Modulation of ribosomal recruitment to 5′-terminal start codons by translation initiation factors IF2 and IF3

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Received 15 March 2001; accepted 22 March 2001

First published online 10 April 2001

Edited by Lev Kisselev

Abstract Sequence determinants and structural features of the RNA govern mRNA–ribosome interaction in bacteria. However, ribosomal recruitment to leaderless mRNAs, which start directly with the AUG start codon and do not bear a Shine–Dalgarno sequence like canonical mRNAs, does not appear to rely on 16S rRNA–mRNA interactions. Here, we have studied the effects of translation initiation factors IF2 and IF3 on 30S initiation at a 5′-terminal AUG and at a competing downstream canonical ribosome binding site. We show that IF2 affects the forward kinetics of 30S initiation complex formation at the 5′-terminal AUG as well as the stability of these complexes. Moreover, the IF2:IF3 molar ratio was found to play a decisive role in translation initiation of a leaderless mRNA both in vitro and in vivo indicating that the translational efficiency of an mRNA is not only intrinsically determined but can be altered depending on the availability of components of the translational machinery. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Initiation factor; Leaderless mRNA; Translation

1. Introduction

Initiation of protein synthesis in prokaryotes proceeds in several interrelated steps during which the translation initiation region of the mRNA and the initiator tRNA, fMet-$tRNA_f^{Met}$, interact with the 30S ribosome to form a ternary complex. The efficiency and fidelity of formation of the 30S initiation complex is promoted by three initiation factors (IF1, IF2, IF3) [1].

IF3 acts as a fidelity factor by destabilizing non-canonical codon–anticodon interactions [1]. The discriminatory function exerted by IF3 appears to result from a conformational change in the ribosome rather than from a direct interaction with fMet-$tRNA_f^{Met}$ [2,3]. While IF3 has been shown to stimulate ternary complex formation on canonical mRNAs, it antagonizes 30S binding on leaderless mRNAs with a 5′-terminal AUG start codon [4].

IF2 accelerates the formation of the codon–anticodon interaction by promoting efficient binding of fMet-$tRNA_f^{Met}$ to the ribosomal P-site [3]. Moreover, a contribution of IF2-dependent GTP hydrolysis in the final adjustment of fMet-$tRNA_f^{Met}$ in the P-site of the 30S subunit has been reported [5]. IF2 has been shown to stimulate translation of leaderless mRNAs which has been interpreted as showing that a ribosome–initiator tRNA complex is required for translation initiation at 5′-terminal start codons [6].

IF1 is essential for cell viability [7] and occupies the ribosomal A-site [8,9]. It has been proposed that IF1 together with IF2 mimics the aminoacyl-tRNA–EF–Tu–GTP ternary complex [10]. Thus, it could serve to prevent binding of the aminoacyl-tRNA as well as initiator tRNA to the A-site during ternary complex formation.

The present study focuses on the effects of both IF2 and IF3 during formation of the 30S initiation complex at 5′-terminal AUGs. We show that the molar ratio of both factors can modulate translation of leaderless mRNAs in vitro as well as in vivo.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain MC4100F′ [11] was lysogenized with λWH103, which harbors a leaderless *tetR-lacZ* translational fusion [12], using standard procedures [13]. Plasmid pAUG*infC* is a derivative of the low copy number plasmid pK184 and encodes an *E. coli infC* allele equipped with an AUG start codon [4]. Plasmid pLBYC8 is a derivative of the high copy number plasmid pTrc99A (Pharmacia Biotech.) and encodes the *E. coli infB* gene [14].

2.2. Primer extension inhibition analysis (toeprinting)

IF depleted 30S subunits were prepared as previously described [15]. They were judged as pure when 23S rRNA was absent from the preparations. The *ompA*Δ117 mRNA (Fig. 1A) was prepared from a PCR template [16] and the *Ava*II primer [17] was used for toeprinting. Charged initiator tRNA was kindly provided by M.V. Rodninia, University of Witten/Herdecke, and by K. Nierhaus, Max-Planck Institute of Molecular Genetics, Berlin, Germany. The *Bacillus staerothermophilus* IF2 and the IF2 mutant protein used in the studies were purified as described [3].

The toeprinting assays in the absence or presence of IF2 or the IF2(R700T) mutant protein were essentially performed as described by Hartz et al. [18]. For the kinetic toeprinting reactions, 2 pmol 30S subunits, 5 pmol IF2 or IF2(R700T) (when present), 0.8 pmol fMet-$tRNA_f^{Met}$ and 1 mM GTP were pre-incubated for 20 s in a 10 μl reaction before the mRNAs were added at a final concentration of 0.005 pmol/μl. The incubation times varied as described in the legend to Fig. 1B. The MMLV reaction was then performed at 37°C for 3 min. The relative toeprints shown in Fig. 2 were calculated as described by Hartz et al. [19]. The toeprinting reactions shown in Figs. 2 and 3 were carried out under the conditions specified in the text and in the corresponding figure legends.

2.3. β-Galactosidase assays

The β-galactosidase activities were determined as described by Miller [13]. Triplicate aliquots were taken of each culture at an OD_{600} of 0.8.

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Abbreviations: IF, translation initiation factor; rbs, ribosome binding site; SD sequence, Shine–Dalgarno sequence

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PII: S0014-5793(01)02378-X

3. Results and discussion

3.1. The stimulatory effect by IF2 on 30S recruitment to 5′-terminal AUGs depends on the fMet-tRNA$_f^{Met}$–IF2 interaction

Since 30S ribosomes were unable to form binary complexes with a leaderless mRNA, we suggested that the 5′-terminal AUG is the only necessary and constant element which is recognized by a 30S initiator tRNA complex [6]. IF2 was shown to stimulate the efficiency and rate of ternary complex formation on a leaderless mRNA to a significantly higher extent than on mRNAs containing a canonical ribosome binding site (rbs). This has been attributed to the stimulation by IF2 on initiator tRNA binding to the 30S subunit.

In this study we assessed by use of kinetic toeprinting assays the influence of *B. stearothermophilus* IF2 and that of an IF2 mutant with a reduced binding activity for fMet-tRNA on the formation of 30S initiation complexes at a 5′-terminal AUG. Toeprinting experiments are suited to localize the position of the initiation complexes since these ternary complexes can stop the progress of a downstream primed reverse transcriptase. Furthermore, the strength of the toeprint signals reflects the efficiency of ternary complex formation at a given start codon. The toeprinting experiments were performed with *ompA*Δ117 mRNA (Fig. 1A) which contains a 5′-terminal AUG codon (AUG1) and a downstream AUG preceded by the canonical Shine–Dalgarno (SD) sequence of *ompA* mRNA (AUGi). This experimental system is suited to study the influence of factors which affect 5′-terminal or internal 30S initiation complex formation since the ribosomal choice of either AUG start codon is mutually exclusive on this mRNA [17].

In the experiment shown in Fig. 1B, the 30S subunits were pre-incubated for 20 s with limiting concentrations of fMet-tRNA$_f^{Met}$ (30S:initiator tRNA ratio was 2.5:1) and, when present, with a 2.5-fold molar excess of *B. staerothermophilus* IF2 or IF2(R700T). *OmpA*Δ117 mRNA was then added and the incubation continued for 10, 20, 30, 60 and 80 s before subjecting the samples to toeprinting analysis. The electrophoretic pattern of the products obtained by reverse transcription (Fig. 1B, lanes 3–7) showed that in the absence of IF2 the ternary complex is readily formed at the internal AUG codon (AUGi) and its amount is increased with incubation time (Fig. 1C). In contrast, no toeprint signal was obtained for the 5′-terminal AUG (AUG1) even after 80 s. However, in the presence of IF2, the 5′-terminal AUG1 was able to compete efficiently with AUGi. The ratio of the toeprint signals remained approximately the same for all times tested with a stronger signal for AUG1 (Fig. 1B, lanes 8–12 and Fig. 1C). The assay was performed under limiting initiator tRNA conditions. As a possible explanation for this result, we considered the IF2 assisted binding of initiator tRNA to the 30S subunit which is brought about by a physical interaction between fMet-tRNA$_f^{Met}$ and IF2 [3].

Next, we used an IF2 mutant protein (R700T) with a ~2.5-fold reduced binding activity for initiator tRNA [20]. The kinetic toeprint experiment on *ompA*Δ117 mRNA was performed as described above. When compared to the reactions performed with wild-type IF2 (Fig. 1B, lanes 8–12), in the presence of the mutant protein IF2(R700T), the toeprint signal corresponding to ternary complex formation at AUG1 was strongly diminished (Fig. 1B, lanes 15–19 and Fig. 1C). These results indicated that the positive effect of IF2 on the ribosomal selection of 5′-terminal start codons requires the interaction of IF2 with fMet-tRNA$_f^{Met}$ and corroborated our hypothesis on the molecular function of IF2 in translation initiation of leaderless mRNAs.

3.2. IF2 stabilizes 30S initiation complexes at 5′-terminal AUGs

It is worth noting that no 30S initiation complexes were formed at AUG1 in the absence of IF2 (Fig. 1B), and that in the presence of IF2 (Fig. 1B) the toeprint signal for AUG1 was ~2.5-fold stronger than for AUGi. Thus, it is conceivable that IF2, besides stimulating the formation of the 30S fMet-tRNA$_f^{Met}$ binary complex, also increases the stability of the 30S initiation complex, wherein a leaderless mRNA is believed to be only tethered to the ribosome via start codon–anticodon base-pairing [6,23]. This effect of IF2 could be mediated through interaction of the factor with fMet-tRNA$_f^{Met}$ and/or the ribosome [20–22].

Since, under the used in vitro toeprinting conditions, IF2 is neither essential to place initiator tRNA into the ribosomal P-site [18] nor for 30S initiation complex formation on leaderless mRNA in the absence of a competing canonical rbs [23], we used *ompA*Δ117 mRNA, and tested the ability of AUG1 to compete for ribosomes with AUGi in the presence of saturating amounts of fMet-tRNA which should compensate for the IF2 mediated stimulation of initiator tRNA binding to ribosomes. When a 5-fold stoichiometric excess of fMet-tRNA was pre-incubated with ribosomes in the absence of IF2, ternary complex formation on *ompA*Δ117 mRNA occurred almost exclusively at AUGi (Fig. 2, lane 2). This finding could have two possible explanations: either AUG1 cannot compete with the canonical start at AUGi for ribosomes under these experimental conditions or the ternary complex at the 5′-terminal AUG has an intrinsically low stability in the absence of IF2. To distinguish between these possibilities, we asked whether ternary complex formation at AUG1 increases when the competition with AUGi is abolished. This condition was obtained by annealing to the ribosomes, prior to the toeprint experiment, an RNA oligonucleotide complementary to the anti-SD domain of 16S rRNA. As expected, increasing amounts of the SD oligonucleotide resulted in a decrease of ternary complex formation at AUGi (Fig. 2, lanes 3–5). When equimolar concentrations of the oligonucleotide were annealed to ribosomes, the toeprint signal for AUGi was almost abolished (Fig. 2, lane 5). However, the toeprint signal for AUG1 did not increase to the same extent as the signal decreased for AUGi. This result, rather than a competition by the downstream canonical start, suggests that the ternary complexes formed at 5′-terminal start codons in the absence of IF2 are intrinsically weak. This explanation is in line with toeprinting and filter binding studies which showed that only ~1.5–2% of leaderless mRNA is bound in ternary complexes in the absence of initiation factors even if ribosomes and fMet-tRNA are present in molar excess [6,23].

IF3 has been shown to antagonize authentic start codon selection on leaderless mRNAs [4]. La Teana et al. [24] have demonstrated that the discriminatory function of IF3 on mRNAs with start codons other than AUG can be compensated for by IF2. To further address the question whether the IF2–fMet-tRNA$_f^{Met}$ interaction contributes to stabilization of 30S initiation complexes at 5′-terminal AUGs, we asked whether altering the ratio of the three initiation factors affects

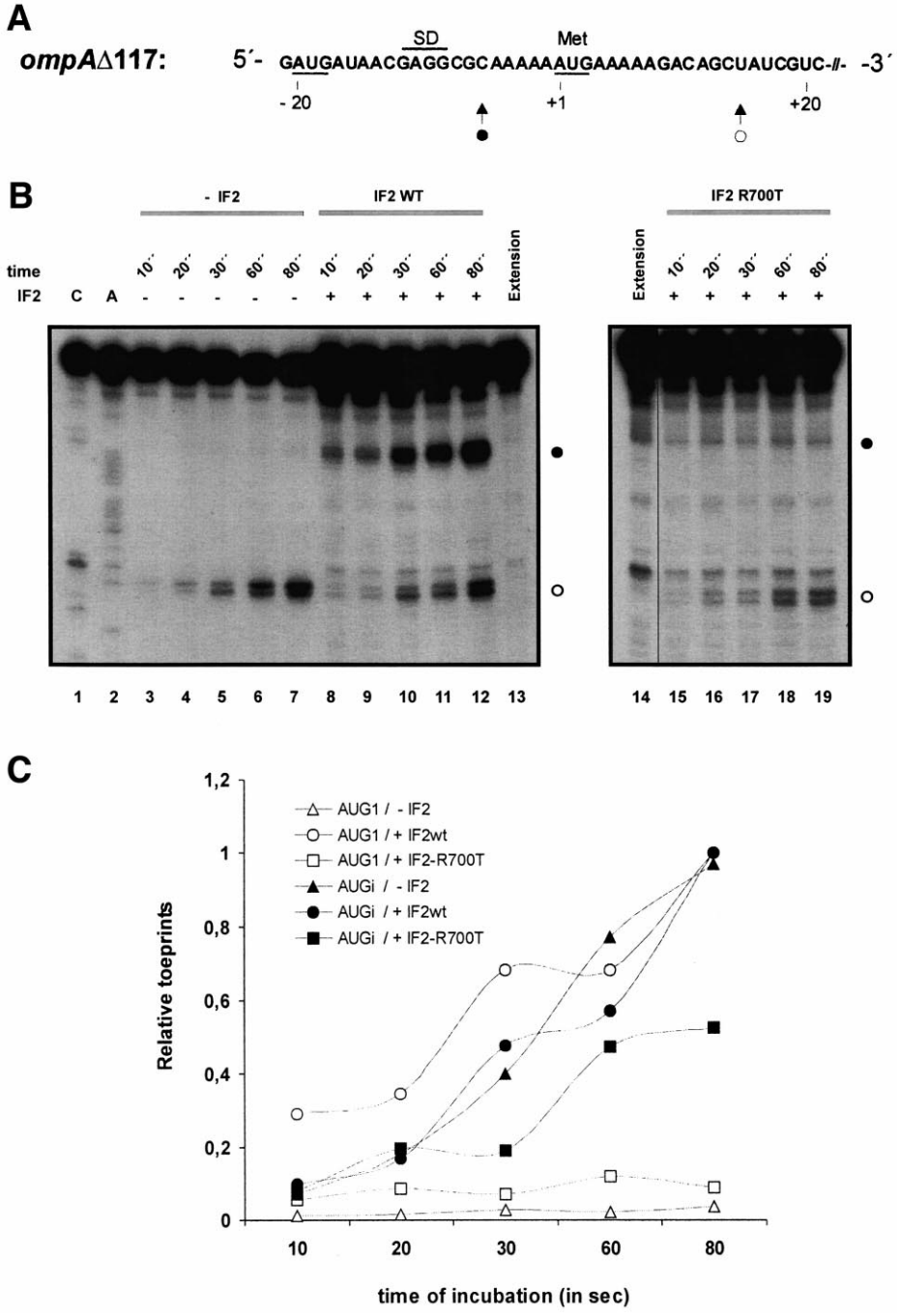

Fig. 1. Selection of the 5′-terminal (AUG1) and the internal AUG (AUGi) on *ompA*Δ117 mRNA in the presence of IF2 and IF2(R700T). A: Depiction of *ompA*Δ117 mRNA. The start codon(s) and the authentic SD sequence of *ompA* mRNA are indicated by bars. For *ompA*Δ117 two toeprint signals are obtained, which result from ternary complex over AUG1 (nt −20–−18) and AUGi (nt 1–3) the latter of which represents the authentic start codon of *ompA* mRNA. The positions of the toeprint signals are indicated by a filled (AUG1) and an open circle (AUGi). B: Kinetic toeprints on *ompA*Δ117 mRNA in the absence or presence of IF2 and IF2(R700T), respectively. Lanes 3–7, kinetic toeprint analysis in the absence of IF2. Lanes 8–12 and 15–19, kinetic toeprint analysis in the presence of IF2 and IF2(R700T), respectively. Lanes 13 and 14, primer extension in the presence of fMet-tRNA$_f^{Met}$ and in the absence of 30S subunits. 30S subunits, fMet-tRNA$_f^{Met}$, IF2 and mRNA were incubated for different times (given in s) as indicated on top. The assay conditions were as described in Section 2. The toeprinting signals obtained for AUG1 and AUGi are indicated by a filled and open circle, respectively. C: Relative toeprints on *ompA*Δ117 mRNA for AUG1 and AUGi. The relative toeprints at either start codon obtained in the absence of IF2 and in the presence of IF2(R700T) were normalized to that of AUG1 and AUGi obtained in the presence of IF2 after 80 s of incubation.

the partitioning of 30S initiation events at AUG1 and AUGi on *ompA*Δ117 RNA. Initiator factor 1 was included in these studies since it apparently interacts with IF2 [9,21], and could thus contribute to IF2 stabilization of 30S initiation complexes on non-canonical starts. First, all three initiation factors were individually included in the toeprinting assay. In the absence of factors and in the presence of only IF1, 30S initiation complex formation occurred exclusively at AUGi while in the presence of IF2 30S initiation complexes were formed at both AUG1 and AUGi (Fig. 3, lane 4). As expected from

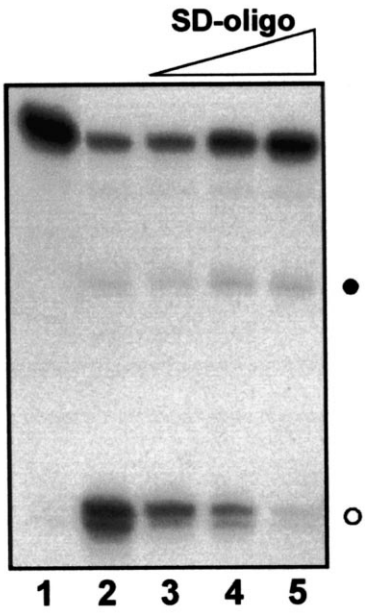

Fig. 2. 30S initiation complexes formed at 5′-terminal start codons are intrinsically weak. Lane 1, primer extension in the absence of initiator tRNA and in the presence of 30S subunits. Lane 2, toeprinting reaction. In the reactions shown in lanes 2–5, the molar ratios of 30S subunits:initiator tRNA:*ompA*Δ117 mRNA was 1:5:0.01. Lanes 3–5, the SD RNA oligonucleotide 5′-UAAGGAGGUG-3′ with complementarity to the 3′-end of 16S rRNA was pre-incubated with 30S subunits for 15 min at 37°C in the same buffer as used for toeprinting. The stoichiometries of 30S subunits:SD oligo used in these assays were 1:0.25 (lane 3), 1:0.5 (lane 4) and 1:1 (lane 5). The toeprinting signals obtained for AUG1 and AUGi are indicated by a filled and open circle, respectively.

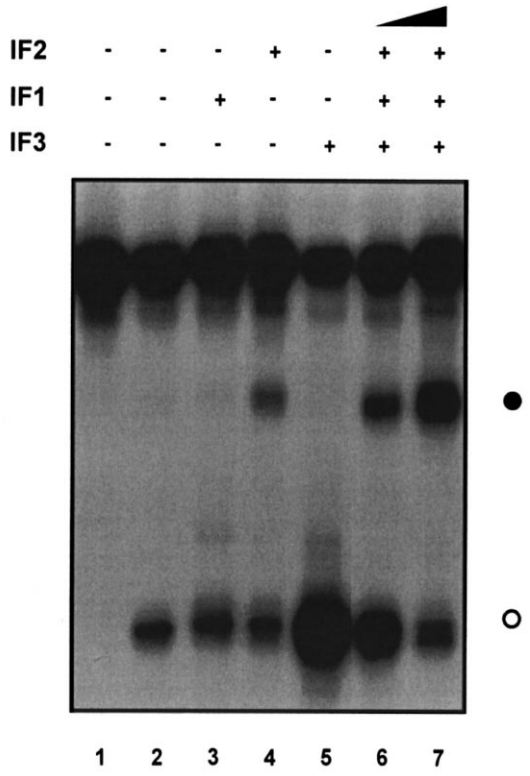

Fig. 3. IF2 can compensate for the discrimination function of IF3 on the 5′-terminal start codon of *ompA*Δ117 mRNA. Lane 1, primer extension in the absence of 30S subunits and fMet-tRNA$_f^{Met}$. Lane 2, toeprinting in the presence of 30S subunits and fMet-tRNA$_f^{Met}$ (molar ratio = 1:2.5). Lanes 3, 4 and 5, toeprinting in the presence of either IF1, IF2 or IF3. The ratio of initiation factors/30S subunits was 2.5:1 in all reactions except for those shown in lane 7. Lane 6, toeprinting in the presence of all three initiation factors (molar ratio of IF1:IF2:IF3 was 1:1:1). Lane 7, toeprinting in the presence of all three initiation factors (molar ratio of IF1:IF2:IF3 was 1:2.5:1). IF1 and IF2 were pre-incubated with 30S subunits for 1 min before IF3 was added. The toeprint signals for AUG1 and AUGi are indicated by a filled and an open circle, respectively.

previous studies performed on different leaderless mRNAs [4], in the presence of IF3 the ternary complexes were formed with a high efficiency at AUGi (Fig. 4, lane 5). Next, the ratio of IF1:IF3 was kept constant and the concentration of IF2 was altered. 30S ribosomes were pre-incubated for 20 s with IF1 and IF2 and fMet-tRNA$_f^{Met}$ before the addition of *ompA*Δ117 RNA and IF3. At a 1:1 ratio of each initiation factor to 30S subunits the initiation complexes were formed preferentially at the internal start codon (Fig. 3, lane 6) with a partitioning of ribosome binding events between AUG1 and AUGi having relative intensities of 1:2. However, when IF2 was added in a 2.5 molar excess over IF1 and IF3 (Fig. 4, lane 7), the partitioning of 30S binding events was reversed, with signal intensities of 4:1 in favor of AUG1. We interpret this as showing that a 30S fMet-tRNA$_f^{Met}$-IF2–mRNA complex formed at a 5′-terminal AUG can counteract the destabilization function exerted by IF3.

3.3. The in vivo translational efficiency of a leaderless reporter gene is correlated with IF2 and IF3 levels

From the study shown in Fig. 3, we concluded that the molar ratio of initiation factors plays a decisive role of whether ribosomes are recruited to 5′-terminal start codons. Next, we studied whether the results obtained in vitro can be verified in vivo by varying the intracellular IF2 and IF3 levels. *E. coli* strain MC4100F′ was lysogenized with λWH103 encoding a leaderless *tetR-lacZ* gene. The lysogen was either transformed with plasmid pKAUG*infC*, in which the *E. coli* *infC* allele is provided with an AUG start codon to avoid

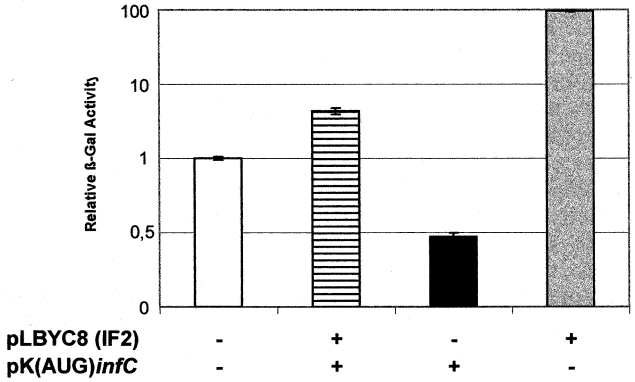

Fig. 4. IF3 and IF2 levels determine the translational efficiency of a leaderless *tetR-lacZ* gene in vivo. The cultures were grown in LB medium [13] at 37°C to an OD_{600} of 0.3 when 1 mM IPTG was added. The β-galactosidase values for each strain were determined in triplicate at an OD_{600} of 0.8. The β-galactosidase value obtained for the control strain MC4100F′λWH103(pK184; pTrc99a) was set to 1 (white bar). The β-galactosidase values for strains MC4100F′λWH103(pAUG*infC*; pLBYC8) (striped bar), MC4100F′λWH103(pAUG*infC*; pTrc99a) (black bar) and MC4100F′λWH103(pK184; pLBYC8) (gray bar), respectively, were normalized to the control strain.

translational autoregulation by IF3 [25,26], with plasmid pLBYC8 harboring the *E. coli infB* gene [14], with both plasmids pKAUG*infC* and pLBYC8, or with both the parental control plasmids pK184 and pTrc99A. The β-galactosidase activity obtained with the control strain MC4100F′λWH103(pK184; pTrc99A) was set to 1. When compared to the control strain, the translational efficiency of the *tetR-lacZ* reporter gene was ∼2-fold decreased and ∼80-fold increased in MC4100F′λWH103(pKAUG*infC*; pTrc99A) and MC4100F′λWH103(pLBYC8; pK184), respectively. Thus, in accordance with the toeprinting experiments shown in Figs. 1 and 3, and with our previous studies [4,6], enhanced levels of IF2 and IF3, respectively, increase and decrease translation events at 5′-terminal start codons in vivo. The presence of both plasmid borne initiation factor genes in strain MC4100F′λWH103(pAUG*infC*; pLBYC8) resulted in a ∼5-fold increase when compared to the control strain. The latter result corroborates the in vitro studies presented in Fig. 3, lanes 6 and 7, in that IF2 can compensate for the discrimination function of IF3 on 5′-terminal start codons.

Howe and Hershey [27] have shown that in exponentially growing cells all three initiation factors are present in approximately equimolar amounts in a ratio of 1:5 with ribosomes. The expression of the genes encoding IF1, IF2 and IF3 is under metabolic control. However, the regulatory mechanisms governing the expression of the IF genes are different [28]. As a consequence, the ratio of IF to each other can vary under certain growth conditions. For instance, when the growth rate increases, a transient elevation of the ribosome/IF3 ratio has been observed [29], whereas IF2 levels were found to be increased under conditions following the cold shock in *E. coli* [30]. It is therefore conceivable that transient oscillations of the relative levels of factors and 30S ribosomes can affect the translational efficiency of leaderless mRNAs.

Acknowledgements: This work was supported by grant P12065-MOB from the Austrian Science Fund (FWF) to U.B. and by grant MURST-PRIN 2000 'Investigating structure–function relationships in translation initiation factor IF2' to C.O.G. We are grateful to Drs. L. Laalami, K. Nierhaus, M. Rodnina and W. Wintermayer for the gift of materials.

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