Molecular Cell
Article

Kinetic Checkpoint at a Late Step in Translation Initiation

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DOI 10.1016/j.molcel.2008.04.014

SUMMARY

The translation initiation efficiency of a given mRNA is determined by its translation initiation region (TIR). mRNAs are selected into 30S initiation complexes according to the strengths of the secondary structure of the TIR, the pairing of the Shine-Dalgarno sequence with 16S rRNA, and the interaction between initiator tRNA and the start codon. Here, we show that the conversion of the 30S initiation complex into the translating 70S ribosome constitutes another important mRNA control checkpoint. Kinetic analysis reveals that 50S subunit joining and dissociation of IF3 are strongly influenced by the nature of the codon used for initiation and the structural elements of the TIR. Coupling between the TIR and the rate of 70S initiation complex formation involves IF3- and IF1-induced rearrangements of the 30S subunit, providing a mechanism by which the ribosome senses the TIR and determines the efficiency of translational initiation of a particular mRNA.

INTRODUCTION

The level of expression of individual gene products can vary considerably depending on the specific need of the cell in any given physiological state. One of the most important types of posttranscriptional regulation is translational control, which can determine whether, when, and how much product is synthesized on a given mRNA. Regulation at the translational level can account for up to three orders of magnitude differences in expression between different genes, although 10-fold differences are most common (McCarthy and Gualerzi, 1990; Voorma, 1996). Temporal regulation of the level of gene expression in bacteria can be modulated by *trans*-acting factors, such as proteins, RNAs, or metabolites that bind to mRNA and can either enhance or repress translation (Gold, 1988; McCarthy and Gualerzi, 1990; Voorma, 1996; Romby and Springer, 2007). A very important role in determining translational efficiency is played by *cis*-acting elements located in the translation initiation region (TIR) of the mRNA. It is generally assumed that, in order to be efficiently translated, an mRNA should lack secondary structure around the AUG initiation codon and possess a Shine-Dalgarno (SD) sequence with maximum complementarity to the anti-Shine-Dalgarno (ASD) sequence of 16S rRNA, enabling the mRNA to form a thermodynamically stable complex with the 30S subunit (Mathews et al., 2007). On the other hand, the level of translation obtainable for a given template in many cases did not correlate with its affinity for the 30S subunit (Calogero et al., 1988; Lang et al., 1989). Not only did the SD-ASD interaction not appear to be mandatory to obtain a high level of translation (Calogero et al., 1988; Melancon et al., 1990), but an extended SD-ASD complementarity, in combination with a short spacer between the SD sequence and the start codon, was found to reduce the level of translation of a number of mRNAs (Lee et al., 1996; Komarova et al., 2002; Skorski et al., 2006). Mutations reducing the SD-ASD complementarity diminished translational efficiency only when ribosome binding was impaired by the mRNA structure; without an inhibitory structure, these mutations appeared to have no effect (de Smit and van Duin, 1994). Efficient initiation generally requires the use of the canonical AUG initiation codon. Nevertheless, codons other than AUG are utilized for initiation in a number of mRNAs, including mRNAs from highly expressed *E. coli* genes such as *tufA* (elongation factor Tu), *hupB* (HUα protein), *rpsT* and *rpsM* (ribosomal proteins S20 and S13), and *infC* (IF3). Thus, the overall structure of the TIR can be thought of as an essential element for determining the "stereochemical fitness" of the mRNA in binding to the 30S subunit.

The mechanism of translation initiation in prokaryotes can be described by a minimal kinetic model (Gualerzi et al., 1977) (Figure 1A). The first phase starts with the binding of the mRNA and initiator fMet-tRNA^{fMet} to the 30S subunit with the help of initiation factors (IFs) and ends with codon-anticodon pairing and the formation of the 30S initiation complex (30S IC) containing the three initiation factors, mRNA, and fMet-tRNA^{fMet}. The second phase comprises the association of the 50S subunit with the 30S IC, GTP hydrolysis, and the dissociation of the initiation factors; this phase is considered irreversible and results in the 70S initiation complex (70S IC) that is ready to enter elongation (Gualerzi et al., 2001). The effect of the thermodynamic properties of the TIR on translation was studied extensively (Lee et al., 1996). By contrast, the importance of the TIR for the irreversible 70S IC formation and the mechanism by which the different elements of the TIR are recognized by the ribosome are poorly understood. Here, we have studied the influence of various structural elements of the TIR (i.e., extent of SD-ASD

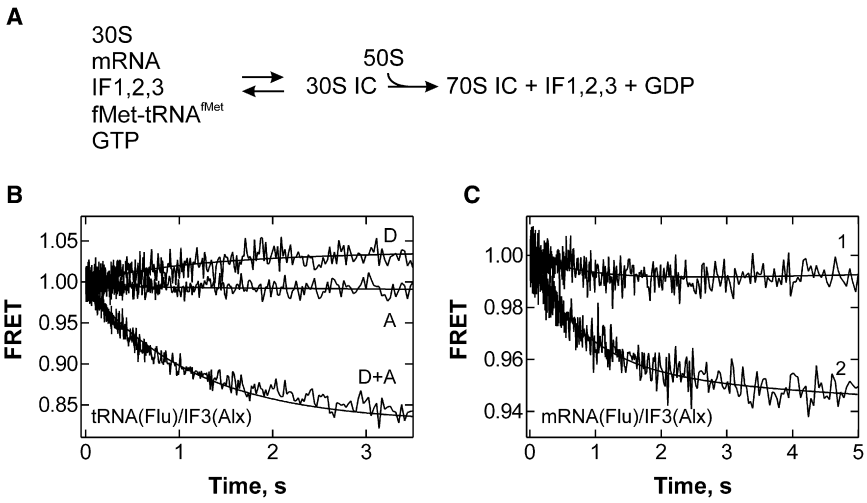

Figure 1. Formation of 70S Translating Ribosomes

(A) Reaction scheme. In the first phase, depicted as a single step, IFs, mRNA, and fMet-tRNA^{fMet} bind to the 30S subunit, forming the 30S IC. In the second phase, the 50S subunit binds to the 30S IC, GTP is hydrolyzed by IF2, and IFs dissociate. Thereby, a functional 70S IC is formed. For kinetic measurements, preformed 30S ICs were rapidly mixed with excess 50S subunits.

(B) Dissociation of IF3 from the ribosome upon 70S IC formation. The reaction was initiated by mixing 50S subunits with 30S ICs containing labeled fMet-tRNA^{fMet}(Flu) and IF3(Alx) (D + A, donor + acceptor). Control measurements were performed with unlabeled fMet-tRNA^{fMet} and labeled IF3 (A, acceptor alone) or with labeled fMet-tRNA^{fMet}(Flu) and unlabeled IF3 (D, donor alone).

(C) Dissociation of IF3 from 30S IC upon fMet-tRNA^{fMet} binding in the absence of 50S subunits (1) or upon addition of 50S subunits (2). FRET between m022 mRNA(Flu) and IF3(Alx) was measured.

complementarity, distance between start codon and SD sequence, and nature of the codon used for initiation) on the kinetics of 70S IC formation using rapid kinetic techniques (stopped-flow and quench-flow).

RESULTS

Experimental Approach

Formation of the 70S IC entails the association of the 50S subunits with the 30S IC and the dissociation of initiation factors. The subunit association step was monitored by light-scattering stopped-flow technique, while the interaction of IF1, IF3, mRNA, and fMet-tRNA^{fMet} with the ribosomes was monitored by FRET between two fluorescent reporter groups (Milon et al., 2007). Fluorescein attached either to 4-thioU8 of the initiator tRNA [fMet-tRNA^{fMet}(Flu)] or to the 3′ terminus of mRNA [mRNA(Flu)] was used as fluorescence donor in combination with Alexa 555, which served as acceptor and was introduced at a cysteine residue engineered at position 4 of IF1 (IF1Alx) or at position 166 of IF3 (IF3Alx), whose native Cys65 was replaced by serine IF3(C65S/E166C). Alternatively, IF3(C65S/E166C) was labeled with Alexa 488 as FRET donor and IF1 with Alexa 555 as an acceptor. Engineering the cysteines in IF3 and fluorescence labeling of IF3 and IF1 did not affect the functional activity of the factors (see Figure S1 available online).

Control experiments showed that FRET signals were observed only when donor and acceptor were simultaneously present on the 30S subunit and that signal changes were very small when either donor or acceptor alone was present (Figure 1B and data not shown). Upon addition of 50S subunits to 30S IC, the efficiency of FRET between the labels in fMet-tRNA^{fMet} and IF3 (Figure 1B, D + A) or mRNA and IF3 (Figure 1C) decreased. As both fMet-tRNA^{fMet} and mRNA are tightly bound to the resulting 70S IC, the decrease of FRET reflects the dissociation of IF3 from the complex. No decrease of FRET between the labels in mRNA and IF3 was observed upon binding of fMet-tRNA^{fMet} to 30S subunits in complex with mRNA and IFs (Figure 1C), indicating that binding of the initiator tRNA alone, in the absence of the 50S subunit, does not cause any significant dissociation of IF3.

Effect of the TIR on Subunit Joining and IF3 Dissociation

The influence of structural elements of the TIR in the mRNA on the initiation efficiency was studied using three model mRNAs (Table S1): m022 containing a four-nucleotide (4 nt) SD sequence and a 9 nt spacer between the SD and the AUG start codon, m002 with a 9 nt SD sequence and a 5 nt spacer, and m003 lacking a SD sequence (La Teana et al., 1993; Brandi et al., 2007). Based on secondary structure predictions, these mRNAs are not expected to have a significant secondary structure in the TIR. In the presence of mRNA, >85% of the 30S subunits were found to bind f[^{3}H]Met-tRNA^{fMet} as measured by nitrocellulose filtration. Upon mixing 50S subunits with 30S ICs, subunit joining can be monitored by an increase in light scattering (Figure 2A). The signal increase was biphasic with any of the mRNAs tested. With m003 or m022 mRNAs, the predominant phase (>80%) had an apparent rate constant of 5 s^{-1} (Table S2). In contrast, the time course with m002 was dominated by a very slow step, 0.03 s^{-1}, which accounted for 75% of the total amplitude (Figure 2A).

When the dissociation of IF3 was monitored by decrease of the FRET efficiency between fMet-tRNA^{fMet}(Flu) and IF3(Alx), the time courses had two exponential terms, the dominant of which comprised >75% of the signal change in all cases (Figure 2B). The rate of IF3 dissociation differed dramatically depending on the mRNA in the 30S IC. While the rate of dissociation was 2 s^{-1} for 30S IC with m003 or m022 mRNAs, it was almost two orders of magnitude slower (0.03 s^{-1}) with m002. The steps with small amplitude most likely reflected a conformational heterogeneity of the 30S IC caused by IF1 (see below).

The 50S subunit association with 30S IC and IF3 dissociation was analyzed not only with model mRNAs but also with natural *E. coli* mRNAs (Table S1). The mRNAs selected for this purpose

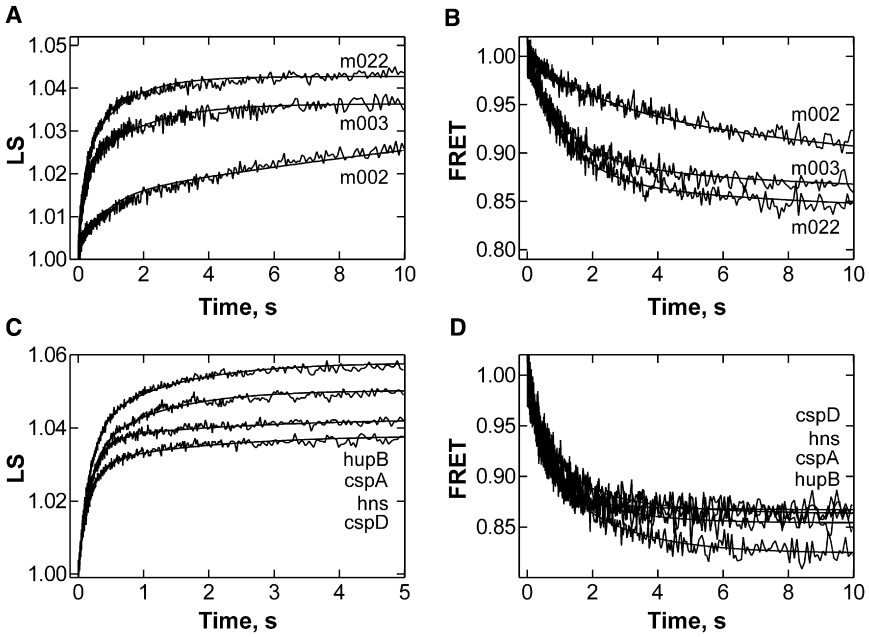

Figure 2. Effect of TIR on 70S IC Formation

(A) 70S IC formation from 30S IC on different model mRNAs (m003, m022, or m002). 50S subunit joining was monitored by light scattering.

(B) Dissociation of IF3 upon formation of 70S IC with m003, m022, or m002 mRNAs. FRET between fMet-tRNA^{fMet}(Flu) and IF3(Alx) was measured.

(C) 50S subunit joining with natural mRNAs (hupB, cspA, hns, and cspD). Light scattering was measured as in (A).

(D) IF3 dissociation upon formation of 70S IC with natural mRNAs. FRET was measured as in (B).

encoded proteins CspA, CspD, HNS, and HUa. With these mRNAs, the association of the 50S subunit with the 30S IC was rapid, with a rate of 5 s^{-1} for the dominant step (>80% of the total signal change) (Figure 2C). The rate of IF3 release was about 2 s^{-1} (Figure 2D). Thus, the rates of 70S IC formation were equally rapid with 30S ICs containing natural mRNAs and the model mRNAs m022 and m003 and much slower with 30S IC containing m002 mRNA. This indicates that the properties of the TIR influence the rates of 50S subunit association and IF3 release from the 70S complex.

To better compare the rates of 50S subunit joining and IF3 dissociation with different mRNAs, we measured the concentration dependencies of the apparent rate constants (k_{app}) of subunit association and factor dissociation in the presence of m022 (Figures 3A and 3B) or m002 (Figures 3C and 3D) mRNA. The k_{app} values of 50S subunit association with 30S IC with m022 were found to increase linearly with the 50S concentration. The slope of the linear dependence yielded association rate constants of $k_1 = 15 \pm 2\ \mu M^{-1}s^{-1}$ in the presence of IF3 and $80 \pm 5\ \mu M^{-1}s^{-1}$ in the absence of IF3 (Figure 3A, inset). In the absence of IF3, the rate of subunit joining was independent of the type of mRNA used (data not shown). The rate constant of the reverse reaction, k_{-1}, indicated by the small y axis intercept, was close to zero with and without IF3. The k_{app} values of IF3 dissociation monitored by the decrease in FRET efficiency between fMet-tRNA^{fMet} and IF3 saturated at high 50S concentrations, as expected for a first-order dissociation step (Figure 3B, inset). From the saturation of k_{app2}, the rate constant of IF3 dissociation was calculated: $k_2 = 3.2 \pm 0.3\ s^{-1}$. Because identical apparent rate constants

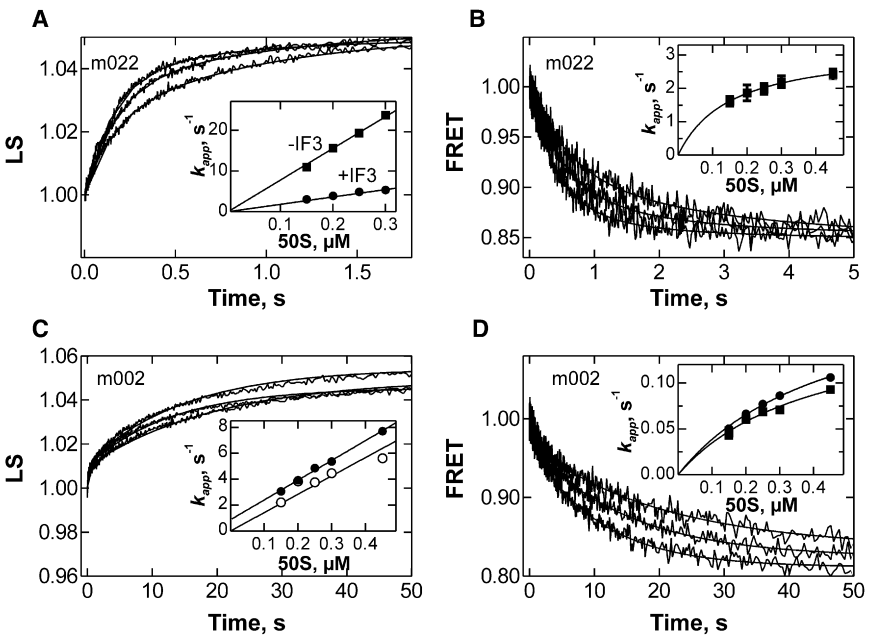

Figure 3. 50S Subunit Joining and IF3 Release from ICs with Different mRNAs

(A) Time courses of 50S subunit joining with 30S IC formed with m022 mRNA (0.05 μM) at different 50S concentrations: 0.15 μM (lower trace), 0.25 μM (middle trace), and 0.45 μM (upper trace). (Inset) Concentration dependencies of k_{app} in the absence (squares) or presence (circles) of IF3.

(B) Time courses of IF3 dissociation upon formation of 70S IC with m022 mRNA at 0.15 μM (upper trace), 0.25 μM (middle trace), and 0.45 μM (lower trace) of 50S subunits. (Inset) Concentration dependence of k_{app} (squares) from IC with m022. Error bars represent standard deviations.

(C) Same as in (A) but for 30S IC formed with m002 mRNA. (Inset) Comparison of the apparent association rate constants obtained with m022 (closed circles) and the fast step observed with m002 (open circles).

(D) Same as in (B) but with m002 mRNA. (Inset) Concentration dependencies of the apparent rate constants of the predominant phase of subunit association (circles) and IF3 release (squares).

for subunit association and IF3 dissociation were determined for all mRNAs except m002 (see below), the constants are probably valid also for the natural mRNAs tested in this work (Figures 2C and 2D).

When the association of the 50S subunit with the 30S IC was measured in the presence of m002 mRNA, the slow step was found to be dominant while the fast step had a rather small amplitude (20% of the total signal change) (Figure 3C). The k_{app} of the fast step increased linearly with the 50S subunit concentration, indicating that it reflected a bimolecular association step, and the values were about the same as observed with 30S IC(m022) (Figure 3C, inset). In contrast, the apparent rate constant of the dominant slow step (Figure 3D, inset) deviated significantly from linearity, suggesting that a first-order reaction (e.g., a conformational rearrangement following binding or the dissociation of the factors) limited the rate. The rate constant of the slow step was estimated by hyperbolic fitting to 0.22 ± 0.02 s^{-1} at saturating concentrations of 50S subunits (Figure 3D, inset). Essentially the same concentration dependence and rate constant at saturation were observed when IF3 dissociation was monitored by FRET (Figure 3D, inset), indicating that IF3 release from ICs was 15-fold slower on m002 mRNA compared to m022 or natural mRNAs.

The Role of IF1

To test whether IF1, in addition to IF3, is involved in TIR selection, joining of the 50S subunit to 30S ICs with m022 or m002, as well as IF3 dissociation during the formation of the corresponding 70S complexes, was monitored in the absence of IF1 (Figure 4A). When IF1 was omitted, 50S subunit joining to 30S IC was equally rapid with both mRNAs. The rate of 50S binding to 30S IC with m002 was increased nearly 200-fold (from 0.03 s^{-1} to 5.3 s^{-1}; Table S2), and IF3 dissociation was found to be 100-fold faster than in the presence of IF1 (from 0.03 s^{-1} to 2.6 s^{-1}). These data suggest that IF1, in addition to IF3, plays an important role in determining the rates of subunit joining and IF3 dissociation, and the effect of IF1 depends on the nature of the TIR. Furthermore, when IF1 was omitted, the two-exponential kinetic behavior was no longer observed. This suggests that the kinetic heterogeneity observed in the experiments with IF1 reflects conformational subpopulations of the 30S IC that are introduced by IF1.

Conformation of the 30S IC

Next, we tested whether the conformation of the 30S IC influences the recognition of the TIR during 70S IC formation. In order to interfere with conformational changes of the 30S subunit, we have used streptomycin, which binds between several helices of 16S rRNA and protein S12 and immobilizes the shoulder relative to the central part of the subunit (Carter et al., 2000). Streptomycin binding did not impair the formation of 30S IC or the stability of fMet-tRNA^{fMet} binding but decreased the affinity of IF1 for the 30S IC ~2-fold (data not shown). To ensure complete occupancy of 30S IC with IF1, the kinetics of 50S subunit joining and IF3 release was monitored at three concentrations of IF1 (Figure 4B), and the simultaneous presence of the two factors on the 30S IC was verified by FRET between IF3 to IF1. In the presence of streptomycin, the kinetic heterogeneity was enhanced

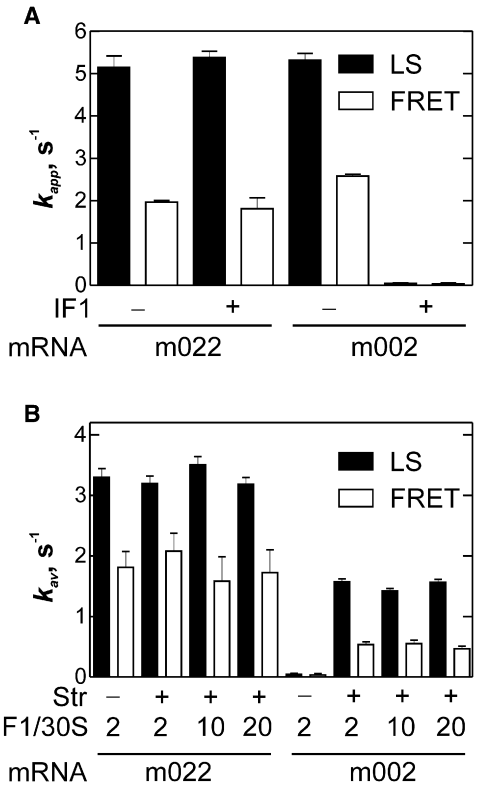

Figure 4. Effect of IF1 and Streptomycin on 70S IC Formation

(A) Effect of IF1 omission on the rates of 50S subunit joining (black bars) and IF3 release (white bars). IF3 dissociation was monitored by FRET between fMet-tRNA^{fMet}(Flu) and IF3(Alx). Error bars represent standard deviations.

(B) Effect of streptomycin on the rates of 50S subunit joining (black bars) and IF3 release (white bars) at different concentrations of IF1.

(Figure S2). For a better comparison, we analyzed average rate constants, k_{av}, corresponding to the observed reaction half-life (see Supplemental Data), rather than k_{app} values of individual exponentials. Subunit association and IF3 dissociation were not affected by streptomycin with 30S IC containing m022 but were accelerated considerably by the antibiotic when the 30S IC was formed with m002 (Figure 4B).

The Choice of the Initiation Codon

To study initiation codon selection, we compared the rates of subunit association and IF3 dissociation using 30S ICs formed with m022 or m002 mRNAs containing either the canonical AUG or the noncanonical AUU start codon. With m022 AUG mRNA, subunit joining and IF3 dissociation were rapid, as described above (Figures 5A and 5B). In contrast, with m022 AUU, both 50S subunit association and IF3 dissociation were very slow (0.06 s^{-1}; Table S2). The rates of these reactions were independent of the 50S subunit concentration (Figures 5C and 5D), suggesting that a first-order reaction (i.e., a conformational rearrangement) was rate limiting. The time course of subunit association comprised a rapid phase with a small amplitude (~20% of total) (Figure 5C), similar to that observed with m002 (Figure 3C), which may indicate the formation of an initial

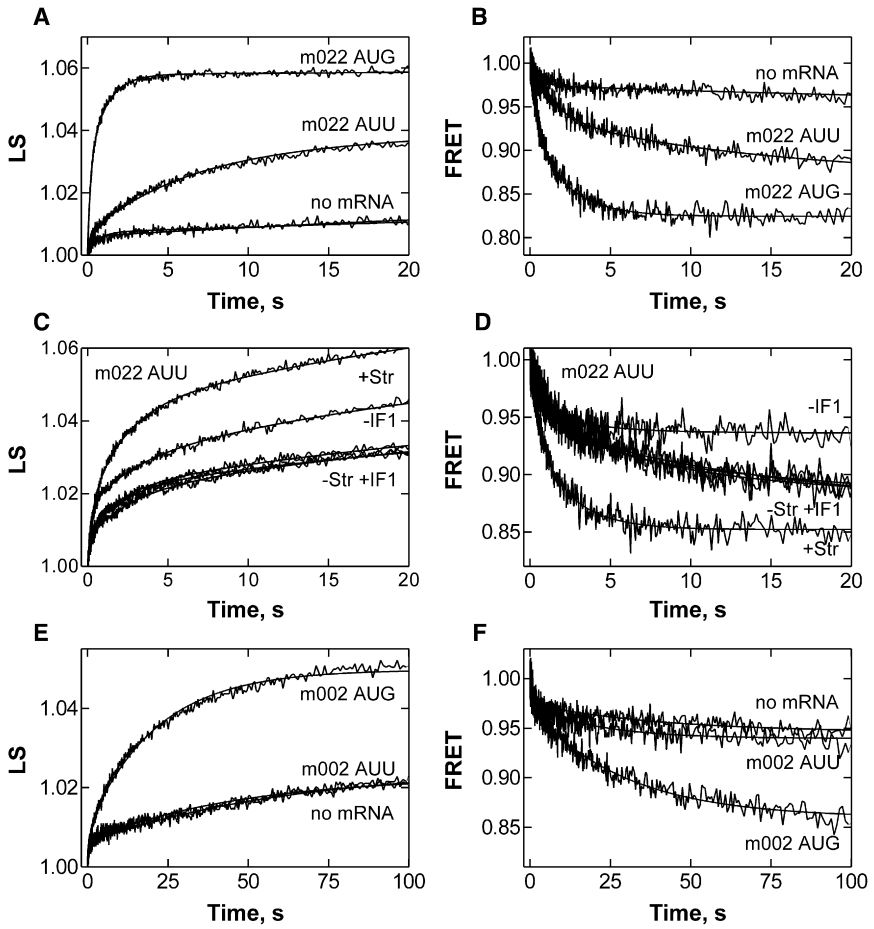

Figure 5. Selection of the Initiation Codon

(A) Time courses of 50S subunit association with 30S IC formed with m022 mRNA with AUG and AUU initiation codons or in the absence of mRNA.

(B) IF3 dissociation from complexes formed with m022 mRNA with AUG and AUU initiation codons or in the absence of mRNA.

(C) Influence of streptomycin (+Str), IF1 omission (−IF1), and 50S concentration (−Str +IF1) on 50S subunit association with 30S IC containing m022 AUU. Traces obtained at different 50S concentrations were indistinguishable (traces obtained with 0.15 μM, 0.25 μM, and 0.45 μM 50S subunits are shown).

(D) Influence of streptomycin (+Str), IF1 omission (−IF1), and 50S concentration (−Str +IF1) on IF3 release upon formation of 70S IC in the presence of m022 AUU. Traces obtained at different 50S concentrations were indistinguishable (concentrations of the 50S subunits as in C).

(E) and (F) Same as panels (A) and (B), respectively, with m002 mRNA.

unstable 70S complex. Based on the amplitude of FRET between fMet-tRNA^{fMet}(Flu) and IF3(Alx) (Figure 5B), the extent of 70S IC formation with the AUU codon was 25% lower compared to that formed with the AUG codon, indicating that 25% less 30S IC was formed on AUU. However, nitrocellulose filtration indicated that only about 25% of the 30S subunits had fMet-tRNA^{fMet} bound (Figure S3), which reflects the fraction of fMet-tRNA^{fMet} that associated with 30S IC stably enough to remain bound during filtration. fMet-tRNA^{fMet} bound to 30S IC in an unstable manner may dissociate upon subunit association without forming a 70S IC.

With m022 AUU-containing 30S IC, subunit association and IF3 release were accelerated by streptomycin to levels comparable to those observed with m022 AUG mRNA (Figure 5). In the presence of streptomycin or in the absence of IF1, the portion of 30S subunits that bound 50S subunits rapidly was larger (Figure 5C) and IF3 dissociation was faster (Figure 5D) than without antibiotic or with IF1. In the absence of IF1, the extent of IF3 release was reduced, probably due to a reduced amount of fluorescent ligands (fMet-tRNA^{fMet} and/or IF3) bound to the 30S IC with m022 AUU mRNA.

With m002 mRNA, subunit joining and IF3 dissociation were slow when the start codon was AUG, and the reaction was essentially abolished with the noncanonical AUU (Figures 5E and 5F), in spite of the fact that the fraction of the 30S subunits carrying stably bound fMet-tRNA^{fMet} was the same with m022 and m002 mRNAs containing the AUU initiation codon (Figure S3).

IF3 Dissociation from the 30S Initiation Complex

Next, we asked whether the differences in the rates of IF3 dissociation observed with mRNAs that have different TIRs are due to different properties of the respective 30S ICs or different properties of the 70S complexes formed by 50S subunit binding. In the absence of the 50S subunit, IF3 has a very high affinity for the 30S IC and does not dissociate to any appreciable extent (Figure 1C, transient 1). Thus, to determine IF3 dissociation rate constants, chase experiments were performed by adding excess unlabeled IF3 to 30S ICs containing fMet-tRNA^{fMet}(Flu) or IF1(Alx) and IF3(Alx) and measuring the decrease of FRET due to dissociation of the labeled factor (Figure 6). The rate constants of IF3 dissociation from 30S IC were found to be ~4 s^{-1} (m022 AUG), 0.6 s^{-1} (m022 AUU), and 0.2 s^{-1} (m002 AUG). These values are somewhat higher than those found for the corresponding 70S complexes, suggesting that the binding of the 50S subunit does not accelerate the release of IF3. Rather, the rate of IF3 dissociation is determined by intrinsic properties of the 30S IC, and the association with the 50S subunit appears to delay IF3 dissociation. Addition of streptomycin or omission of IF1 increased the rate of IF3 dissociation from the 30S IC, indicating that IF1 and the conformation of the 30S subunit are important regulatory factors.

Transition to Translation

To determine whether the differences in the rates of 70S IC formation observed with different mRNAs are also reflected in the rates by which translation is initiated, the rates of fMetPhe

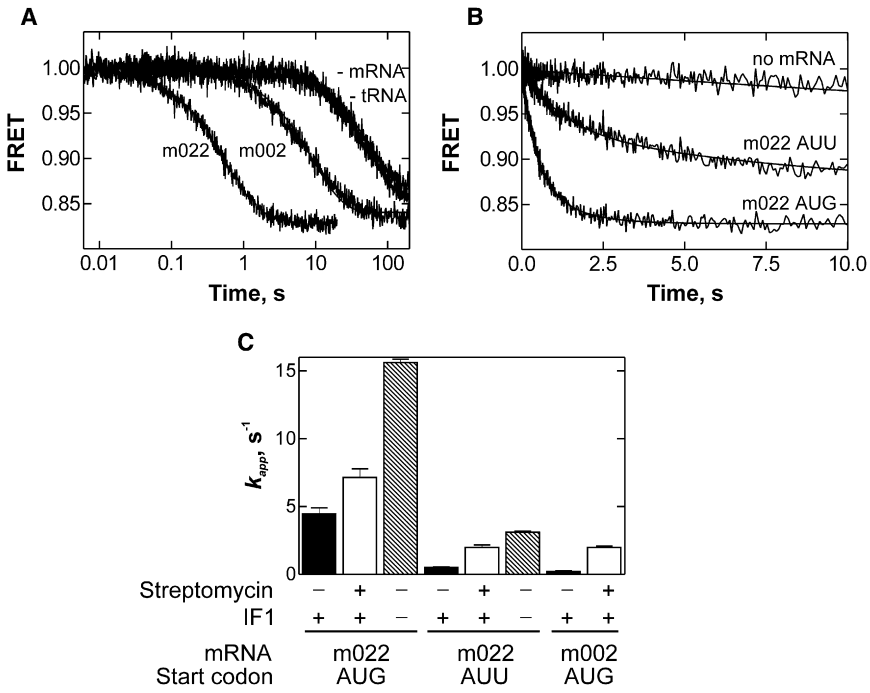

Figure 6. IF3 Dissociation from 30S IC

(A) Time courses of IF3(Alx) dissociation upon addition of excess unlabeled IF3 (1.2 μM) from 30S IC (0.1 μM) formed with m022 or m002 mRNA in the absence of mRNA or in the absence of initiator tRNA; the latter two curves are indistinguishable. FRET between IF3(Alx488) and IF1(Alx555) was monitored.

(B) Time courses of IF3 dissociation from 30S IC containing m022 AUG, m022 AUU, or in the absence of mRNA. FRET between IF3(Alx488) and IF1(Alx555) was monitored.

(C) Effect of TIR, initiation codon, addition of streptomycin, or omission of IF1 on IF3 dissociation from the 30S IC measured by FRET from fMet-tRNA^{fMet}(Flu) to IF3(Alx). Error bars represent standard deviations.

dipeptide formation were measured. With either m022 or m002 mRNA, the rate of dipeptide formation on preformed 70S ICs was about 2 ± 0.2 s^{-1} (Figure 7A), indicating that the nature of the mRNA per se does not influence the rate of peptide bond formation. When 30S ICs containing m022 AUG were rapidly mixed with 50S subunits and EF-Tu·GTP·Phe-tRNA^{Phe} ternary complex, the rate of dipeptide formation was 0.9 ± 0.1 s^{-1}. This rate should reflect the time required to form the 70S IC, which is determined by IF3 dissociation and takes place at a rate of about 2 s^{-1} (Figure 3B, inset) and for Phe-tRNA delivery and peptide bond formation on fully formed 70S IC, also at 2 s^{-1} (Figure 7A). Thus, the overall rate of peptide bond formation expected upon mixing 30S IC with 50S subunits and ternary complex is 1/(1/2 + 1/2) = 1 s^{-1}, in agreement with the measured value of 0.9 s^{-1}. The rate of dipeptide formation with 30S IC containing m002 AUG mRNA was 0.27 ± 0.07 s^{-1}, and the dipeptide appeared only after a substantial time delay (Figure 7A). However, when IF3 was omitted, the rate of dipeptide formation was increased to 0.8 ± 0.1 s^{-1}, which is almost as rapid as the reaction with 30S IC containing m022 AUG mRNA. It should be noted that the final extent of dipeptide formation was similar in all cases, indicating that the control of translation efficiency is predominantly kinetic and takes place during the transition to the 70S translating ribosomes, while the amount of potentially reactive 30S IC is the same with m022 and m002 mRNAs. With 30S IC containing m022 AUU mRNA and IF3, the rate of dipeptide formation was slow, 0.1 ± 0.01 s^{-1} (Figure 7B), a value very close to that found for 50S subunit joining and IF3 dissociation from these complexes. The reaction rate in the absence of IF3 was 0.6 ± 0.1 s^{-1}, i.e., almost the same as with the mRNA containing the AUG codon. The reaction in the presence of IF3 is clearly limited by steps on the pathway from 30S IC to 70S IC, because the intrinsic rate of peptide bond formation of the preformed 70S IC with m022 AUU mRNA was 2.2 ± 0.2 s^{-1}. In addition, the final level of dipeptide formation on AUU was generally lower than on the AUG codon or when IF3 was absent. This can be explained by the weaker binding of fMet-tRNA^{fMet} on AUU in the presence of IF3, consistent with the small fraction (~25%) of 30S IC that had stably bound fMet-tRNA^{fMet}, as assessed by nitrocellulose filtration (Figure S3).

DISCUSSION

Selection of the TIR during Initiation

The present results suggest that the efficiency of initiation on a particular mRNA depends on the properties of the TIR and is controlled at two stages. During the first stage, i.e., the formation of the 30S IC, parameters such as the secondary structure of the TIR, the strength of the SD-ASD interaction, and the quality of codon-anticodon interaction determine the efficiency of 30S IC formation. In the cell, the first step of mRNA selection is likely to operate when mRNAs with different TIRs compete for 30S subunits (Calogero et al., 1988). The efficiency of the first selection step is probably not very high, because all mRNAs, even those with a significant secondary structure around the start codon or a weak SD-ASD interaction, exhibit fast association and slow dissociation kinetics and form stable 30S ICs (Studer and Joseph, 2006), while the presence of a noncanonical initiation codon decreases the level of stable 30S IC formation by up to a factor of five (Figure S3).

The second important step, which modulates the efficiency of mRNA selection, entails the kinetic control of the transition from the 30S IC to the translating 70S ribosome. The present work suggests that this type of control accounts for variations in mRNA translation depending on both the SD region and the nature of the initiation codon. Those mRNAs that contain a favorable TIR enter translation more efficiently, because the conformation of the 30S IC favors rapid binding of the 50S subunit and dissociation of IF3, which, in turn, leads to the rapid formation of the functional 70S IC that enters elongation. In contrast,

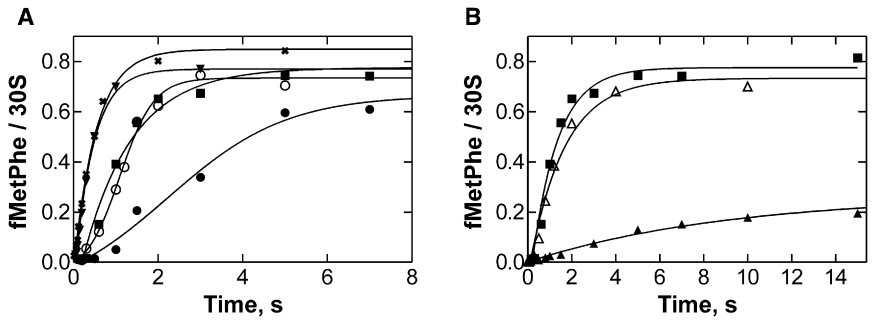

Figure 7. Formation of fMetPhe Dipeptide

(A) Time courses measured with 70S IC with m022 (triangles) or m002 (exes), 30S IC with m022 (closed squares) or m002 (closed circles), and mRNA prepared with all three IFs or with m002 mRNA in the absence of IF3 (open circles). Reactions were initiated by mixing 30S IC or 70S IC (0.1 μM) with EF-Tu·GTP·Phe-tRNA^{Phe} ternary complexes (0.4 μM) and 50S subunits (0.3 μM) in a quench-flow apparatus.

(B) Time courses measured with 30S IC with m022 AUU mRNA formed in the presence of IF3 (closed triangles) and in the absence of the factor (open triangles) compared to the reaction with m022 AUG (closed squares). Concentrations of components were as indicated in (A).

those 30S ICs that contain an mRNA with unfavorable TIR or are formed on a noncanonical initiation codon appear to assume a conformation that does not allow for rapid adjustment of the subunits. The dissociation of IF3 from these complexes is slow, thereby inhibiting the transition from initiation to elongation. This type of selection is effective also for mRNAs present in high concentrations and is not affected by competition between mRNAs. The present data indicate that variations in the rate of 70S IC formation could account for a 10-fold difference in the efficiency of translation of different mRNAs, consistent with the average differences of translation efficiency observed in vivo.

The Mechanism of TIR Sensing upon 70S Formation

Sensing the structure of the TIR depends on the presence of IF3 and IF1. In principle, IF3, which binds at the platform of the 30S subunit, could directly act as a sensor for the TIR by responding to conformational changes induced by the SD-ASD interaction or to misalignments of contacts between the ribosome, tRNA, and IF2 that may be caused by mismatches between the initiation codon and the anticodon of the initiator tRNA. By contrast, IF1 binds at the A site on the 30S subunit, which is distant from both the P site and the SD-ASD duplex. The conformation of the 30S subunit induced by the binding of IF1 and IF3 (Shapkina et al., 2000; Carter et al., 2001) may be crucial for the discrimination between different mRNAs in the transition from initiation to elongation. The conformation of the 30S IC that binds the 50S subunit rapidly is predominant with m022 mRNA in the presence of IF1 or with any mRNA in the absence of IF1, but it is found in only a minor fraction of the ribosomes with m002 mRNA in the presence of IF1 or with mRNAs that have AUU as a start codon. In the absence of IF1 or when streptomycin had stabilized a particular conformation of the 30S subunit, sensing of the unfavorable TIR was impaired, whereas the rates of 70S IC formation with an mRNA optimal for initiation, such as m022 mRNA, did not change. This may indicate, somewhat unexpectedly, that in the presence of all three IFs, fMet-tRNA^{fMet}, and an optimal TIR, the conformation of 30S IC may be similar to that favored in the absence of IF1. The IF1-induced rearrangement of the 30S subunit into a conformation that is unfavorable for further reaction is likely to be transient and to take place during the early stages of initiation; it should be reversed when the 30S IC is formed on an mRNA with favorable TIR and canonical AUG. In contrast, in complexes with unfavorable TIR or noncanonical initiation codon, the conformation induced by IF1 persists and impairs initiation.

The nature of the signals that induce the presumed 30S IC rearrangements is unknown. The formation of the productive conformation probably entails mRNA movements relative to the 30S subunit (La Teana et al., 1995; Yusupova et al., 2006). This would explain why m002 mRNA, which is capable of forming an extended SD-ASD interaction and has a short spacer between SD sequence and initiation codon, is impaired in undergoing these rearrangements and forming a 30S IC capable of rapidly entering the 70S IC pool. A strong SD-ASD interaction and a short spacer between SD sequence and initiation codon may additionally stabilize the IF1-induced conformation of the 30S subunit, which would normally be switched back when the correct codon-anticodon interaction is established. On the other hand, a noncanonical codon-anticodon pairing may either be thermodynamically insufficient to induce the release of the IF1-induced 30S subunit conformation or result in a position of the tRNA that stabilizes the IF1-induced conformation. The 50S subunit may bind to any type of the 30S IC; however, it requires a particular conformation of the 30S IC to start forming the intersubunit bridges, and this process seems to be slow when the structure of the 30S IC is unfavorable. Thus, the efficiency with which an mRNA enters the pool of translating ribosomes is controlled by the conformation of the 30S IC, while the 50S subunit appears to be a sensor that provides the irreversibility of the reaction.

EXPERIMENTAL PROCEDURES

Biochemical Methods

All measurements were performed in buffer A (50 mM Tris-HCl [pH 7.5], 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$) at 20°C. Ribosomal subunits were prepared from purified 70S ribosomes (Rodnina and Wintermeyer, 1995; Milon et al., 2007) by sucrose gradient centrifugation in a zonal rotor (Ti 15, Beckman); the activity of reassociated 30S and 50S subunits was >95% in fMet-tRNA^{fMet} binding and peptide bond formation. 30S subunits were reactivated in buffer A with 20 mM $MgCl_2$ for 1 hr at 37°C. fMet-tRNA^{fMet} was purified by HPLC (Milon et al., 2007) and was 95% aminoacylated and formylated. mRNA was prepared by T7 RNA polymerase transcription or purchased from Dharmacon (USA) or Curevac (Germany). Preparation of single-cysteine mutants of IF1 and IF3 and fluorescence labeling of all components was carried out as described by Milon et al. (2007). EF-Tu, [^{14}C]Phe-tRNA, and the ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} were prepared as described by Rodnina et al. (1994), except that the ternary complex was used without

purification. 30S ICs were formed by incubating 30S subunits (0.1 μM), IF1 (0.2 μM), IF2 (0.2 μM), IF3 (0.2 μM), f[^{3}Met]-tRNA^{fMet} (0.2 μM), mRNA (0.8 μM), and GTP (0.25 mM) in buffer A for 30 min at 37°C. When the effect of IF1 omission was studied, 30S ICs were prepared in the same way, except that IF1 was omitted and IF2 was added in 4-fold excess over 30S subunits. The extent of f[^{3}H]Met-tRNA^{fMet} binding to the 30S subunit was measured by nitrocellulose filtration.

Kinetic Experiments

Fluorescence stopped-flow measurements were performed using a SX-18MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK). Experiments were carried out by rapidly mixing equal volumes (60 μl each) of reactants, typically containing 30S IC (0.1 μM) and 50S (0.3 μM), respectively, at 20°C. In a single experiment, 1000 data points were acquired in logarithmic sampling mode. Excitation wavelengths were 465 nm for fluorescein and Alx488. Fluorescence emission from FRET was measured after passing a KV590 cutoff filter (Schott). To measure light scattering, the excitation wavelength was set to 430 nm, and the scattered light was measured at an angle of 90° to the incident beam without a filter. The experiments were carried out at pseudofirst order conditions, i.e., at least at a 3-fold excess of 50S subunits over 30S ICs. Data were evaluated by fitting the exponential function $F = F_\infty + A \times \exp(-k_{app} \times t)$ with a characteristic time constant, k_{app}; the amplitude of the signal change, A; the final signal, F_∞; and the fluorescence at time t, F. If necessary, additional exponential terms were included. Calculations were performed using TableCurve software (Jandel Scientific) or Prism (Graphpad Software). Standard deviations of all values were calculated from seven to ten time courses. To determine average rate constants, k_{av}, which are the inverse of the time at which a reaction reached 50% completion, all k_{app} values and their respective amplitudes were taken into account. The weighted average was calculated as $k_{av} = (k_{app1} \times A_1 + k_{app2} \times A_2)/(A_1 + A_2)$, where k_{app1} and k_{app2} and A_1 and A_2 are the apparent rate constants and the respective amplitudes estimated from two-exponential fitting of the time courses.

Dipeptide formation was measured in a quench-flow apparatus (KinTek RQF-3). 30S ICs containing f[^{3}H]Met-tRNA^{fMet} were rapidly mixed with 50S subunits and EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} ternary complex (14 μl each), and the reaction was stopped with 0.5 M KOH. The amount of fMetPhe dipeptide was analyzed by HPLC as described by Tomsic et al. (2000). Rate constants were determined by single-exponential fitting; when a significant delay in dipeptide formation was observed, the rate constant was derived by numerical integration using Scientist software (Micromath, St. Louis, MO).

SUPPLEMENTAL DATA

The Supplemental Data for this article, which include Supplemental Figures and Tables, can be found online at: http://www.molecule.org/cgi/content/full/30/6/712/DC1/.

ACKNOWLEDGMENTS

We thank Wolfgang Wintermeyer for critically reading the manuscript and Carmen Schillings, Astrid Böhm, Simone Möbitz, and Petra Striebeck for expert technical assistance. The work was supported by grants of the Deutsche Forschungsgemeinschaft (M.V.R) and Italian MIUR (C.O.G, PRIN 2005).

Received: September 3, 2007
Revised: December 7, 2007
Accepted: April 16, 2008
Published: June 19, 2008

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