

# Ribosomal selection of mRNAs with degenerate initiation triplets

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## 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 AUA) 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-

ciated 70S ribosomes is restricted to the cases in which the template is a polynucleotide such as polyuridylic acid or a leaderless mRNA (3) or when the subunits are artificially prevented from dissociating as a result of crosslinking (4).

Formation of a 30S translation initiation complex (30SIC) represents a key step within the whole process of protein synthesis. The small ribosomal subunit bearing one copy each of the three initiation factors IF1, IF2 and IF3 binds in stochastic order an mRNA and an initiator tRNA (fMet-tRNA) molecule (for reviews see 5–7). Together, these ribosomal ligands are assembled in a 30S pre-initiation complex (30S preIC) in which codon-anticodon interaction has not yet occurred or is incomplete (8–10). A first-order transition which likely involves a structural modification of the highly conserved GGAA tetraloop of h45 (G1516–A1519) and the conversion of the h44/h45/h24a interface from an 'open' to a 'closed' conformation accompanies codon-anticodon interaction in the P-site and marks the transformation of the 30S preIC into a 30SIC (8,10). A 30SIC containing canonical ligands and endowed with a canonical structure is amenable for docking by the 50S subunit and enters the subsequent stages of the translation initiation pathway whose epilogue consists in the formation of a 70S initiation complex (70SIC) and of the first peptide bond yielding the initiation dipeptide (11).

The 30S preIC → 30SIC transition is under the kinetic control of the initiation factors and represents the first checkpoint of translational fidelity (8,11,12). In fact, both *on*- and *off*-rates of the transition are increased by IF3 but the *off*-rates are affected to different extents, depending upon the nature of the 30S ligands and upon the structural properties of the resulting complex. In this way non-canonical complexes are dissociated and discriminated against. Indeed, at least four different types of 30S complexes regarded as non-canonical are rejected by IF3 (5,13,14) with the assistance of IF1 (11,15) and have little or no chance to enter the later stages of translation initiation. In particular, the nature of the initiation triplet

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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 AUC 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 AUA 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 NH<sub>4</sub>Cl; 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 EcoRI and HindIII digestion, purified by 1.2% agarose gel electrophoresis, electroelution followed by ethanol precipitation (36) and cloned into the pSelect<sup>TM</sup>-1 (Promega) phagemid to yield pSelectAUG*infAWT*. This plasmid was then subjected to site-directed mutagenesis using appropriate oligonucleotides whereby the AUG initiation triplet was changed to GUG, UUG, AUU and AUA to yield pSelectGUG*infAWT*, pSelectUUG*infAWT*, pSelectAUU*infAWT* and pSelectAUA*infAWT*, respectively. Another manipulation yielded pSelectAUG*infAD34H* in which a single base substitution was introduced into the coding sequence of pSelectAUG*infAWT* 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 H<sub>34</sub>D) 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 EcoRI-HindIII digestion and the resulting DNA fragments cloned into pTZ18 (Pharmacia) downstream of the phage T7 RNA polymerase promoter to yield pTZ18::AUG*infAWT*, pTZ18::AUG*infAD34H*, pTZ18::GUG*infAWT*, pTZ18::UUG*infAWT*, pTZ18::AUU*infAWT* and pTZ18::AUA*infAWT*.

### 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× 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 bentonite, 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  $\mu$ l of buffer A: 5 mM DTT; 2 mM adenosine 5'-triphosphate (ATP); 0.4 mM Guanosine 5'-triphosphate; 10 mM phosphoenolpyruvate; 1.25  $\mu$ g pyruvate kinase; 0.12 mM folic acid; 50  $\mu$ g of total tRNA; 0.2 mM of each non-radioactive amino acid but methionine that was present as a mixture of [<sup>35</sup>S] methionine (0.4  $\mu$ M, 1 Ci/Mole) and non-radioactive methionine (39.6  $\mu$ M); 16 pmoles 70S ribosomes; 8 pmoles each of IF1, IF2 and IF3; post-ribosomal supernatant (60  $\mu$ g protein) and 40 pmoles 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 pmoles) of AUG*infAD34H* mRNA and increasing amounts (from 0 to 40 pmoles) of one of the other mRNAs (i.e. AUG*infAWT*; GUG*infAWT*; UUG*infAWT*; AUU*infAWT* or AUA*infAWT*). 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  $\mu$ g pyruvate kinase; 0.12 mM folic acid; 50  $\mu$ g of total tRNA; 0.2 mM of each non-radioactive amino acid but methionine that was present as a mixture of [<sup>35</sup>S] methionine (0.4  $\mu$ M, 1 Ci/Mole) and non-radioactive methionine (39.6  $\mu$ M). 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 RNaseA and 0.2 mM DTT.

### Electrophoretic method

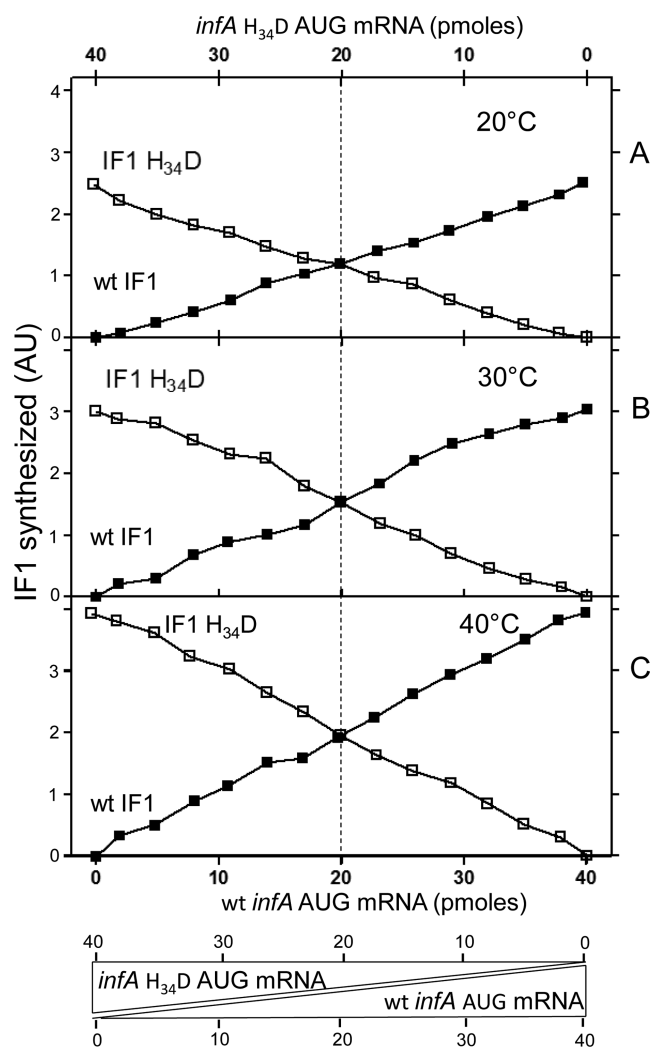
The translation products were separated on 15% polyacrylamide in Buffer B containing 8M corresponding to the first dimension of the electrophoretic method developed to separate ribosomal proteins (41), using buffers C and D as upper and lower buffers, respectively; the radioactive products were detected 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 His<sub>34</sub> causes the substitution of this residue with Asp to generate 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 H<sub>34</sub>D) 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 pmoles and the amount of *infA* H<sub>34</sub>D correspondingly decreasing from 40 to 0 pmoles. 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 H<sub>34</sub>D with decreasing amounts of *infA* H<sub>34</sub>D mRNA. The same amounts of wt IF1 and IF1 H<sub>34</sub>D 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 pmoles 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-



**Figure 1.** Competition between wt and mutant *infA* mRNAs at three different temperatures. Translation was carried at 20°C (panel A), 30°C (panel B) and 40°C (panel C). As indicated in the scheme presented at the bottom of the figure, the reaction mixtures contained from 0 to 40 pmoles of (■) wt *infA* mRNA and from 40 to 0 pmoles of (□) *infA* H<sub>34</sub>D mRNA so that each tube contained a total amount of 40 pmoles mRNA, both beginning with AUG. The reaction mixtures also contained a limiting amount (16 pmoles) of 70S ribosomes. The other reaction conditions are described in ‘Materials and Methods’ section. At the end of the incubation, [<sup>35</sup>S] Methionine-labeled wt IF1 and mutant IF1 H<sub>34</sub>D were separated by electrophoresis as described in ‘Materials and Methods’ section. The values reported in the ordinate, expressed as AU = Arbitrary Units, represent the amount of each protein synthesized as quantified with a Personal Molecular Imager FX and Quantity One software (Bio-Rad).

tional activity of the mutated mRNA which is recruited by the ribosomes with the same efficiency as the wt mRNA. Therefore, we concluded that it was possible to use the mutated mRNA beginning with AUG to determine the efficiency by which mRNAs starting with non-AUG initiation triplets compete for recruitment by a limiting amount of ribosomes. Furthermore, the differences in the translational activity observed at different temperatures indicate that our experimental design is suitable to investigate the effects which different environmental conditions may have

on the ribosomal selection of mRNAs having different initiation triplets.

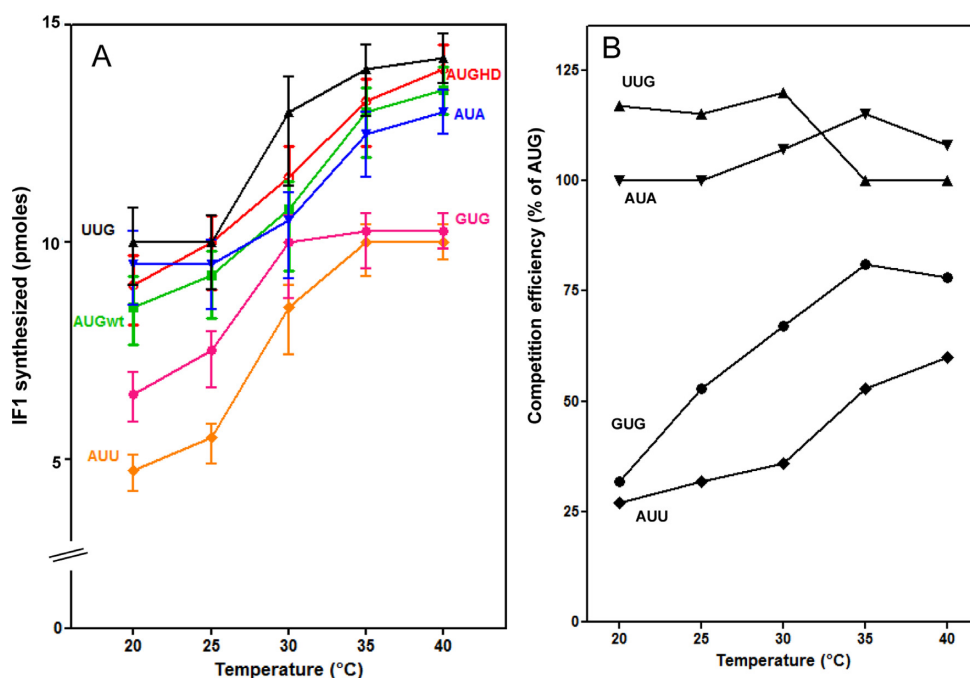
### Ribosomal recruitment of mRNAs with different initiation codons as a function of temperature

*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, AUA 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 AUA 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 H<sub>34</sub>D as a function of the amount of wt *infA* AUGmRNA and *infA* H<sub>34</sub>D AUGmRNA offered to the translational systems. The amounts of the two forms of IF1 produced are the same when same amounts (20 pmoles each) of the two mRNAs were present in the reaction mixture (Figure 3). This finding indicates that the mRNAs beginning with AUA 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 H<sub>34</sub>D, 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* H<sub>34</sub>D mRNA) (Figure 2B). As seen from this figure, the efficiency of GUG and AUUmRNAs 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 AUA and UUGmRNAs 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 pmoles of only one type of mRNA: (green) wt AUG *infA* mRNA; ○ (red) AUG *infA* H<sub>34</sub>D 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 of ribosomes between pairs of mRNA consisting of AUG *infA* H<sub>34</sub>D 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* H<sub>34</sub>D 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.

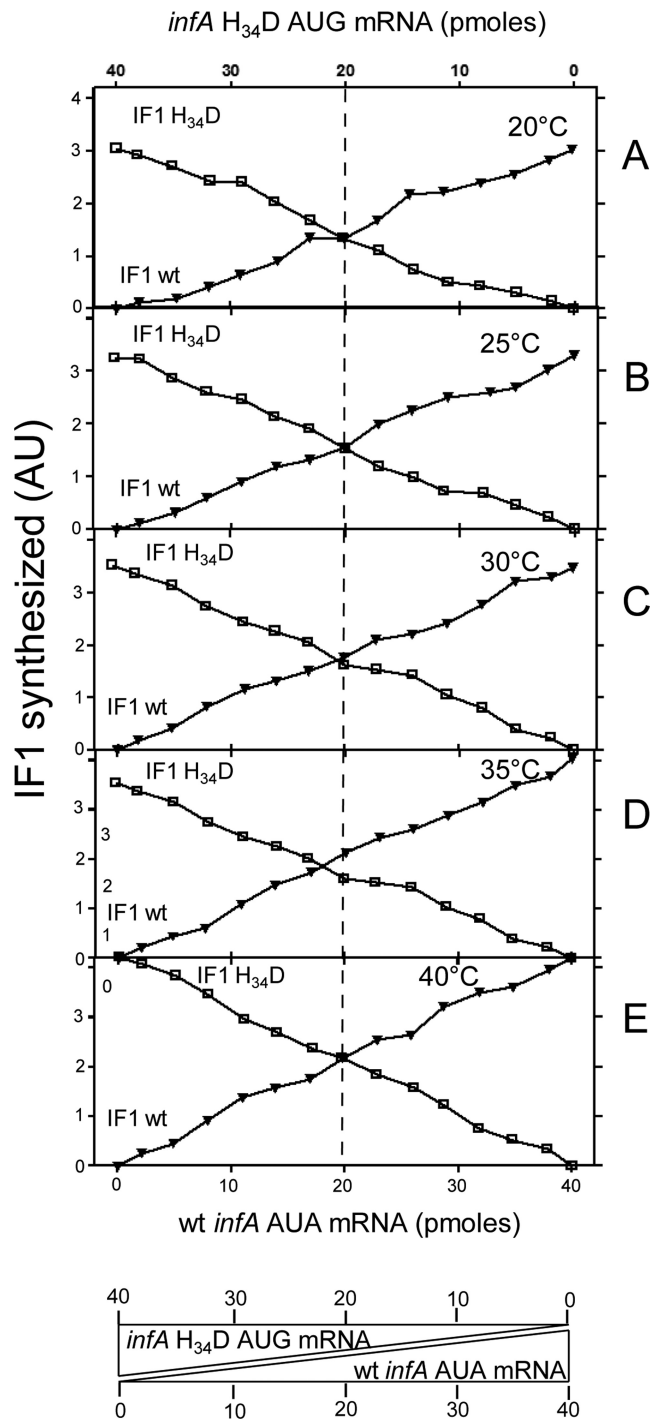
### Ribosomal recruitment of mRNAs with different initiation codons as a function of the metabolic state of the cells

Whether the availability of nutrients in the growth medium might influence competition between the mRNAs beginning with non-AUG start codons and the AUG mRNA 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 H<sub>34</sub>D synthesized by these extracts, normalized for their ribosome content and programmed with different amounts of mRNAs, were determined as indicated above.

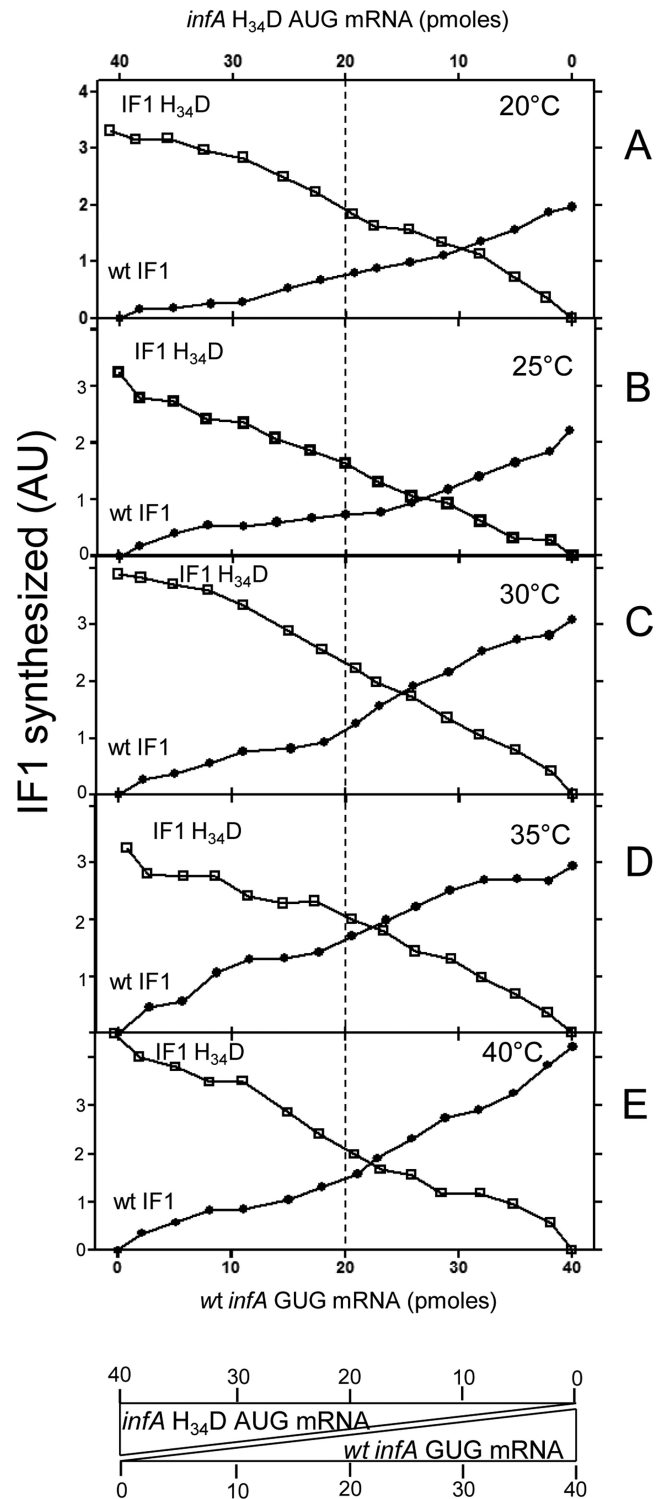
*Translational activity of non-competed mRNAs.* Under conditions of non-competition in the extracts of cells grown in TB the mRNAs beginning with AUA, UUG and GUG codons synthesized wt IF1 with an efficiency comparable to that displayed by the two AUG mRNAs (i.e. wt *infA* and *infA* H<sub>34</sub>D mRNAs) whereas the activity of the AUU mRNA was ~25% lower (Figure 7A). Furthermore, all mRNAs, including the AUU mRNA, 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

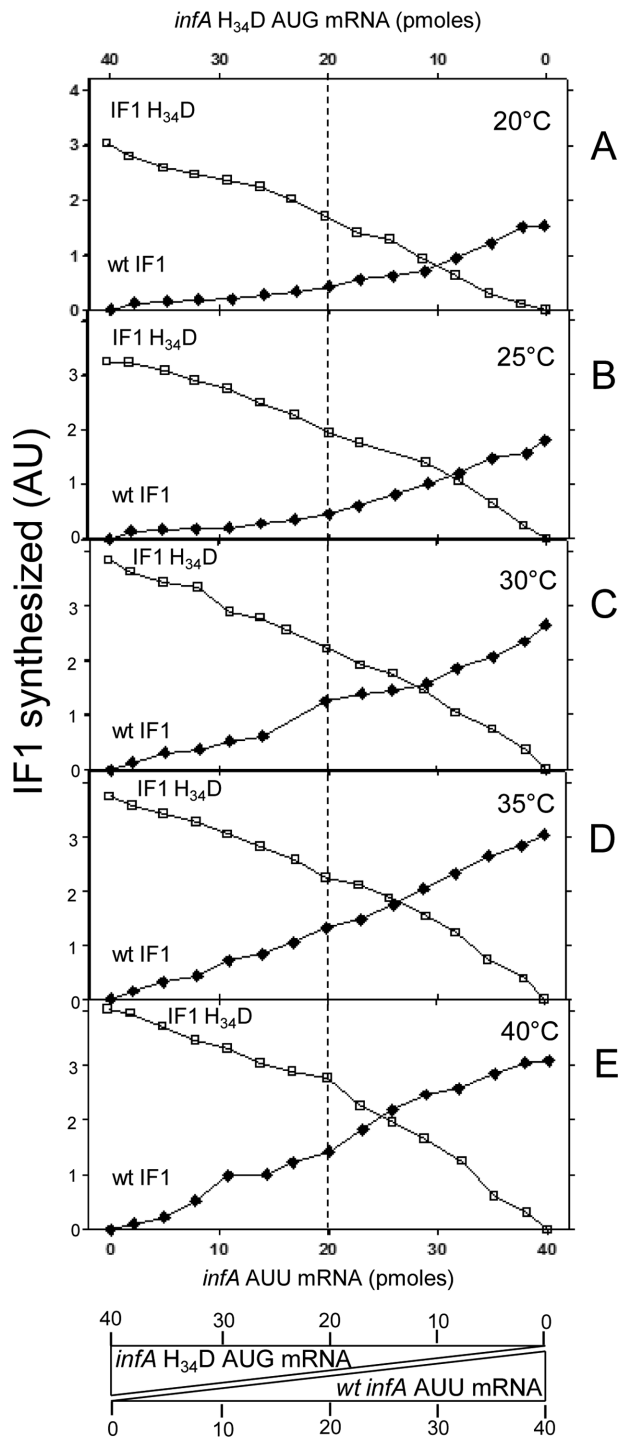
of cells grown in M9; indeed, with the exception of the UUG mRNA, whose activity was only marginally affected (i.e. ~20%), all the other non-AUG mRNAs translated substantially less IF1 than the two AUG mRNAs; as seen from the results of Figure 7B, the amount of IF1 synthesized with AUU mRNA, GUG mRNA and AUU mRNA was reduced respectively by ~50, 40 and 30% compared to that synthesized with the AUG mRNAs. 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 AUU mRNA 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 AUU mRNA drops from ~42% of that displayed by the AUG mRNA in the extracts of cells in early exponential growth to ~28% in stationary phase extracts (Figure 7B). Thus, aside from the case of the AUU mRNA, 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 incubation



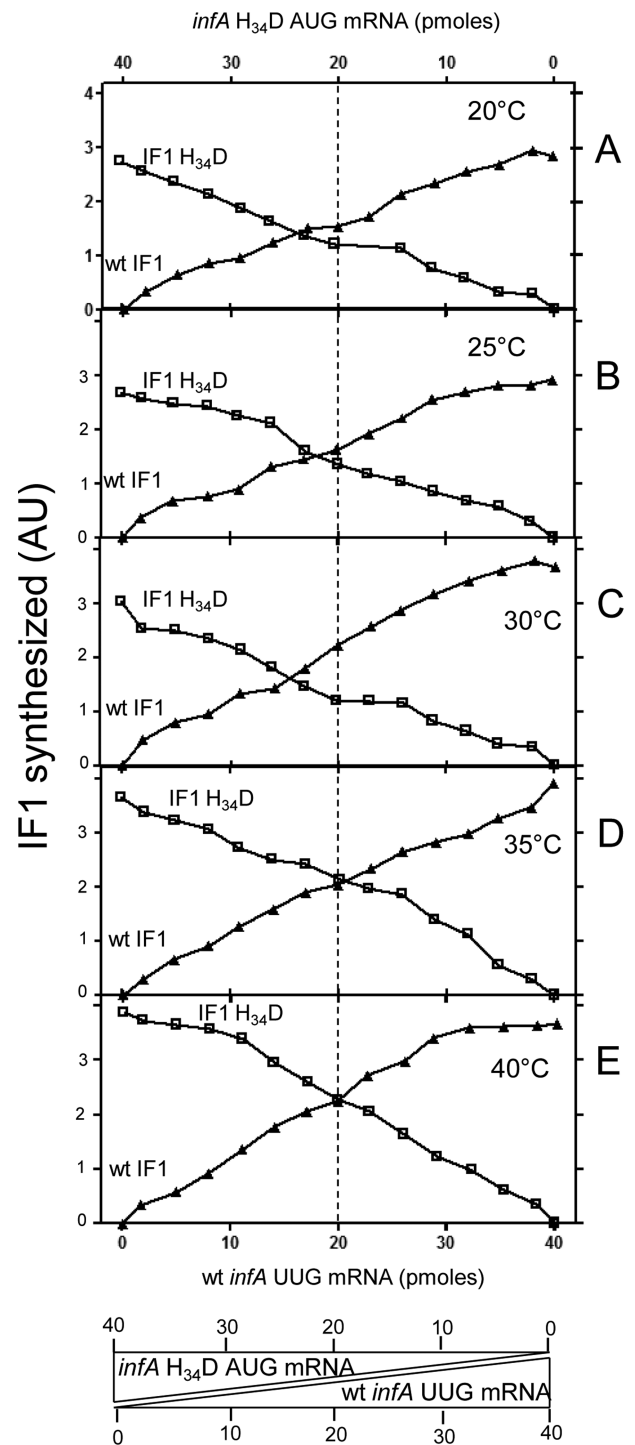
**Figure 3.** Competition between mRNAs beginning with AUG and AUA for translation by a limiting amount of ribosomes as a function of temperature. The translation experiments were carried out as described in the legend of Figure 1 at the temperatures indicated in each panel (from A to E: 20, 25, 30, 35 and 40°C) with reaction mixtures containing a total amount of 40 pmoles mRNA constituted by a mixture of (▼) *wt infA* AUA mRNA (0–40 pmoles) and (□) *infA* H<sub>34</sub>D AUG mRNA from (40–0 pmoles) as indicated in the scheme presented at the bottom of the figure.



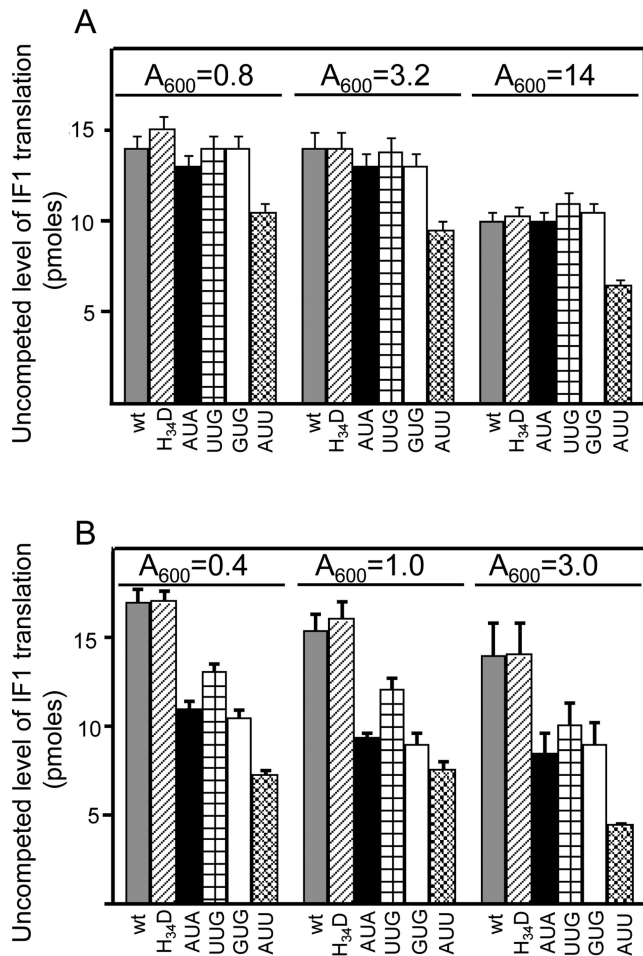
**Figure 4.** Competition for translation by a limiting amount of ribosomes between mRNAs beginning with AUG and GUG as a function of temperature. The translation experiments were carried out as described in the legend of Figure 1 at the temperatures indicated in each panel (from A to E: 20, 25, 30, 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* H<sub>34</sub>D 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 AUA 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* AUA mRNA (0–40 pmoles) and (□) *infA* H<sub>34</sub>D 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* H<sub>34</sub>D AUG mRNA from (40–0 pmoles) as indicated in the scheme presented at the bottom of the figure.



**Figure 7.** Influence of mRNA initiation triplet on the level of IF1 synthesis by non-competed mRNAs as a function of growth stage in rich and poor medium. The histogram bars indicate the amount of IF1 synthesized in crude extracts of cells growing in (A) rich (i.e. TB) and (B) minimal (i.e. M9) medium. The S30 cell extracts, prepared from cultures harvested at the optical density ( $A_{600}$ ) indicated above each group of bars, were programmed with different mRNAs bearing the non-AUG start codons reported in the abscissa. The first two bars of each group, marked as wt and H<sub>34</sub>D are the two mRNAs with AUG start codon. Translation reactions were carried out at 35°C under the reaction conditions described in M&M. The results shown in the graph represent the average of five independent experimental points. Error bars are drawn to represent the upper and lower values of each point obtained in the five replicates.

tion mixture contained the same amount of ribosomes and the reaction mixtures were supplemented with ATP, GTP, folic acid, 20 amino acids and tRNAs. Thus, our experimental conditions were made independent of many parameters whose growth-phase-dependent variations could influence translational efficiency; in light of this, it can be surmised that in addition to the nature of their start codons the observed differences in translational efficiency of the mRNAs can be attributed exclusively to differences in the intrinsic property of the ribosomes and/or in the protein composition and possible presence of regulatory molecules (e.g. ppGpp, as it will be shown below) in the extracts.

*Competition between mRNAs with non-AUG and AUG initiation codons.* The activity of the various 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 AUA (Supplementary Figure S3) UUG (Supplementary Figure S4) and GUG (Supplementary Figure S5) competed for translation with the AUG *infA* H<sub>34</sub>D 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 AUUmRNA, whose competition capacity was reduced by ~30% (Supplementary Figure S6).

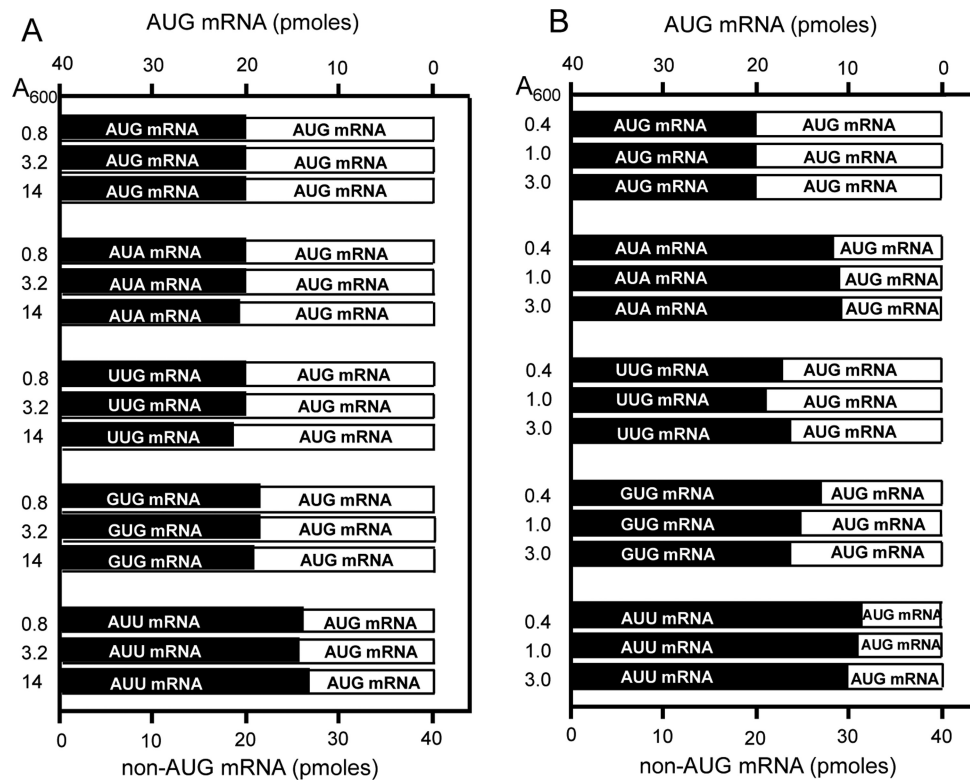
In the extracts of cells grown in minimal medium the competition between the two AUG mRNAs was not affected (Supplementary Figure S7) whereas the competitiveness displayed by the other mRNAs was strongly reduced, namely by 30, 45 and 60% for GUG, AUA and AUU mRNAs, respectively (Supplementary Figures 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 *cyaA* mRNA suitable for translation under conditions of nutritional stress (see ‘Discussion’ section).

A quantification of the results of 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* H<sub>34</sub>D 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 H<sub>34</sub>D 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.  $A_{600} = 0.8, 3.2$  and 14 in TB and  $A_{600} = 0.4, 1.0$  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).

#### **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 (Supplementary Figure S12) and AUAmRNA (Supplementary Figure S13) whereas the competitiveness of the





**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 ( $A_{600}$ ) 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* H<sub>34</sub>D AUG 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 H<sub>34</sub>D and wt IF1).

UUGmRNA 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* H<sub>34</sub>D 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 H<sub>34</sub>D and wt IF1, respectively.

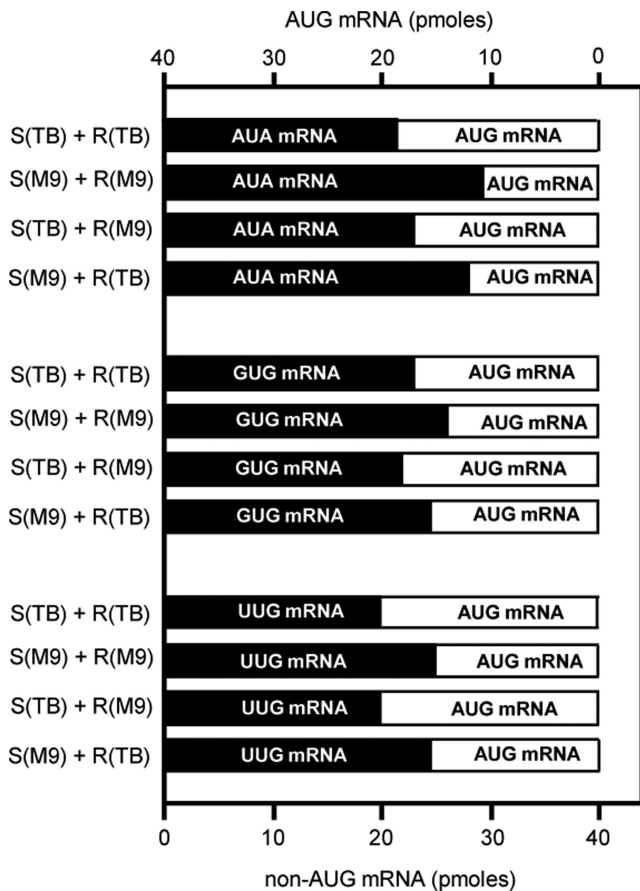
Taken together, these results indicate that the origin of the ribosomes plays only a minor role in the discrimination between different mRNAs, whereas the presence or absence of some key component(s) in the post-ribosomal supernatant of cells grown in minimal medium is responsible for a reduced ribosomal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

tors 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 mRNA 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-



**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  $A_{600} = 14$  [R(TB) and S(TB)] or in M9 up to  $A_{600} = 3$  [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* H<sub>34</sub>D 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 whereas 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 H<sub>34</sub>D and wt IF1).

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 AUAmRNA and 0.48 for AUUmRNA. 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 AUA 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).

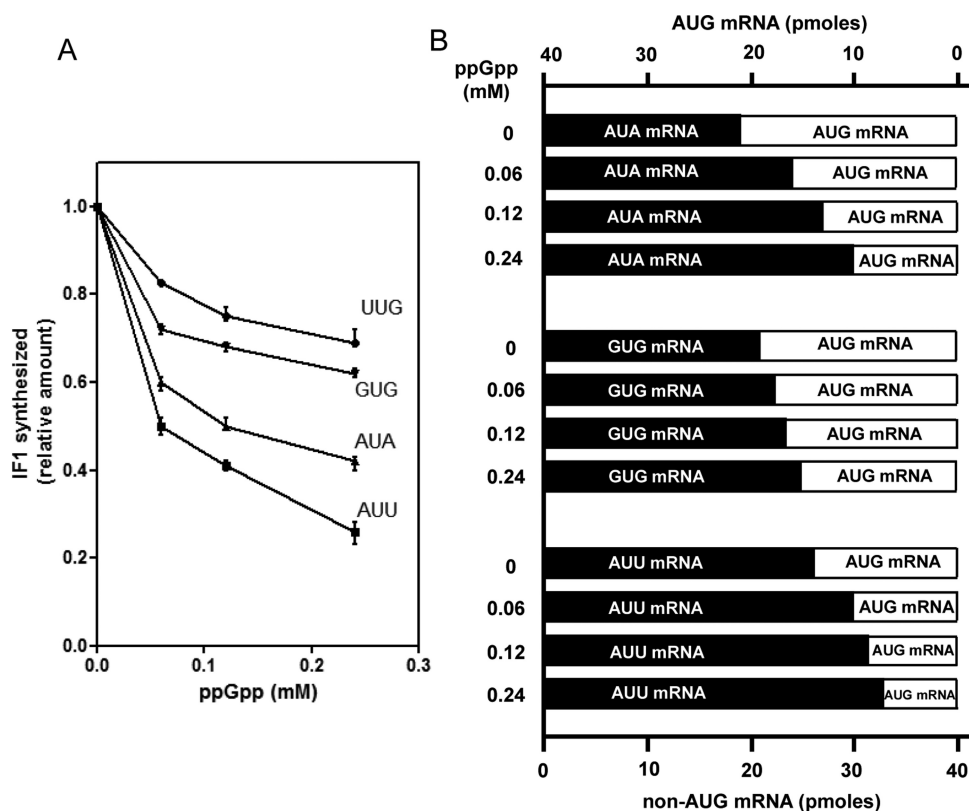
In summary, the above data indicate that the efficiency by which mRNAs beginning with AUA, GUG and UUG are translated is not altered by increasing amounts of IF3 in the reaction mixtures prepared with extracts of TB cells and is only marginally reduced in the M9 cell extracts. The activity of the AUUmRNA, 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 AUA, 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 AUA mRNAs 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, AUA 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 10.** Effect of ppGpp on the translational activity and on the competition capacity of mRNAs with different initiation codons. (A) Reaction mixtures were prepared as described in 'Materials and Methods' section using the extracts of cells grown in TB up to  $A_{600} = 14$  and programmed with 40 pmoles of a single wt *infA* mRNA beginning with: • UUG, ▼ GUG, ▲ AUA or ■ AUU. Before starting the reactions the mixtures were supplemented with the amounts of ppGpp indicated in the abscissa. Identical reaction mixtures programmed with *infA* H<sub>34</sub>D AUG mRNA served as controls. The levels of translation obtained for each mRNA at each ppGpp concentration after 30 min incubation at 37°C were quantified by the hot-TCA method (33) and compared to the levels obtained with the control AUG mRNA under the same conditions. The relative translational activities of each non-AUG mRNA with respect to the AUG mRNA are reported in the ordinate. The results shown in the graph represent the average of three independent experimental points. Error bars are drawn to represent the upper and lower values of each point obtained in the three replicates. (B) The reaction mixtures, prepared and processed as described above were programmed with a mixture of two mRNAs (40 pmoles total) constituted in all cases by *infA* H<sub>34</sub>D AUG mRNA and by a second wt *infA* mRNA with initiation triplets AUA, GUG or UUG. The black and white bars in the scheme 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 H<sub>34</sub>D and wt IF1). The ppGpp concentrations in each reaction mixture are indicated on the left side of the panel.

start codons which must be present in the reaction mixtures together with *infA* H<sub>34</sub>D AUG mRNA to allow the synthesis of the same amounts of wt IF1 and IF1 H<sub>34</sub>D. 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 AUUmRNA. 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 a low level the expression of some genes (27–29,50). Indeed, an AUG to AUA mutation caused by a base transition in the bacteriophage T4 *rIIB* 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 murein 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 *enterobacteriales* and other *gammaproteobacteria*. 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'-GAAUUCGGCCCUUG UUAACAAUUAAGGAGGUUAUACUAUGGCGAAA GAAGAUAAUUAUGAA...-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

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

may happen in nature (62). For instance, the nature of the +2 codon was shown to be one of the determinants for the high translational efficiency of an mRNA beginning with UUG but not of a GUG start codon (62); likewise, a change in the initiation codon from AUG to AUA, which did not affect mRNA binding by ribosomes in the case of phage T7 0.3 gene (52), reduced the binding to the Q $\beta$  coat protein cistron to <10 and to 30% if the initiation triplet was followed by a G and by an A, respectively (63). Finally, the relative amounts of adenylate cyclase synthesized from initiation codons UUG, GUG and AUG is 1:2:6 when the triplets are located within the *cyaA* mRNA TIR but become 1:2:3 when the translation is driven from a *lacZ* fusion (53).

In any event, before attributing the different translational properties of the mRNAs observed in this study exclusively to the nature of their start codons, it was necessary to ascertain that the single base changes within the start codon did not alter the RNA structure so as to influence translational efficiency. However, since our RNA structure predictions (64) did not detect any major initiation codon-dependent structural change which could account for the different activity levels displayed by the mRNAs tested, we could not find any obvious rationale to attribute the different behaviors 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 double stranded helix in the mRNAs with AUG, AUA, UUG, AUU whereas this helix was predicted to be less stable in the GUG mRNA, a circumstance which could favor the recruitment of this mRNA by the 30S subunit. Likewise, the structural predictions place the bases of all initiation triplets in a single stranded conformation or involved in non-WC pairings such as the G:U wobble pairing.

The results presented here indicate that the four tested mRNAs bearing a non-AUG start codon are translationally active and capable of competing, albeit to different extents, with an AUGmRNA. Furthermore, in light of the evidence that factors such as temperature (51) and phase of bacterial growth may affect the efficiency of mRNAs starting with non-AUG codons (53) we introduced similar environmental variations and found that parameters such as temperature and availability of nutrients in the culture medium can strongly affect the mRNA activities. In particular, it was observed that at 35–40°C under conditions of non-competition the activities of the mRNAs beginning with AUA and UUG are similar to those of the two mRNAs beginning with AUG, whereas both GUGmRNA and AUUmRNA are ca. 30% less active. Upon lowering the temperature to 20–30°C all mRNAs, including the AUGmRNAs become less active but the differences in the translational efficiency between GUGmRNA and AUUmRNA on one hand and the AUGmRNAs on the other is magnified. On the contrary, the AUAmRNA was found to be at least as active in the cold as the two AUGmRNAs whereas the UUGmRNA was even more active than the latter. These differences in translational efficiency were much more pronounced when the non-AUG mRNAs were tested in competition with the AUG mRNA for recruitment by a limiting amount of ribosomes. Both GUGmRNA and AUUmRNA were very poor competitors of the AUGmRNA, espe-

cially at the lower temperatures whereas the UUGmRNAs and the AUAmRNA were very efficient competitors of the AUGmRNA at all temperatures and the UUGmRNA was actually favored over the AUGmRNA at 20–30°C

As mentioned above, mutations of the AUG start codons into AUA were reported to confer a severe cold-sensitivity to the expression of the resulting mRNAs. However, our results obtained at different temperatures with the AUAmRNA indicate that the phenotypes described in the literature cannot be attributed to the start codon itself, but must instead stem from the particular context in which the mutated initiation codon is present, as discussed above. Fully compatible with this premise is the different severity of the cold-sensitive phenotype reported for the AUG to AUA mutations (51,52) and the excellent performance of the AUA 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 GUGmRNA and even more so by the AUUmRNA 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 *infC* genes which begin with these triplets. Thus, while the extensive phylogenetic conservation of the AUU triplet at the beginning of *infC* mRNAs is justified by the mechanism of translation autoregulation based on the discrimination of this non-canonical initiation codon by IF3, the efficient translation of *infC* mRNA at the cold-stress temperature 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 function 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 participate in the structural organization of the nucleoid (68) and also to play a role in translation regulation (67). In particular, 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 HU $\beta$  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 HU $\beta$  in cells entering stationary phase is compatible with the modest inhibition produced by ppGpp on the translation of the GUGmRNA, as observed in this study. However, the cold stress which stimulates HU $\beta$  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.

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