by a base transition in the in bacteriophage T4 rifB gene resulted in a substantial reduction (−85%) of translation at 25°C and an even more severe temperature sensitive phenotype (51). Likewise, in vitro and in vivo synthesis of phage T7 0.3 protein was severely reduced following an AUG to ACG transition, although recruitment of the mutated mRNA by the ribosomes was not affected (52). Mutagenesis of the UUG initiation triplet of the cyaA gene has shown that initiation codons UUG, GUG and AUG directed the synthesis of adenylate cyclase at relative levels of 1:2:6, respectively (28). In another case, lacZ translation from GUG and UUG (53) or AUC (54) codons instead of AUG was reduced two- to three-times; likewise, a truncated form of bacteriophage HB-3 murine hydrolase was synthesized in E. coli with an efficiency of 7.5 and 5% when instead of AUG the initiation codon was AUA and AUU or AUC (54).

Nevertheless, although the above explanations for the occurrence of the rare codons are likely correct in some cases, they appear overall too simplistic to satisfy entirely our curiosity. Indeed, genes like tufA and hupB are expressed at very high levels despite the fact that their mRNAs start with a non-AUG triplet; the products of these genes (i.e. elongation factor EF-TuA and nucleoid-associated protein HU-β) are among the most abundant proteins in the cell. Each E. coli cell was estimated to contain 74 981 molecules of EF-TuA and 87 672 molecules of EF-TuB translated from approximately the same number (53 molecules/cell) of mRNAs starting with a GUG and an AUG initiation codon, respectively (55). Likewise, HU-β is present with 5891 molecules whereas HU-α is present with 5025 molecules/cell despite the fact that the cellular level of hupA mRNA is more than twice that of hupB mRNA (12 versus 27 molecules/cell (55). Finally, also infC, which begins with AUU, is expressed to fairly high levels since there are 5488 IF3 molecules in E. coli cells (55).

Taken together, these data challenge the premise that the purpose of the rare codons is to limit the level of translation suggesting instead that at least in some cases the presence of a non-AUG start codon serves a more complex role in translational regulation of gene expression. For instance, it is well established that the AUU or AUC initiation triplets found in infC represent the target of translational autoregulation by initiation factor IF3 (19–22) and several clues suggest that, like in eukarya, at least some of the bacterial non-AUG start codons might be instrumental in supporting some kind of complex regulatory mechanism beyond the simple limitation of gene expression. This premise is strengthened by evidence that at least in some cases the occurrence of non-AUG codons is non-random but is phylogenetically conserved at the beginning of specific genes or at the beginning of at least one of the genes belonging to a given operon (Table 1). For instance, the cyaA gene, whose expression is subjected to complex regulations (56), begins with the degenerate initiation triplet UUG in a large number of enterobacteria and other gamma-proteobacteria. Likewise, in the majority of gammaproteobacteria either tufA, or fus (encoding elongation factor EF-G) or both belonging to the str operon begin with GUG (Table 1). Furthermore, in most of the gammaproteobacteria which like E. coli possess two genes encoding nucleoid associated protein HU, one gene (hupA) begins with AUG whereas the other (hupB) begins with GUG (Table 1) and it is noteworthy that at least in E. coli the two genes are subjected to different and somewhat complementary types of regulation, whereby the levels of the two proteins change in response to environmental changes. HUβ is expressed mainly during mid-late exponential phase, during stationary phase (57,58) and following a cold stress (59).

**DISCUSSION**

In light of the above considerations and of the key role in mRNA initiation site and reading frame selection played by start codon decoding by fMet-tRNA (33,34,60), the aim of this study was to determine the efficiency by which mRNAs beginning with non-AUG start codons are recruited by bacterial ribosomes.

To tackle this problem, we have analysed the efficiency by which mRNAs bearing degenerate initiation codons compete for a limiting amount of ribosomes with an mRNA starting with the common AUG triplet. It should be stressed, in this connection, that we have focused on the mRNA properties attributable exclusively to their start codon present within an optimal TIR region. Indeed, the mRNAs used in this study are derived from infA+, the synthetic gene which was constructed to optimize translational expression of initiation factor IF1 (35) whose TIR was designed based on the consensus sequence derived from the comparison of E. coli translation initiation sites available at that time (61). The sequence of this TIR with the indication in bold letters of the SD sequence and the original initiation triplet is 5'-GAAUUCCGGCCCUCUG UUAACAAUUAGGAGGUAUAUCAUUGGCAGAA GAAGAUAUUUGGA...-3'. Thus, the conclusion of this study should not be extended to all genes beginning with a non-AUG triplet, but instead should be taken as a general reflection of the influence which the rare start codons may have on the ribosomal selection of an mRNA within the aforementioned optimal context. It is quite possible that mRNAs bearing the same rare start triplets within a different context may display different translational efficiencies. There is indeed evidence that this
### Table 1. Examples of non-AUG initiation triplets in gammaproteobacterial genes

| Organisma | Geneb | cyaA | tuf or fus (Str operon) | tuf or fus (not Str operon) | hup | spoT |
|-----------|-------|------|------------------------|----------------------------|-----|------|
| *Escherichia coli* K-12 MG1655 | ttg | tuf: gtg | tuf: atg | hupA: atg | hupB: gtg | ttg |
| *Salmonella enterica* subsp. *enterica* serovar *Typhi* CT18 | ttg | tuf: gtg | tuf: atg | hupA: atg | hupB: gtg | ttg |
| *Pasteobacterium* *aeroasepticum* *SCRI1043* | ttg | tuf: gtg | tuf: atg | hupA: atg | hupB: gtg | ttg |
| *Yersinia pestis* CO92 (biovar *Orientalis*) | ttg | tuf: gtg | tuf: atg | hupA: atg | hupB: gtg | ttg |
| *Buchnera aphidicola* APS (Acythosiphon pismum) | - | tuf: atg | tuf: atg | hupA: atg | - | - |
| *Pasteurella multocida* subsp. *multocida* Pm70 | ttg | tuf: gtg | tuf: atg | hupA: atg | - | ttg |
| *Haemophilus ducreyi* | atg | tuf: gtg | tuf: atg | hupA: atg | hupB: atg | ttg |
| *Vibrio cholerae* O1 biovar *El* *Tor* N16961 | ttg | tuf: gtg | tuf: atg | hupA: atg | hupB: gtg | ttg |
| *Aeromonas hydrophila* subsp. *hydrophilia* ATCC 7966 | ttg | tuf: gtg | tuf: atg | hupA: atg | hupB: atg | ttg |
| *Psychromonas ingrahamii* | atg | tuf: gtg | tuf: atg | hupA: atg | hupB: atg | atg |
| *Shewanella oneidensis* | atg | tuf: gtg | tuf: atg | hupA: atg | hupB: atg | ttg |
| *Pseudoalteromonas atlantica* | - | tuf: atg | tuf: atg | hupA: atg | - | ttg |
| *Idiomarina loihiensis* L2TR | - | tuf: atg | tuf: atg | hupA: atg | - | gtg |
| *Pseudomonas fluorescens* | atg | tuf: gtg | tuf: atg | hupA: atg | atg |
| *Pseudomonas aeruginosa* | atg | tuf: gtg | tuf: atg | hupA: atg | hupB: gtg | ttg |
| *Marinobacter hydrocarbonoclasticus* VT8 | ttg | tuf: gtg | - | hupA: atg | hupB: gtg | gtg |
| *Saccharophagus degradans* | atg | tuf: gtg | - | hupA: atg | - | ttg |
| *Acinetobacter baumannii* ATCC 17978 | atg | tuf: gtg | - | hupA: atg | - | atg |
| *Psychrobacter cryohalolentis* | - | tuf: atg | - | hupA: atg | - | atg |
| *Francisella tularensis* subsp. *tularensis* SCHU S4 | - | - | - | hupA: atg | - | ttg |
| *Legionella pneumophila* subsp. *pneumophila* Philadelphia 1 | - | tuf.: atg | tuf.: atg | - | hupA: atg | atg |
| *Methylococcus capsulatus* | - | tuf.: gtg | tuf.: atg | - | hupB: atg | atg |
| *Xylella fastidiosa* 9a5c | - | tuf.: atg | tuf.: atg | - | hupB: atg | ttg |

a. The listed organisms, sorted in increasing evolutionary distance from *E. coli*, are representatives of the main subgroups of the *Gammaproteobacteria* class (66)
b. The initiation codon of the orthologous genes was retrieved from the KEGG gene database. The absence of orthologs in the genome is indicated by a dash.
ally active and capable of competing, albeit to different ex-
pairings such as the G:U wobble pairing. Structural predictions place the bases of all initiation triplets cruitment of this mRNA by the 30S subunit. Likewise, the GUG mRNA, a circumstance which could favorthere-
AUU whereas this helix was predicted to be less stable in the initiation triplet itself. In particular, the SD sequence GGAGG was predicted to be within the same short dou-
ors of the tested mRNAs to anything but the nature of the initiation triplet itself. In particular, the SD sequence GGAGG was predicted to be within the same short dou-
the initiation triplet under most of the translational conditions tested here in contrast to the low level of translation of AUAmRNAs reported in the literature (54).

For similar reasons, the cold-sensitivity displayed by the GUgRNA and even more so by the AUuRNA lead us to conclude that neither GUG nor AUU start codons can be regarded as determinants for the preferential translation at low temperature in the cold-stress-inducible hupB and inf/C genes which begin with these triplets. Thus, while the extensive phylogenetic conservation of the AUU triplet at the beginning of inf/C mRNAs is justified by the mech-
nism of translation autoregulation based on the discrim-
ation of this non-canonical initiation codon by IF3, the efficient translation of inf/C mRNA at the cold-stress tem-
perature could simply result from the reduced efficiency by which IF3 discriminates against its AUU target at low temperature. Indeed, it has been reported that the fidelity func-
tion of this factor, which stems from the dissociation rate of the non-canonical 30S initiation complexes, is strongly temperature-sensitive (65).

It is more difficult to find a plausible explanation for the fairly conserved presence of a GUG initiation codon in gammaproteobacterial (66) hupB genes. Protein HU does not bind exclusively to the DNA, but binds with very high affinity also to different types of RNA (67,68), to partici-
pate in the structural organization of the nucleoid (68) and also to play a role in translation regulation (67). In partic-
ular, HU was shown to bind specifically to the TIR of rpoS mRNA and to stimulate its translation. HU seems to bind preferentially to nucleic acids endowed with particular structures such as kinked four way junctions in DNA (69) and three-way junctions in RNA (67). It is therefore possible that HUβ expression, in addition to being under well-
established transcriptional controls (57), might also be subjected to some kind of translational autoregulation and that the presence of the GUg initiation triplet might represent part of a structural element of the hupB mRNA which might be targeted by HU itself. The increased level of HUβ in cells entering stationary phase is compatible with the mod-
est inhibition produced by ppGpp on the translation of the GUgRNA, as observed in this study. However, the cold stress which stimulates HUβ production is associated with a ‘relaxed state’ of the cell and with a reduction of the ppGpp production (70).

In addition to temperature, also the nutritional state of the cell was found to influence the ribosomal recruitment of
non-AUG mRNAs. Indeed, we show that, with the exception of UUGmRNA, which is fairly active under all conditions, the other non-AUG mRNAs display translational activity comparable to that of the AUGmRNA and compete very efficiently with the latter only when tested in extracts of cells growing in rich medium; however, in translational systems based on extracts of cells growing in poor medium the translational efficiency and the competition capacity these non-AUG mRNAs is strongly reduced. This is one of the most interesting and yet intriguing findings of this study. After ruling out that some compositional and/or chemical modification of the ribosomes occurring in cells growing in poor medium might be at the root of the observed phenomenon, we concluded that the presence or absence of some compound in the post-ribosomal supernatant was responsible for the reduced activity of the non-AUG mRNAs. Limiting amounts of IFs and initiator tRNA or an excess of IF3 were shown to play only a very marginal role in determining the competitiveness of non-AUG mRNAs vis-à-vis the AUGmRNA. However, because we were able to reproduce the reduction of activity and competitiveness of these mRNAs in extracts of cells grown in TB by additions of increasing amounts of ppGpp, at concentrations comparable to those attained in cells undergoing nutrient limitations (71–73), we conclude that the concentration of this alarmone can modulate the efficiency by which mRNAs bearing non-AUG start codons are translated. This conclusion is full of agreement with the finding that mRNAs with non-AUG start codons are more IF2-dependent than AUG mRNAs (21) and that ppGpp can act as a specific inhibitor of the IF2 function (74).

Among the non-AUG start codons, UUG was the one whose activity proved to be less sensitive to the cold and to the nutritional state of the cell and to ppGpp inhibition. These findings are compatible with the phylogenetic conservation of this initiation triplet in the cyaA genes and spoT (encoding bifunctional (p)ppGpp synthetase) (Table 1), two gene products involved in the cellular response to nutritional stress (e.g. carbon deprivation, inhibition of fatty acid metabolism and membrane perturbation) by synthesizing cAMP and ppGpp, respectively.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**FUNDING**

Initial Research Foundation of Jilin Agricultural University [201616 to H.C.]; Education Department of Jilin Province [NO.JJKH20170317K to H.C.]; Ministero dell’Istruzione, Università e Ricerca (Italy), through the “Ruolo della struttura dell’RNA messaggero nel determinare efficienza e fedeltà dell’inizio della sintesi proteica in batteri ed archéi” [PRIN-2009 to C.O.G.]. Funding for open access charge: Initial Research Foundation of Jilin Agricultural University.

Conflict of interest statement. None declared.

REFERENCES

1. Guthrie, C. and Nomura, M. (1968) Initiation of protein synthesis: a critical test of theS subunit model. Nature, 219, 232–235.
2. Blumberg, B.M., Nakamoto, T. and Goldberg, I.S. (1975) Kinetic evidence for the obligatory formation of a 30S initiation complex in polyphenylalanine synthesis with N-acetylphenylalanyl-5RNA. Biochemistry, 14, 2889–2894.
3. Moll, I., Hirokawa, G., Kiel, M.C., Kaji, A. and Blasi, U. (2004) Translation initiation with 70S ribosomes: an alternative pathway for leaderless mRNAs. Nucleic Acids Res., 32, 3354–3363.
4. Orell, C., Carlson, E.D., Szal, T., Florin, T., Jewett, M.C. and Mankin, A.S. (2015) Protein synthesis by ribosomes with tethered subunits. Nature, 524, 119–124.
5. Gualerzi, C.O., Brandi, L., Caserta, E., Garofalo, C., Lamm, M., La Teana, A., Petrelli, D., Spurio, R., Tomasic, J. and Pon, C.L. (2001) Role of the initiation factors in the early events of mRNA translation in bacteria. Cold Spring Harb. Symp. Quant. Biol., 66, 363–376.
6. Gualerzi, C.O., Fabbretti, A., Brandi, L., Milon, P. and Pon, C.L. (2010) Role of the initiation factors in mRNA start site selection and fMet-tRNA recruitment by bacterial ribosomes. Isr. J. Chem., 50, 80–94.
7. Gualerzi, C.O. and Pon, C.L. (2015) Initiation of mRNA translation in bacteria—structural and dynamic aspects. Cell. Mol. Life Sci., 72, 4341–4367.
8. Gualerzi, C.O., Risuleo, G. and Pon, C.L. (1977) Initial rate kinetic analysis of the mechanism of initiation complex formation and the role of initiation factor IF3. Biochemistry 16, 1684–1689.
9. Milon, P., Carotti, M., Konevega, A.L., Wintermeyer, W., Rodnina, M.V. and Gualerzi, C.O. (2010) The ribosome bound initiation factor 2 recruits initiator tRNA to the 30S initiation complex. EMBO Rep., 11, 312–316.
10. Fabbretti, A., Scheidbauer, A., Brandi, L., Kaminichi, T., Giuliani, A.M., Galafaro, R., Ochoa-Lizaralde, B., Takemoto, C., Yokoyama, S., Connell, S.R. et al. (2016) Inhibition of translation initiation complex formation by GER81112 unravels a 16S RNA structural switch involved in P-site decoding. Proc. Natl. Acad. Sci. U.S.A., 16, E2286–E2295.
11. Milon, P., Konevega, A.L., Gualerzi, C.O. and Rodnina, M.V. (2008) Kinetic checkpoint at a late step in translation initiation. Mol. Cell, 30, 712–720.
12. Wintermeyer, W. and Gualerzi, C. (1983) Effect of E. coli initiation factors on the kinetics of NaPhe-tRNA\(^{Phe}\) binding to 30S ribosomal subunits. A fluorescence stopped-flow study. Biochemistry, 22, 690–694.
13. Gualerzi, C., Pon, C.L. and Kaji, A. (1977) Initiation factor dependent release of aminocyl-tRNA from complexes of 30S ribosomal subunits, synthetic polynucleotides and aminocyl-tRNA. Biochem. Biophys. Res. Comm., 45, 1312–1319.
14. Risuleo, G., Gualerzi, C. and Pon, C.L. (1976) Specificity and properties of the destabilization, induced by initiation factor IF3, of ternary complexes of 30S, aminocyl-tRNA and polynucleotides. Eur. J. Biochem., 67, 603–613.
15. Qin, D. and Fredrick, K. (2009) Control of translation initiation involves a factor-induced rearrangement of helix 44 of 16S ribosomal RNA. Mol. Microbiol., 71, 1239–1249.
16. Hartz, D., McPheeters, D.S. and Gold, L. (1989) Selection of the initiator tRNA by Escherichia coli initiation factors. Genes Dev., 3, 1899–1912.
17. Bacserd, C., Chiariutini, C., Enz, K., Graeffe, S., Milet, M., Mathy, N., Dondon, J. and Springer, M. (1996) The role of the AUA initiation codon in the negative feedback regulation of the gene for translation initiation factor IF3 in Escherichia coli. Mol. Microbiol., 21, 331–346.
18. Sussman, J.K., Simons, E.L. and Simons, R.W. (1996) Escherichia coli translation initiation factor 3 discriminates the initiation codon in vivo. Mol. Microbiol., 21, 247–260.
19. Brombach, M. and Pon, C.L. (1987) The unusual translation initiation codon AUU limits the expression of infC (initiation factor IF3) gene of Escherichia coli. Mol. Gen. Genet., 208, 94–100.
20. Butler, J.S., Springer, M. and Grunberg-Manago, M. (1987) AUU to AUG mutation in the initiator codon of translation initiation factor IF3 abolishes translational control of its own gene (infC) in vivo. Proc. Natl. Acad. Sci. U.S.A., 84, 4022–4025.

Downloaded from https://academic.oup.com/nar/article-abstract/45/12/7309/3855591 on 28 July 2018 by guest
23. Villegas,A. and Kropinski,A.M. (2008) An analysis of initiation codon utilization in the Domain Bacteria—concerns about the quality of bacterial genome annotation. Microbiology, 154, 2559–2561.

24. Blattert,F.R., Plunkett,G. III, Bloch,C.A., Perna,N.T., Burland,V., Riley,M., Collado-vides,J., Glaser,J.D., Rode,C.K., Mayhew,G.F. et al. (1997) The complete genome sequence of Escherichia coli K-12. Science, 277, 1453–1462.

25. Binns,N. and Masters,M. (2002) Expression of the Escherichia coli penB gene is translationally limited using an inefficient start codon: a second chromosomal example of translation initiated at AUU. Mol. Microbiol., 44, 1278–1298.

26. Hecht,A., Glasgow,J., Jaschke,P.R., Bawazer,L., Munson,M.S., Cochran,J., Endy,D. and Salit,M. (2017) Measurements of translation initiation from all 64 codons in E. coli. Nucleic Acids Res., 45, 3615–3626.

27. Roy,A., Haziza,C. and Danchin,A. (1983) Regulation of adenylate cyclase synthesis in Escherichia coli: nucleotide sequence of the control region. EMBO J., 2, 791–797.

28. Reddy,P., Peterkofsky,A. and McKinney,K. (1985) Translational efficiency of the Escherichia coli adenylate cyclase gene: mutating the UUG initiation codon to GUG or AUG results in increased gene expression. Proc. Natl. Acad. Sci. U.S.A., 82, 5656–5660.

29. Danchin,A. and Lenzen,G. (1988) Structure and evolution of bacterial adenylate cyclase: comparison between Escherichia coli and Erwinia chrysanthemi. Second Messengers Phosphoproteins, 12, 7–28.

30. Gerashenko,M.V., Su,D. and Gladyshev,V.N. (2010) CUG start codon generates thioredoxin expression. FEBS Lett., 584, 147–153.

31. Touriol,C., Bornes,S., Bonnal,S., Audigier,S., Prats,H., Prats,A.C., Bockmann,J., Kinder,S. and Kreienkamp,H.J. (2014) A non-canonical initiation site is required for efficient translation of the dendratically localized Shank1 mRNA. PLoS One, 9, e88518.

32. Trotot,P., Sismeiro,O., Vivarès,C., Glaser,P., Bresson-Roy,A. and Danchin,A. (1990) Comparative analysis of the cya locus in enterobacteria and related gram-negative facultative anaerobes. Biochimie, 78, 277–287.

33. Belin,D., Hedgepeth,J., Selzer,G.B. and Epstein,R.H. (1979) Temperature-sensitive mutation in the initiation codon of the rIB gene of bacteriophage T4. Proc. Natl. Acad. Sci. U.S.A., 76, 700–704.

34. Dunn,J.J., Buzhash-Pollert,E. and Studier,F.W. (1978) Mutations of bacteriophage T7 that affect initiation of synthesis of the gene 0.3 protein. Proc. Natl. Acad. Sci. U.S.A., 75, 2741–2745.

35. Khudyakov,Yu.E., Nepluyevas,S., Kalinina,T.I. and Smirnov,V.D. (1988) Effect of structure of the initiator codon on translation in E. coli. FEBS Lett., 232, 369–371.

36. Romero,A. and Garcia,P. (1991) Initiation of translation at AUG and AUU codons in Escherichia coli. FEMS Microbiol. Lett., 86, 325–330.

37. Lu,P., Vogel,C., Wang,R., Yao,X. and Marcotte,E.M. (2007) Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. Nat. Biotechnol., 25, 117–124.

38. Thorsen,L.K., Fandi,J.P. and Artz,S.W. (1990) Analysis of sequence elements important for expression and regulation of the adenylate cyclase gene (Cya) of Salmonella Typhimurium. Genetics, 125, 709–717.

39. Clare,J. and Rouviere-Yaniv,J. (1996) Regulation of HUα and HUβ by CRP and FIS in Escherichia coli. J. Mol. Biol., 263, 126–139.

40. Clare,J. and Rouviere-Yaniv,J. (1997) Variation in HU composition during growth of Escherichia coli: the heterodimer is required for long term survival. J. Mol. Biol., 273, 93–104.

41. Giorgetti,M., Giuliadori,A.M., Gualerzi,C.O. and Pon,C.L. (2002) Selective expression of the β3-subunit of nucleoid-associated protein HU during cold shock in Escherichia coli. Mol. Microbiol., 44, 205–216.

42. Taniguchi,T. and Weissmann,C. (1979) Escherichia coli ribosomes bind to non-initiator sites of Q beta RNA in the absence of formylmethionyl-tRNA. J. Mol. Biol., 128, 481–500.

43. Fritsch,C., Herrmann,A., Nothnagel,M., Szafranski,K., Huse,K., Schumann,F., Schreiber,S., Platter,M., Krawczak,M., Hampe,J. et al. (2012) Genome-wide search for novel human uORFs and N-terminal protein extensions using ribosomal footprinting. Genome Res., 22, 2208–2218.

44. Lee,S., Liu,B., Lee,S., Huang,S.-X., Shen,B. and Qian,S.-B. (2012) Global mapping of translation initiation sites in mammalian cells at single-nucleotide resolution. Proc. Natl. Acad. Sci. U.S.A., 109, E2424–E2432.

45. Danchin,A. and Lenzen,G. (1988) Structure and evolution of bacterial adenylate cyclase: comparison between Escherichia coli and Erwinia chrysanthemi. Second Messengers Phosphoproteins, 12, 7–28.

46. Gehardenko,M.V., Su,D. and Gladyshev,V.N. (2010) CUG start codon generates thioredoxin expression. FEBS Lett., 584, 147–153.

47. Jay,G. and Kaempfer,L. (1974) Sequence of events in initiation of translation: a role for initiator transfer RNA in the recognition of messenger RNA. Proc. Natl. Acad. Sci. U.S.A., 71, 3199–3203.

48. Calogero,R.A., Canonaco,M.A., Gualerzi,C.O. and Pon,C.L. (1988) Selection of the mRNA translational initiation region by Escherichia coli ribosomes. Proc. Natl. Acad. Sci. U.S.A., 85, 6427–6431.

49. Calogero,R., Pon,C.L. and Gualerzi,C.O. (1987) Chemical synthesis and in vivo expression of Escherichia coli translation initiation factor IF1. Mol. Gen. Genet., 208, 63–69.

50. Sambrook,J., Maniatis,T. and Fretsch,E.F. (1989) Molecular Cloning. CSHL Press, NY.

51. Gualerzi,C.O., Spurio,R., La Teana,A., Calogero,R., Celano,B. and Pon,C.L. (1989) Site-directed mutagenesis of Escherichia coli translation initiation factor IF1. Identification of the amino acid involved in its ribosomal binding and recycling. Protein Eng., 3, 133–138.

52. Brandi,L., Fabbretti,A., Milon,P., Carotti,M., Pon,C.L. and Gualerzi,C.O. (2007) Methods for identifying compounds that specifically target translation. Meth. Enzymol., 431, 229–267.

53. Pawlik,R.T., Littlechild,J., Pon,C.L. and Gualerzi,C. (1981) Purification and properties of E. coli initiation factors. Biochem. Internat., 2, 421–428.

54. Milón,P., Tischken,E., Tomsic,J., Caserta,E., Folkers,G., La Teana,A., Rodinia,M.V., Pon,C.L., Boeens,R. and Gualerzi,C.O. (2006) The nucleotide binding site of bacterial translation initiation factor IF2 as a metabolic sensor. Proc. Natl. Acad. Sci. U.S.A., 103, 13962–13967.

55. Mets,L.J. and Bogorad,L. (1974) Two dimensional polyacrylamide electrophoresis. An improved method for ribosomal proteins. Anal. Biochemistry, 57, 200–210.
63. Taniguchi, T. and Weissmann, C. (1978) Site-directed mutations in the initiator region of the bacteriophage Qβ coat cistron and their effect on ribosome binding. J. Mol. Biol. 118, 533–565.
64. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res., 31, 3406–3415
65. Gualerzi, C., Risuleo, G. and Pon, C.L. (1979) Mechanism of the spontaneous and IF3 induced dissociation of 30S-aminoacyl-tRNA mRNA ternary complexes. J. Biol. Chem., 254, 44–49.
66. Williams, K.P., Gillespie, J.J., Sobral, B.W., Nordberg, E.K., Snyder, E.E., Shalomi, J.M. and Dickerman, A.W. (2010) Phylogeny of gammaproteobacteria. J. Bacteriol., 192, 2305–2314.
67. Balandina, A., Claret, L., Hengge-Aronis, R. and Rouviere-Yaniv, J. (2001) The Escherichia coli histone-like protein HU regulates rpoS translation. Mol. Microbiol., 39, 1069–1079.
68. Macvanin, M., Edgar, R., Cui, F., Trostel, A., Zhurkin, V. and Adhya, S. (2012) Noncoding RNAs binding to the nucleoid protein HU in Escherichia coli. J. Bacteriol., 194, 6046–6055.
69. Pontiggia, A., Negri, A., Beltrame, M. and Bianchi, M.E. (1993) Protein HU binds specifically to kinked DNA. Mol. Microbiol., 7, 343–350.
70. Jones, P.G., Cashel, M., Glaser, G. and Neidhardt, F.C. (1992) Function of a relaxed-like state following temperature downshifts in Escherichia coli. J. Bacteriol., 174, 3903–3914.
71. Buckstein, M.H., He, J. and Rubin, H. (2007) Characterization of nucleotide pools as a function of physiological state in Escherichia coli. J. Bacteriol., 190, 718–726.
72. Potrykus, K. and Cashel, M. (2008) (p)ppGpp: still magical? Annu. Rev. Microbiol., 62, 35–51.
73. Srivatsan, A. and Wang, J.D. (2008) Control of bacterial transcription, translation and replication by (p)ppGpp. Curr. Opin. Microbiol., 11, 100–105.
74. Milón, P., Tischenko, E., Tomšiè, J., Caserta, E., Folkers, G., La Teana, A., Rodnina, M.V., Pon, C.L., Boelens, R. and Gualerzi, C.O. (2006) The nucleotide binding site of bacterial translation initiation factor IF2 as a metabolic sensor. Proc. Nat. Acad. Sci. U.S.A., 103, 13962–13967.