

## REVIEW ARTICLE

# Kinetic control of translation initiation in bacteria

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### Abstract

Translation initiation is a crucial step of protein synthesis which largely defines how the composition of the cellular transcriptome is converted to the proteome and controls the response and adaptation to environmental stimuli. The efficiency of translation of individual mRNAs, and hence the basal shape of the proteome, is defined by the structures of the mRNA translation initiation regions. Initiation efficiency can be regulated by small molecules, proteins, or antisense RNAs, underscoring its importance in translational control. Although initiation has been studied in bacteria for decades, many aspects remain poorly understood. Recent evidence has suggested an unexpected diversity of pathways by which mRNAs can be recruited to the bacterial ribosome, the importance of structural dynamics of initiation intermediates, and the complexity of checkpoints for mRNA selection. In this review, we discuss how the ribosome shapes the landscape of translation initiation by non-linear kinetic processing of the transcriptome information. We summarize the major pathways by which mRNAs enter the ribosome depending on the structure of their 5' untranslated regions, the assembly and the structure of initiation intermediates, the individual and synergistic roles of initiation factors, and the mechanisms of mRNA and initiator tRNA selection.

**Keywords:** protein synthesis, translation initiation, ribosome function, mRNA selection, regulation of gene expression, RNA, RNA-protein complex assembly, translation initiation factors

## Overview of translation initiation

During initiation, the ribosome, assisted by initiation factors, recruits the mRNA to be translated and defines the reading frame by identifying a start codon. Initiation is often referred to as the rate-limiting step of translation (Jacques & Dreyfus, 1990; Laursen *et al.*, 2005; Kudla *et al.*, 2009); however, other reports suggested that initiation is rapid compared to the elongation phase of protein synthesis (Mitarai *et al.*, 2008). In bacteria, newly synthesized mRNA can be recruited into translation while still being transcribed. However the predominant mRNA species is the full-length transcript, which is apparent from the quantitative RNA sequencing of an entire bacterial transcriptome (Passalacqua *et al.*, 2009). Furthermore, the duration of transcription elongation, and hence the phase at which coupled transcription-translation is possible, is much shorter (about 25 s for a 1 kb gene; (Proshkin *et al.*, 2010)) than the known mRNA lifetimes (between 4 and 11 min for *Escherichia coli* transcripts (Bernstein *et al.*, 2002)), indicating that a bulk of mRNAs are translated post-transcriptionally. Usually

one mRNA is translated by multiple ribosomes, which successively load on the mRNA with an interval of 2–4 s to form polysomes (Mitarai *et al.*, 2008). At conditions where the availability of ribosomes is limited, assembly of the initiation complex may depend on the efficient ribosome recycling, which is facilitated by the ribosome recycling factor (RRF) and elongation factor G (EF-G). As the recovery of ribosomes after termination of translation on a given mRNA is quite slow ( $<1 \text{ s}^{-1}$  measured with components from *E. coli* (Karimi *et al.*, 1999; Peske *et al.*, 2005)), ribosome recycling may limit the effective rate of initiation. Exactly which step of translation is rate-limiting for the translation of a given mRNA may depend on the efficiency of its translation initiation region (TIR), the codon usage along the coding sequence, and growth conditions.

In bacteria, translation initiation requires the action of three initiation factors, IF1, IF2, and IF3, and the initiator tRNA, fMet-tRNA<sup>fMet</sup>. Initiation proceeds through three main phases (Figure 1). During the first phase, mRNA, IFs, and fMet-tRNA<sup>fMet</sup> bind to the small (30S)

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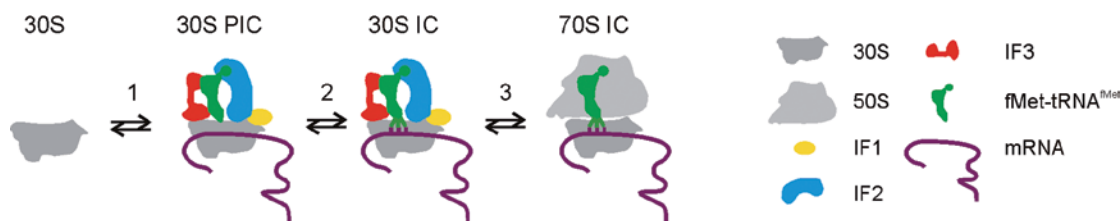


Figure 1. Schematic of the three main phases of translation initiation. Phase 1: Assembly of the 30S PIC upon recruitment of initiation factors, mRNA, and fMet-tRNA<sup>Met</sup> to the 30S subunit. Phase 2: Conversion of 30S PIC to 30S IC after start codon recognition by fMet-tRNA<sup>Met</sup>. Phase 3: Formation of the 70S IC following 50S subunit joining and release of initiation factors. (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

ribosomal subunit to assemble into a transient 30S pre-initiation complex (30S PIC). The 30S PIC is converted to a functional 30S initiation complex (30S IC) when the interaction between the start codon and the anticodon of fMet-tRNA<sup>Met</sup> in the P site is established. At the last phase, the large (50S) ribosomal subunit joins the 30S IC forming the 70S initiation complex (70S IC), which is poised for the translation of the selected mRNA. Each of the three phases entails a number of distinct elemental steps, providing a complex network of interactions that contribute to translational control of gene expression and prevent the synthesis of aberrant polypeptides due to out-of-frame mRNA decoding.

## Initiation machinery

In recent years, a number of structures of 30S and 70S ICs have been reported which revealed the positions and orientations of the initiation components. Bioinformatics provided unprecedented insights into the diversity of mRNAs in different organisms. Rapid kinetics and single-molecule FRET studies unraveled the timing of events and the molecular choreography of initiation. In this chapter, we summarize recent advances in structural and functional studies of IF1, IF2, IF3 and the mRNA recognition elements that define the start codon selection. The descriptions of structures of the ribosomal subunits and fMet-tRNA<sup>Met</sup> can be found in earlier reviews (Laursen *et al.*, 2005; Allen & Frank, 2007; Myasnikov *et al.*, 2009; Simonetti *et al.*, 2009).

### Initiation factors

IF1 is a small (71 amino acid residues) basic protein comprised of a single domain that is formed by a five-stranded  $\beta$ -barrel belonging to the OB-fold family of proteins (Sette *et al.*, 1997) (Figure 2A). The factor binds at the A site of the 30S subunit in the vicinity of ribosomal protein S12, the 530 loop and helix 44 of 16S rRNA (Carter *et al.*, 2001). IF1 stabilizes the binding of IF2 and IF3 to the 30S subunit and modulates the selection of mRNA and fMet-tRNA<sup>Met</sup> by controlling the conformational dynamics of the 30S subunit (see below) (Gualerzi *et al.*, 1977; Milon *et al.*, 2008; Milon *et al.*, 2010).

IF2 is a multi-domain GTPase that plays a key role in the recruitment of fMet-tRNA<sup>Met</sup> to the 30S PIC and in regulating the formation of the 70S IC. Based on the

results of partial proteolysis and sequence alignments, the structure of IF2 was divided in the N-terminal domain (N-domain, further divided into subdomains N1 and N2), the GTP-binding domain (G-domain, consisting of G1, G2, and G3), and the C-terminal domain (C-domain, subdomains C1 and C2) (Figure 2B). Subdomains G2, G3, C1, and C2 are highly conserved. G2 is the GTP-binding subdomain of IF2, whereas G3 is characteristic for all translational GTPases and is homologous to domain 2 of EF-Tu and EF-G. C2 contains the binding site for the 3'-end and the fMet moiety of fMet-tRNA<sup>Met</sup> (Guenneugues *et al.*, 2000). The N-domain may act as an additional anchor for IF2 on the 30S subunit (Moreno *et al.*, 1999; Caserta *et al.*, 2006; Julian *et al.*, 2011). The subdomains N1, N2, and G1 vary both in amino acid composition and length among different bacterial species and are absent in archaea (Roll-Mecak *et al.*, 2000).

The crystal structure of a full-length bacterial IF2 is not known so far. The structures of the C1 and C2 subdomains and of a small fragment of the N-domain are available (Meunier *et al.*, 2000; Laursen *et al.*, 2003; Wienk *et al.*, 2005). Recently, the structure of the G2 subdomain in the apo form and in complex with GDP has been solved by NMR (Wienk *et al.*, 2012). Crystals of *T. thermophilus* IF2 have been reported (Simonetti *et al.*, 2011), but the structure has not been released so far (as of January 2012). Otherwise, the knowledge on the IF2 structure is limited to homology models (Allen *et al.*, 2005; Simonetti *et al.*, 2009) derived from the crystal structure of the archaeal ortholog, eIF5B, from *Methanobacterium thermoautotrophicum* (Roll-Mecak *et al.*, 2000). Recently, the structure of full-length *E. coli* IF2 was modeled using the density attributed to the factor in the cryo-EM structure of the *E. coli* 30S IC (Julian *et al.*, 2011) (Figure 2B). The model suggests that the three N-terminal subdomains in IF2 fold into compact structures, rather than being disordered as thought previously (Allen & Frank, 2007). On the 30S subunit, the N domain of IF2 contacts IF1 and S12 (Julian *et al.*, 2011), in agreement with the biochemical evidence suggesting that the isolated N-domain of *E. coli* IF2 can bind to the 30S subunit (Mortensen *et al.*, 1998; Moreno *et al.*, 1999).

For a GTP-binding protein, IF2 is quite unusual. The affinity of GTP and GDP binding to IF2 is low, ranging between 1  $\mu$ M (GDP) (Haurlyliuk *et al.*, 2009) and 10–40  $\mu$ M (GTP) (Milon *et al.*, 2006; Haurlyliuk *et al.*, 2009).

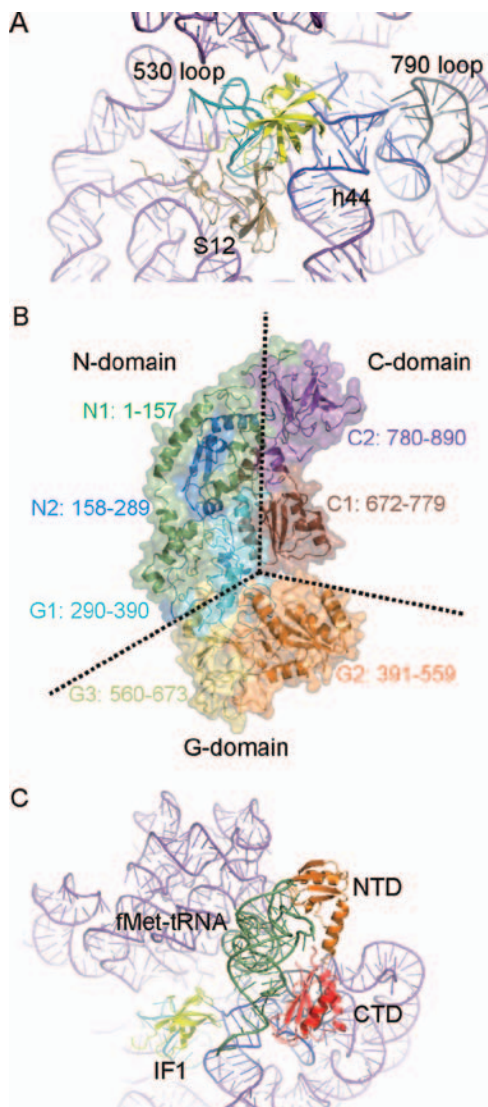


Figure 2. Structures of the initiation factors bound to the 30S subunit. (A) IF1 (yellow; PDB 1AH9) bound at the A site of the 30S subunit in the vicinity of the ribosomal protein S12 (sand), the 530 loop (cyan) and helix 44 (blue) of 16S rRNA (purple). The 790 loop (gray) is the binding site of IF3 (adapted from (Carter *et al.*, 2001; Julian *et al.*, 2011); PDB 1HRO). (B) Model of full-length *E. coli* IF2 in the 30S IC (adapted from (Julian *et al.*, 2011)). IF2 subdomains are indicated; dotted lines mark the boundaries between the N-terminal domains, the G2/G3 functional unit conserved in all translational GTPases, and the C-terminal domains of the protein. (C) IF3 in the 30S IC (adapted from (Julian *et al.*, 2011)). IF3-NTD (orange; PDB 1TIF) contacts the elbow region of fMet-tRNA<sup>Met</sup> (green, PDB 2FMT). IF3-CTD (red, PDB 2IFE) is bound to the 790 loop of 16S rRNA. The position of IF1 (yellow) is also indicated. (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

Nucleotide exchange is spontaneous due to the high rate of nucleotide dissociation (Milon *et al.*, 2006). Isothermal titration calorimetry and small-angle X-ray scattering analyses suggested that IF2 changes conformation when going from ligand-free to the GDPNP- or GDP-bound forms (Pon *et al.*, 1985; Haurlyuk *et al.*, 2009; Vohlander Rasmussen *et al.*, 2011), although the magnitude of these changes remains uncertain. IF2 binds fMet-tRNA<sup>Met</sup> with

micromolar affinity forming a labile complex that dissociates rapidly ((Milon *et al.*, 2010) and references therein). In contrast to other translation factors capable of forming complexes with tRNAs, such as EF-Tu, SelB, or eIF2, the formation of the IF2-fMet-tRNA<sup>Met</sup> complex does not require GTP (Mitkevich *et al.*, 2010), which would be quite unusual if the complex were the physiological vehicle for the delivery of the tRNA to the ribosome. In fact, the complex has to dissociate before the tRNA can be recruited to the 30S PIC (Milon *et al.*, 2010). Rather, the complex IF2-fMet-tRNA<sup>Met</sup> might be important as a storage reservoir to protect the tRNA from degradation before being recruited to the 30S PIC.

IF3 consists of two domains, the N-terminal (NTD) and C-terminal (CTD), connected by a linker (Figure 2C). The structures of the separate domains are available; however, their arrangement in full-length IF3 is not known. The N-terminal domain of IF3 has a globular  $\alpha/\beta$  fold, whereas the IF3-C domain is built of a two-layered  $\alpha/\beta$  sandwich (Biou *et al.*, 1995; Garcia *et al.*, 1995). Single-molecule FRET experiments with fluorescence reporter groups introduced in the IF3-NTD and IF3-CTD suggest that free IF3 fluctuates between two conformations, a closed one with the two domains packed against one another and an open conformation with the domains adopting an extended arrangement (D. Rodnin, P. Milon, C. Seidel, M. V. Rodnina, unpublished data). Binding of IF3 to the 30S subunit leads to the stabilization of the open conformation of IF3. The binding site for the factor is located at the platform of the 30S subunit (McCutcheon *et al.*, 1999; Dallas & Noller, 2001; Fabbretti *et al.*, 2007). Hydroxyl radical probing (Dallas & Noller, 2001), mutational analysis (Tapprich *et al.*, 1989), and a recent cryo-EM reconstruction of the 30S IC (Julian *et al.*, 2011) placed IF3-CTD at the 790 loop of 16S rRNA, whereas IF3-NTD was located in the close proximity to fMet-tRNA<sup>Met</sup> (Julian *et al.*, 2011). The functional role of the IF3-NTD and the linker region is not known. IF3 carrying mutations in the NTD and the linker region could bind normally to 30S subunits, but were defective in start codon and fMet-tRNA<sup>Met</sup> selection (Sussman *et al.*, 1996; Sette *et al.*, 1999; Maar *et al.*, 2008) and in the discrimination against leaderless mRNA (Maar *et al.*, 2008), which suggests a role of the IF3 linker in maintaining initiation fidelity.

IF3 has several functions during translation initiation. It interferes with ribosomal subunit association (Debey *et al.*, 1975), affects the rates of tRNA association to and dissociation from the P site (Gualerzi *et al.*, 1977; Wintermeyer & Gualerzi, 1983; Antoun *et al.*, 2006a), ensures the fidelity of translation initiation (Hartz *et al.*, 1989; Hartz *et al.*, 1990; Antoun *et al.*, 2006a; Lomakin *et al.*, 2006; Milon *et al.*, 2008) and is crucial for the discrimination against mRNAs with unfavorable TIRs (La Teana *et al.*, 1993; Grigoriadou *et al.*, 2007b; Milon *et al.*, 2008).

### Bacterial mRNAs

Bacterial mRNAs are normally polycistronic and possess characteristic signals for initiation of protein synthesis



in the TIR which spans nucleotides  $-20$  to  $+15$  around the translation start codon (Dreyfus, 1988). The components of the TIR that are known to affect the initiation efficiency are: (i) the start codon; (ii) the Shine–Dalgarno (SD) sequence which is located approximately 7–10 nucleotides upstream of the initiator codon and pairs with a complementary sequence near the 3' end of 16S rRNA (anti-Shine–Dalgarno sequence, aSD); (iii) the thermodynamic stability of the mRNA fold near the start site; and (iv) A/U-rich elements in the mRNA which are recognized by protein S1 of the 30S ribosomal subunit. Usually a combination of these elements contributes to the recruitment of an mRNA to the ribosome and thus the overall structure of the TIR can be thought as being an essential element determining the efficiency with which a given mRNA is recruited for translation (Gualerzi *et al.*, 2001; Jin *et al.*, 2006). Although AUG is most commonly used as initiation codon (83% in *E. coli*), GUG and UUG are also used rather frequently (14% and 3%, respectively, in *E. coli* and up to 40% in other organisms) (Ma *et al.*, 2002). Non-canonical AUU, AUC, CUG initiation codons have been found in *infC* (encoding IF3) (Polard *et al.*, 1991; Baudet *et al.*, 2010), *pcnB* (encoding *E. coli* poly(A) polymerase) (Binns & Masters, 2002), *dnaA* (encoding the protein involved in initiation of DNA replication in *E. coli* and *Deinococcus radiodurans*) and *rpsL* (the ribosomal protein S12 in *D. radiodurans*) (Baudet *et al.*, 2010). Depending on the sequence of the TIR, most of the mRNAs can be classified into one of three main groups, those (i) containing the Shine–Dalgarno sequence (SD-led), (ii) lacking the SD (non-SD-led), and (iii) leaderless mRNAs (Figure 3). In addition, initiation on polycistronic mRNAs can proceed through the re-initiation mechanism (Figure 3). After completion of translation of an upstream open reading frame (ORF) the ribosome can remain bound to the mRNA and slide in a bi-directional manner (see (Yoo & Rajbhandary, 2008) for references) until an AUG codon is found, usually in close proximity to the preceding termination codon (Yoo & Rajbhandary, 2008). The mechanism of re-initiation has received little attention in recent years, therefore in the following we

focus on the mechanisms of *de novo* initiation on the leading ORF.

### SD-led mRNAs

Most common (and best studied) are the mRNAs that use the SD sequence, typically GGAGG, in their 5' untranslated region (5' UTR) to anchor the mRNA on the 30S subunit through association with the aSD sequence in the 16S rRNA. Sequence analysis of a large number of bacterial genomes suggested that an SD sequence is present in more than 40% of all genes (Ma *et al.*, 2002). In general, fast-growing bacteria, gram-negative thermophiles, spirochetes, methanogens, and hyperthermophilic archaea have a relatively high proportion of SD-containing genes (up to 90%), while in obligate intracellular parasites, surface parasites, pathogens, and cyanobacteria this proportion is diminished (to as little as 10–20%) (Ma *et al.*, 2002; Chang *et al.*, 2006). The spacing between the SD sequence and the initiation codon varies from 5 to 13 bases, with optimal spacing of about 7–10 bases in *E. coli* mRNAs. The SD–aSD interaction leads to the placement of the initiation codon in the P site of the ribosome, where it is recognized by fMet-tRNA<sup>fMet</sup>. The importance of the SD–aSD pairing has been conclusively demonstrated by experiments showing that mRNA translation can be impaired by mutations within the SD sequence and then restored by compensatory mutations in the aSD (Hui & de Boer, 1987; Jacob *et al.*, 1987). However, overall, the strength of the SD correlates only moderately with the efficiency of translation (Calogero *et al.*, 1988; Melancon *et al.*, 1990; de Smit & van Duin, 1994; Lee *et al.*, 1996; Ma *et al.*, 2002; Nakamoto, 2006). When expressed at a moderate level (30–40% of total ribosomes), specialized ribosomes with an altered aSD sequence are only two-fold less active than normal ribosomes for the translation of bulk cellular mRNAs (Skorski *et al.*, 2006). Ribosomes lacking the 3' end of 16S rRNA (and therefore the aSD sequence) correctly select the translation start site in some natural mRNAs, suggesting that the SD sequence is not the only determinant of the translational initiation (Melancon *et al.*, 1990). Together, these findings suggest that the SD–aSD

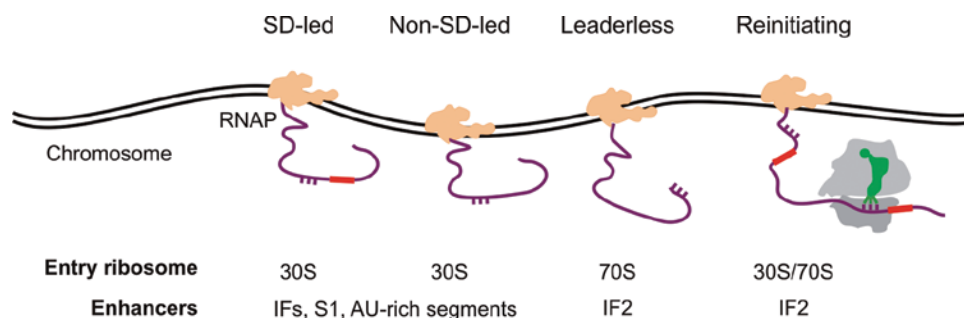


Figure 3. Diversity of initiation mechanisms for bacterial mRNAs. mRNAs emerging from the elongating RNA-polymerase (RNAP) display the TIR for ribosome binding. SD-led and non-SD-led mRNAs are recruited to the 30S subunit. The leaderless mRNA enters translation through the 70S ribosome, whereas the state of the ribosome (30S subunit or 70S ribosome) acting in mRNA re-initiation is controversial (Yoo & Rajbhandary, 2008). The potential secondary structure at the TIR (not shown) modulates the efficiency of initiation and may play a particularly important role during translation of mature full-length mRNAs uncoupled to transcription (not shown). (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

interaction plays an important but not essential role for the synthesis of bulk cellular proteins.

### Non-SD-led mRNAs

mRNAs that lack the SD sequence exist in most bacteria and archaea (Tolstrup *et al.*, 2000; Weiner *et al.*, 2000; Ma *et al.*, 2002; Chang *et al.*, 2006). Genes with an AUG start codon are more likely to have an SD sequence than genes with either GUG or UUG (Ma *et al.*, 2002). In archaea and some bacteria internal genes of operons have a much higher SD presence than those of leading genes (Ma *et al.*, 2002; Chang *et al.*, 2006). Essentially nothing is known about the mechanism of initiation on non-SD-led mRNAs. They are generally less structured in their TIR; in fact, the lack of local RNA secondary structure around the start codon is crucial for the expression of non-SD-led reporter gene constructs in bacteria (Scharff *et al.*, 2011). mRNAs lacking the SD sequence bind to the 30S subunit rapidly and form a stable complex committed to translation (Milon *et al.*, 2008); however, the mechanisms by which the start codon is found is not clear. AUG itself is an important recognition element. Another potential mediator is protein S1, which may be an important enhancer of mRNA binding of both SD-led and non-SD-led mRNAs (see below).

One of the most curious examples of a non-SD-led mRNA in *E. coli* is *rpsA*; it codes for the protein S1, which on the ribosome recognizes A/U-rich elements of the mRNA. The TIR of the S1 mRNA shows a number of unusual features. It extends far upstream (to position -91) of the initiator AUG, lacks a canonical SD sequence, and can fold into three successive hairpins that are essential for its high translational activity (Boni *et al.*, 1991; Skorski *et al.*, 2006). Interestingly, the efficiency of *rpsA* translation is strongly repressed by free S1, suggesting a mechanism of translation autoregulation (Boni *et al.*, 1991).

### Leaderless mRNAs

Leaderless genes that lack their 5' UTR are widespread in a variety of bacteria, e.g. in *Actinobacteria*, *Deinococcus*, and *Thermus* more than 20% of genes are leaderless (Zheng *et al.*, 2011). Leaderless mRNAs have a very short or no 5'-UTR and directly bind to 70S ribosomes, recruiting fMet-tRNA<sup>fMet</sup> to the AUG codon with the help of IF2 (Grill *et al.*, 2000). Close proximity of the start codon to the 5' terminus is a strong determinant of both ribosome binding and expression (Krishnan *et al.*, 2010). Curiously, translation of leaderless mRNAs requires the AUG codon as initiation signal, in addition to the codon-anticodon complementarity (Van Etten & Janssen, 1998). 30S proteins S1 and S2, which modulate the recruitment of leader-containing mRNA, are dispensable for the translation of leaderless RNA (Moll *et al.*, 2002). Moreover, a number of other ribosomal proteins are also dispensable: S6, S12, S18, and S21 (Kaberina *et al.*, 2009).

Notably, leaderless mRNAs may have an important role in stress adaptation in bacteria and may be much more widespread than previously thought. Recently,

a stress-induced endo-ribonuclease activity of an *E. coli* toxin, MazF, was reported to cleave single-stranded mRNAs at ACA sequences closely upstream of the AUG start codon of some specific mRNAs, thereby generating leaderless mRNAs (Vesper *et al.*, 2011). Furthermore, MazF also targets 16S rRNA, removing the sSD sequence from the 3' terminus. This produces a subpopulation of ribosomes that selectively translate leaderless mRNAs under conditions of stress (Vesper *et al.*, 2011). Two reports pointed out that translation of mRNAs with and without a leader sequence responds differently to temperature changes (Grill *et al.*, 2002; Vimberg *et al.*, 2007), further linking the type of mRNA entry to the adaptation to environment changes.

## Assembly of the 30S PIC

### Recruitment of initiation factors and fMet-tRNA<sup>fMet</sup>

In the first phase of translation initiation, initiation factors, fMet-tRNA<sup>fMet</sup>, and mRNA are recruited to the 30S subunit. Early work showed that initiation factors can interact with the 30S subunit independent of each other, suggesting a random order of binding with a number of potential alternative pathways towards 30S PIC assembly (Gualerzi & Pon, 1990). However, recent kinetic data revealed that the arrival times of the three initiation factors and fMet-tRNA to the 30S subunit are quite different, about 30 ms for IF1, 1.3 ms for IF2, <1 ms for IF3, and 100 ms for fMet-tRNA<sup>fMet</sup>, suggesting a kinetically favored route of the 30S PIC assembly (Figure 4A) (Milon *et al.*, 2012). The arrival times imply that IF3 and IF2 are the first factors to bind to the 30S subunit, followed by IF1, which binds significantly more slowly, and finally by fMet-tRNA<sup>fMet</sup>, which docks to IF2 bound to the 30S subunit (Milon *et al.*, 2010). The early intermediate of the 30S PIC assembly, the 30S-IF2-IF3 complex, is formed very rapidly but is rather short-lived (lifetime of about 30 ms; (Milon *et al.*, 2012)). The following recruitment of IF1 leads to a strong stabilization of IF2 and IF3 in the 30S PIC, with the residence times of the factors up to 1500-fold longer than in the initial complex. Docking of fMet-tRNA<sup>fMet</sup> is the slowest reaction in the 30S PIC assembly. This final assembly step may be delayed even further (by up to 25 ms) when fMet-tRNA<sup>fMet</sup> is stored in the complex with IF2-GTP (Milon *et al.*, 2010).

Translation initiation is tightly coupled to ribosome recycling, which replenishes the pool of ribosomal subunits available for initiation. In this case, the predominant pathway of the 30S PIC formation is somewhat different from the one prevailing with free ribosomal subunits (Figure 4B). After the termination of mRNA translation, the 70S post-termination complexes are split into 30S-mRNA-tRNA complexes and 50S subunits by the concerted action of RRF and EF-G (Karimi *et al.*, 1999; Peske *et al.*, 2005). Re-association of the subunits is prevented by IF3 binding to the 30S complex (Debey *et al.*, 1975). Kinetically, the recruitment of IF3 is limited by the rate of the subunit splitting by EF-G and RRF, which is

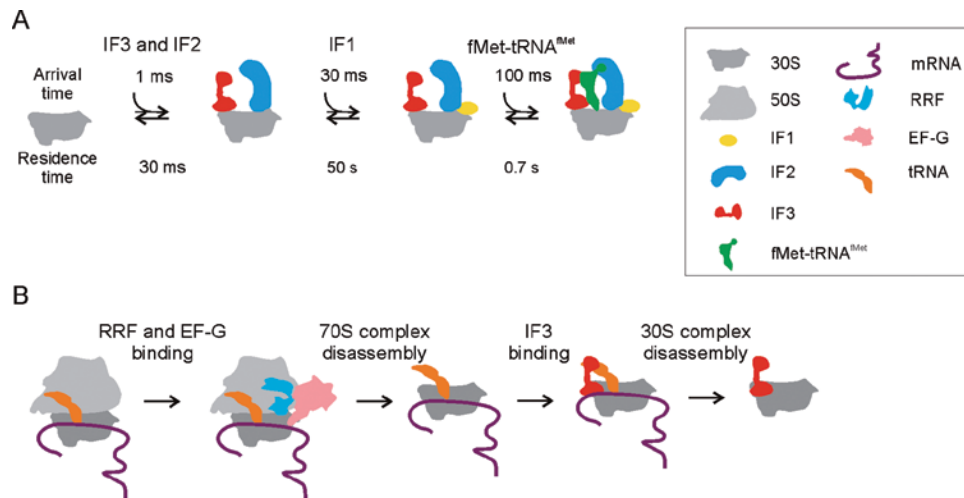


Figure 4. Elemental steps of 30S PIC assembly. (A) Assembly on vacant 30S subunits. The kinetically favored order of initiation factors and fMet-tRNA<sup>Met</sup> arrivals and the residence times of the respective complexes are indicated. (B) Assembly of 30S PIC coupled to ribosome recycling. (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

slow. However, after the ejection of the 50S subunit, IF3 can bind to the 30S-mRNA-tRNA complex with the same rate as to vacant ribosomes (Milon *et al.*, 2012). Binding of other initiation components, particularly of fMet-tRNA and the new mRNA, may be delayed until deacylated tRNA and the translated mRNA have been released from the 30S subunit, which is promoted by IF3 (Karimi *et al.*, 1999; Peske *et al.*, 2005).

The 30S PIC formation provides an important checkpoint for fMet-tRNA<sup>Met</sup> selection. 30S-bound IF2 specifically recognizes fMet-tRNA<sup>Met</sup> and favors kinetically the binding of aminoacyl-tRNAs that have the  $\alpha\text{NH}_2$  group blocked more than it does with non-blocked elongator tRNAs (Canonaco *et al.*, 1986; Gualerzi & Wintermeyer, 1986; Boelens & Gualerzi, 2002; Antoun *et al.*, 2006a). A unique feature of initiator tRNA<sup>Met</sup> is a mismatch at the bottom of the acceptor stem (C1-A72). When the activity of formyl-methionyl-transferase is impaired, non-formylated Met-tRNA<sup>Met</sup>, as well as elongator aa-tRNAs, can be recruited to the 30S PIC, albeit very slowly (Antoun *et al.*, 2006a; Pavlov *et al.*, 2011), probably because interactions with IF2 are lacking or impaired.

### Maturation of the 30S PIC

The initial recruitment of initiation factors to the 30S subunit is followed by conformational rearrangements which collectively can be referred to as 30S PIC maturation. Some of the rearrangements depend on the synergistic action of initiation factors, in particular IF1 and IF3, and are essential for the ability of the ribosome to discriminate against non-optimal TIRs and a non-cognate start codon during the following steps of initiation (La Teana *et al.*, 1993; Grigoriadou *et al.*, 2007b; Milon *et al.*, 2008). Cryo-EM structures indicated that IF3 induces a rearrangement within the 30S subunit towards the conformation that is usually observed in the rotated form of the 70S ribosome, i.e. with the 30S head tilted towards the platform (Julian *et al.*, 2011), whereas in the absence

of IF3 a classic, non-rotated structure of the 30S subunit is found (Simonetti *et al.*, 2008). The importance of the dynamics of the 30S subunit is further supported by the mutational analysis of 16S rRNA which identified a number of mutations in the neck of the 30S subunit and in the vicinity of the conserved A1413:G1487 base pair of h44 that affected the initiation fidelity (Qin & Fredrick, 2009). Crystal structures showed that IF1 promotes the displacement of one strand of h44 disrupting the A1413:G1487 base pair (Carter *et al.*, 2001), suggesting an active role for IF1 in modulating the 30S subunit conformation by remodeling the 16S rRNA. More recently, structural probing of 30S subunits showed that the 1408 region of h44 changed the conformation upon IF1 binding, whereas formation of the correct codon-anticodon interaction reverted the observed effect (Qin *et al.*, 2012). Furthermore, interfering with the conformational mobility of the 30S subunit by adding the antibiotic streptomycin or omitting IF1 decreased the fidelity of initiation during subunit joining (Milon *et al.*, 2008).

Further rearrangements that occur upon maturation of the 30S PIC appear to involve conformational adjustments of IF2. The comparison of cryo-EM structures of the 30S IC with and without IF3 indicated that the CTD of IF2 may be positioned in a different way, depending on the presence of IF3 (Simonetti *et al.*, 2008; Julian *et al.*, 2011). Kinetic experiments also indicated IF2 rearrangements (Grigoriadou *et al.*, 2007a), although the correspondence between the fluorescence changes of IF2 observed in that work and the rearrangements observed in cryo-EM (Myasnikov *et al.*, 2005) is not clear. Recent NMR studies on isolated IF2 domains suggested that the C2 subdomain has an intrinsic high mobility which may be independent of the G2 conformation (Wienk *et al.*, 2012). Together, the data suggest the propensity of IF2 CTD to move, presumably, in order to optimize the alignment of the fMet-tRNA<sup>Met</sup> with respect to the AUG codon and to the incoming 50S subunit.



## Recruitment of mRNA

The timing and stability of mRNA binding to the 30S PIC prior to start codon selection is independent of the composition of the 30S complex, i.e. the presence of the initiation factors and fMet-tRNA<sup>fMet</sup> (Studer & Joseph, 2006; Milon *et al.*, 2012). The initial recruitment of mRNA to the 30S subunit is modulated by four parameters: (i) the abundance of a given mRNA in the total mRNA pool (Passalacqua *et al.*, 2009); (ii) the secondary structure of the TIR (de Smit & van Duin, 1994; Studer & Joseph, 2006); (iii) the presence of an SD sequence, the strength of the SD-aSD complex, and the length of the linker between the SD sequence and the start codon; and (iv) the accessibility of single-stranded A/U sequences that can bind to the protein S1 (Komarova *et al.*, 2002; Skorski *et al.*, 2006). These factors determine whether a particular mRNA docks to the ribosome prior to, simultaneously with, or subsequently to the arrival of IFs and fMet-tRNA<sup>fMet</sup>. Concentrations of mRNAs in the cell may vary by orders of magnitude, and not all mRNAs are expressed in all cells in a bacterial population at a given time (Passalacqua *et al.*, 2009). Variations in the mRNA secondary structures range from weakly folded mRNAs to compact, tightly regulated riboswitches (Geissmann *et al.*, 2009).

Secondary structure at the TIR of mRNAs has been long recognized to have a strong influence on protein expression by hampering mRNA loading onto the ribosome (Nakamoto, 2006). Base exchanges in the TIR and manipulation of its G+C content have a profound effect on translational efficiency, indicating that mRNA secondary structure in this region impairs translation (Voges *et al.*, 2004). Bioinformatic analysis indicated that mRNA secondary structure content is particularly low at the beginning of genes in *E. coli* and *Salmonella typhimurium* (Katz & Burge, 2003; Tuller *et al.*, 2010). The notion that there is a selection for structures with weak folding at the beginning of genes has been supported by several studies (Kudla *et al.*, 2009; Allert *et al.*, 2010; Tuller *et al.*, 2010); however, the implications of this finding for gene expression remained controversial. mRNA folding and the associated rates of translation initiation were suggested to play a predominant role in shaping expression levels of individual genes (Kudla *et al.*, 2009; Allert *et al.*, 2010). In contrast, other studies indicated that the selection for weak secondary structures at the TIR is global and not related to protein abundance or mRNA levels (Tuller *et al.*, 2010).

Although weak secondary structure at the TIR favors efficient initiation (Kudla *et al.*, 2009), most of the mRNAs in the cell are probably folded to some extent. Furthermore, also highly structured mRNAs can be recruited to the ribosome. Structural work suggested the existence of a docking site for folded mRNAs at the platform of the 30S subunit in the close proximity of proteins S2, S7, S11, S18, and S21 (Allen *et al.*, 2005; Jenner *et al.*, 2005; Marzi *et al.*, 2007). The proteins provide patches of positive charges which might bind different

folded mRNAs regardless of their sequence (Marzi *et al.*, 2007). Notably, also poly(A) and poly(U) extensions 5' to the coding region of mRNAs formed stable contacts to protein S2 (Yusupova *et al.*, 2006), suggesting that also unfolded mRNAs can bind to the same region. One can speculate that these proteins, together with S1 (see below), which is located in the vicinity, form a universal initial docking site for all mRNAs, although the details of recruitment may be different depending on the features of the mRNA (Figure 5A).

Many mRNAs possess an A/U-rich region 5' of the start codon and the SD region (if present). A/U-rich sequences are recognized by protein S1 (Komarova *et al.*, 2002), which is present in many, albeit not all, bacteria. S1 might facilitate the initial docking of mRNAs or assist mRNA unfolding by entrapping melted single-stranded regions. Cryo-EM structures provided evidence for direct interactions of S1 with 11 nucleotides of the mRNA immediately upstream of the SD sequence (Sengupta *et al.*, 2001). The location of S1 at the platform of the 30S subunit suggests that S1 may constitute a part of the stand-by site for mRNA recruitment (Sengupta *et al.*, 2001; Marzi *et al.*, 2007) (Figure 5B). In fact, mRNA selection was found to be dominated by S1, rather than by the SD-aSD interaction (Ringquist *et al.*, 1995). SD and A/U-rich enhancer stimulate translation co-operatively: initiation on strong SD sequences is stimulated by the enhancer much more than on weak SD sequences (Vimberg *et al.*, 2007). Disruption of the *E. coli* gene coding for S1 is lethal (Kitakawa & Isono, 1982). A decreased level of

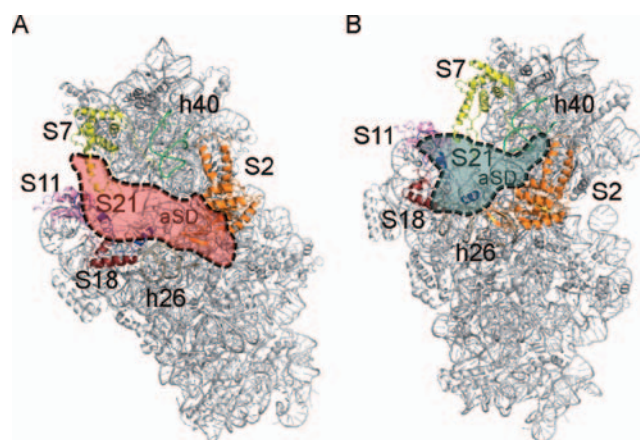


Figure 5. The mRNA docking site at the platform of the 30S subunit. (A) The location of the universal mRNA docking site (semitransparent salmon) as suggested by cryo-EM (adapted from (Marzi *et al.*, 2007)). Indicated are the positions of the aSD region, of the proteins S2 (orange), S7 (yellow), S11 (pink), S18 (brown), and S21 (magenta), and h26 (sand) and h40 (green) of 16S rRNA. (B) The location of protein S1 suggested by cryo-EM. The position of S1 bound to 11 nucleotides immediately upstream of the SD sequence of the mRNA is indicated semitransparent light teal (redrawn from (Sengupta *et al.*, 2001) using the structure of the 30S subunit from *E. coli*, PDB 2AW7; (Schuwirth *et al.*, 2005)). Note the different orientations of the 30S subunit in A and B. (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

S1 protein in the cell leads to a rapid decrease in total protein synthesis (Sorensen *et al.*, 1998), which may suggest a crucial role of S1 in translation initiation.

The initial recruitment of mRNA to the 30S subunit is followed by conformational adjustments which depend on the properties of the TIR. A structured mRNA attached at the docking platform of the 30S subunit has to unfold, which would expose the SD sequence and the start codon (Marzi *et al.*, 2007). Weak secondary structure elements are likely to fluctuate spontaneously between the folded and unfolded state; the latter may be trapped by the ribosome (de Smit & van Duin, 1994; Studer & Joseph, 2006). The SD-aSD interaction positions the mRNA in such a way that the start codon is placed in the P site ready to interact with the anticodon of fMet-tRNA<sup>fMet</sup>. The final adjustment may require movements of the mRNA from the stand-by to the initiation site (Canonaco *et al.*, 1989; La Teana *et al.*, 1995; Studer & Joseph, 2006; Yusupova *et al.*, 2006). The efficiency of unfolding can be mediated by ligands and environmental conditions (metabolites, proteins, non-coding RNA, thermoswitches) by a variety of potential mechanisms, such as entrapment of inactive mRNA folds by the ribosome or competition between a trans-acting ligand and the 30S subunit for binding to the mRNA (Geissmann *et al.*, 2009).

## Transition to the 30S IC

### Start codon selection

The major rearrangement that converts the 30S PIC into the mature 30S IC is the start codon recognition by the anticodon of fMet-tRNA<sup>fMet</sup>. In the SD-led mRNAs, formation of the SD-aSD complex is expected to be sufficient to bring the start codon into the P site (Gold, 1988). However, as the length of the spacer varies considerably, sense codons on either side of the start codon may become accessible for IF2-mediated recruitment of fMet-tRNA<sup>fMet</sup> to a near-cognate codon or for elongator tRNA binding (Gold, 1988; Hartz *et al.*, 1989; Antoun *et al.*, 2006a). Both scenarios can lead to synthesis of a protein with an aberrant N-terminal amino acid sequence or to out-of-frame mRNA reading. However, in reality the SD-aSD pairing is not essential for the selection of the reading frame (Calogero *et al.*, 1988) and the error frequency of initiation is very low, indicating that the ribosome controls the quality of codon-anticodon interaction and reject poor initiation substrates. Initiation on near-cognate codons (i.e. those that are not fully complementary to the anticodon of initiator tRNA, such as CUG, AUU, AUA, or AUC) by fMet-tRNA<sup>fMet</sup> is 100–1000 times less frequent than on AUG, even if those codons are placed in an mRNA context that is optimal for initiation (O'Connor *et al.*, 1997; O'Connor *et al.*, 2001; Qin *et al.*, 2007). Erroneous initiation at internal GUG or UUG codons, which are used as initiation codons, may occur at a somewhat higher frequency of  $(1-6) \times 10^{-2}$ ; initiation at other internal codons is negligible (O'Connor *et al.*, 2001).

Initiation factors play an important role in tuning the fidelity of start codon recognition (Hartz *et al.*, 1989; Hartz *et al.*, 1990; Petrelli *et al.*, 2001; Antoun *et al.*, 2006a). Binding of IF3 to the ribosome increases the rate of association and dissociation of any tRNA by several orders of magnitude, and this effect is amplified by IF1 (Wintermeyer & Gualerzi, 1983; Antoun *et al.*, 2006a). This ensures that incorrect tRNAs are rapidly rejected from the complex and can be replaced by the correct initiator tRNA. The latter may be specifically stabilized through the three adjacent G-C base pairs in the anticodon stem, which is a characteristic feature of initiator tRNA (Seong & Rajbhandary, 1987; Mandal *et al.*, 1996; Lancaster & Noller, 2005). Two of these G-C base pairs (G29-C41 and G30-C40) are particularly important for efficient initiation, and interact with 16S rRNA residues G1338 and A1339, respectively (Mandal *et al.*, 1996; Qin *et al.*, 2007). Correct start codon recognition results in a major stabilization of fMet-tRNA<sup>fMet</sup>, IF2, and IF1 binding to the 30S subunit, whereas the binding of IF3 is destabilized (Milon *et al.*, 2008; Milon *et al.*, 2012). The affinity switch induced by codon recognition comprises an important checkpoint that prepares the 30S IC for the following transition to the 70S IC and commits the mRNA for translation (Milon *et al.*, 2008; Milon *et al.*, 2012).

### Structure of the 30S IC

The structure of the mature 30S IC with all components in place has been recently revealed by cryo-EM studies (Julian *et al.*, 2011) (Figure 6A). As mentioned above, in the 30S IC the head of the 30S subunit was found in a rotated conformation with respect to the body (Figure 6B). Taking into account the position of the SD-aSD duplex, the rotation of the 30S subunit head moves the mRNA in the direction which would place the start codon closer to the P site. This finding agrees well with crosslinking studies which suggested that the formation of the SD-aSD complex in the absence of initiation factors places the mRNA in a “standby” position, from which it is shifted backward, i.e. closer to the P site, before the large subunit joins the complex (La Teana *et al.*, 1995). Similarly, crystal structures of the 70S IC indicate that the mRNA has moved in the 3'→5' direction with simultaneous clockwise rotation and lengthening of the SD duplex, bringing it into contact with ribosomal protein S2 (Yusupova *et al.*, 2006). The conformation of the 30S subunit in the 30S IC is the same as in the 70S IC, both stalled in the presence of IF2-GDPNP, where the 30S subunit is also found in a rotated orientation with respect to the 50S subunit (Allen *et al.*, 2005). Notably, the conformation is not induced by the 50S subunit and does not occur in the absence of IF3 (Simonetti *et al.*, 2008). This suggests that binding of IFs, in particular IF3, could induce or stabilize the altered 30S subunit conformation (Julian *et al.*, 2011). The rotated state of the 30S subunit appears to be retained upon 50S subunit joining until GTP hydrolysis and dissociation of IF2 (Marshall *et al.*, 2009).



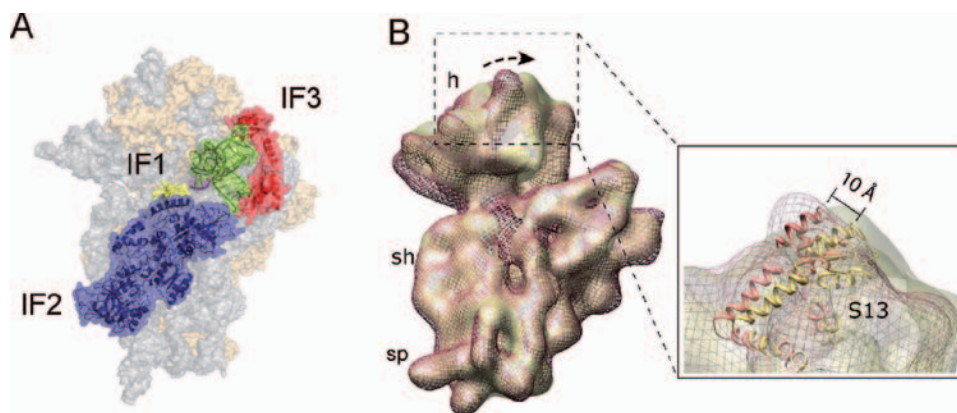


Figure 6. Structure of the 30S IC. (A) Cryo-EM reconstruction of a complete 30S IC with IF1 (yellow), IF2 (blue), IF3 (red) and fMet-tRNA<sup>Met</sup> (green) bound to the 30S subunit (16S rRNA, gray; ribosomal proteins, sand). (B) 30S subunit conformations in the complex with mRNA in the absence of initiation factors or tRNA (red mesh) and in 30S IC (semitransparent yellow). The arrow indicates the clockwise rotation of the 30S subunit head. The inset shows the positions of protein S13 in the two maps for comparison (reproduced from (Julian *et al.*, 2011) with permission). (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

In the 30S IC, fMet-tRNA<sup>Met</sup> is held in its characteristic position by two interactions: one involving the anticodon stem which is buried in the P site of the 30S subunit, and the other between IF2 and the acceptor end of the tRNA (Simonetti *et al.*, 2008; Julian *et al.*, 2011). The orientation of fMet-tRNA<sup>Met</sup> in the known structures of initiation complexes deviates from the canonical P site position and is referred to as a P/I state (Allen *et al.*, 2005). However, the details of the fMet-tRNA<sup>Met</sup> orientation are not identical in the available 30S IC and 70S IC structures; the differences may be related to various functional states of the complexes (30S IC with or without IF3, 30S IC vs. 70S IC). In all structures, the anticodon stem of the tRNA is buried in the P site of the 30S subunit, whereas the position of the tRNA CCA end varies. In the 30S IC from *E. coli* prepared with the full set of initiation factors and the non-hydrolyzable GTP analog, GDPNP, the CCA end points towards the A site of the peptidyl transferase center (Julian *et al.*, 2011). A somewhat different P/I state was reported for the 30S IC from *T. thermophilus* formed with GTP in the absence of IF3 (Simonetti *et al.*, 2008), which may reflect the known effect of IF3 on the stability of fMet-tRNA<sup>Met</sup> binding to the 30S IC. Comparison of the 30S IC with the 70S IC from *E. coli* (both with GDPNP) suggested that, upon binding of the 50S subunit, the CCA end moves towards the E site (70S IC; (Allen *et al.*, 2005)). The position of the C2 domain of IF2, to which the fMet moiety of fMet-tRNA<sup>Met</sup> is bound, changes accordingly. It is possible that all described P/I positions are sampled during the transition of the initiator tRNA towards the final P-site-bound state and that they are important for discrimination of mRNAs with unfavorable TIR.

## Formation of the 70S IC

### The sequence of events

The formation of the 70S IC is the final, quite complex step of initiation which commits an mRNA for

translation (Figure 7). The 50S subunit binds to the 30S IC carrying all three initiation factors, mRNA, and fMet-tRNA<sup>Met</sup> (Grigoriadou *et al.*, 2007a; Milon *et al.*, 2008). The ribosome-bound IF2, together with fMet-tRNA<sup>Met</sup> attached to its C2 domain, provide a large surface area for the docking of the 50S subunit. Interestingly, rapid subunit association depends on a specific interaction between IF2 and the protein L12 of the 50S subunit (Huang *et al.*, 2010; Mandava *et al.*, 2012). Association of the 50S subunit triggers GTP hydrolysis by IF2 (Tomsic *et al.*, 2000; Grigoriadou *et al.*, 2007a; Huang *et al.*, 2010) and the release of IF3 and IF1 from the complex (Milon *et al.*, 2008). IF3 seems to prevent the accommodation of fMet-tRNA<sup>Met</sup> in the P site (Allen *et al.*, 2005) and slows down 50S subunit joining (Antoun *et al.*, 2006a; Milon *et al.*, 2008), but is required to control the efficiency and fidelity of initiation on a given mRNA (Milon *et al.*, 2008).

GTP hydrolysis and conformational change from the GTP-bound to the GDP-bound form of IF2 involves substantial rearrangements of the factor (Roll-Mecak *et al.*, 2000) and the entire ribosome. In the presence of a non-hydrolyzable GTP analog, IF2 interacts mostly with the 30S subunit and extends to fMet-tRNA<sup>Met</sup>, whereas in the GDP-bound state the factor moves away from the tRNA, loses part of the interactions with both ribosomal subunits and adopts a “ready-to-leave” conformation (Myasnikov *et al.*, 2005). Furthermore, GTP hydrolysis facilitates the rearrangement that promotes the transition of the ribosome from the rotated to the non-rotated state (Myasnikov *et al.*, 2005; Marshall *et al.*, 2009). Dissociation of IF2 is the final step of complex maturation resulting in the 70S IC complex that is committed to translation.

Joining of the 50S subunit with the 30S IC may itself be a stepwise process (Grigoriadou *et al.*, 2007a; Fabbretti *et al.*, 2007; Milon *et al.*, 2008). The stepwise nature of docking explains how the 50S subunit can bind to the 30S IC while IF3 is still present, even though the factor

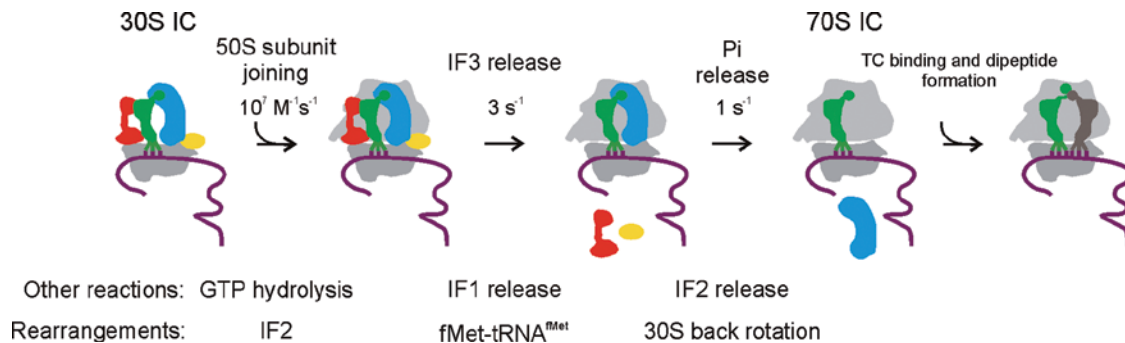


Figure 7. Formation of the 70S IC. Step 1, docking of the 50S subunit to the 30S IC containing all three initiation factors (Milon *et al.*, 2008). Joining of the 50S subunit triggers GTP hydrolysis by IF2, which promotes conformational rearrangements of the factor (Tomsic *et al.*, 2000; Grigoriadou *et al.*, 2007a). Step 2, dissociation of IF3 and IF1 (Milon *et al.*, 2008). The position of fMet-tRNA<sup>fMet</sup> likely changes (Allen *et al.*, 2005; Myasnikov *et al.*, 2005; Grigoriadou *et al.*, 2007a; Julian *et al.*, 2011). Step 3, release of Pi from IF2 (Tomsic *et al.*, 2000; Grigoriadou *et al.*, 2007a), dissociation of IF2, and the rearrangements of the 30S subunit from the rotated to non-rotated state (Marshall *et al.*, 2009), resulting in a mature 70S IC. The final step, binding of the ternary complex (TC) EF-Tu•GTP•aminoacyl-tRNA is followed by the formation of the first peptide bond, completing the transition from initiation to elongation. The reaction rate constants are given for an mRNA which forms a moderate-strength SD-aSD interaction and has an optimal spacer between the SD sequence and the AUG codon, which is representative for many *E. coli* mRNAs (Tomsic *et al.*, 2000; Grigoriadou *et al.*, 2007a; Milon *et al.*, 2008). Alterations in the strength of the SD-aSD complex, the length of the spacer or the identity of the start codon affect (usually reduce) the arrival time of the 50S subunit (Grigoriadou *et al.*, 2007b; Milon *et al.*, 2008). (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))

interferes with the formation of an important intersubunit bridge (McCutcheon *et al.*, 1999; Dallas & Noller, 2001; Julian *et al.*, 2011). Results of time-resolved chemical probing indicate that (i) different interface regions of the ribosomal subunits associate sequentially (Hennelly *et al.*, 2005); (ii) subunit joining entails intermediate stages in which the interactions between IF3 and the ribosome are only partly dissolved; and (iii) the adjustment of the ribosomal subunits precedes the final ejection of IF3 (Fabbretti *et al.*, 2007). Alternatively, based on indirect estimations, Antoun *et al.* suggested that the binding of fMet-tRNA<sup>fMet</sup> to the 30S PIC causes the release of IF3 which, in turn, is required for subunit docking (Antoun *et al.*, 2006b). However, direct measurements of IF3 release indicate that binding of fMet-tRNA<sup>fMet</sup> to a cognate start codon the affinity of IF3 for the complex decreases radically; however, the factor's residual affinity for the 30S IC (in the range of 10 nM) is still high enough, such that at the cellular concentrations, the factor remains attached to the 30S subunit. However, the 50S subunit, once docked, prevents IF3 from re-binding at the interface side of the 30S subunit (McCutcheon *et al.*, 1999; Dallas & Noller, 2001), rendering the release of the factor practically irreversible (Milon *et al.*, 2008).

### Role of the GTP-bound form of IF2 and of GTP hydrolysis

GTP binding and hydrolysis have a global effect on initiation, even though the effects on each step alone may not be large. The affinity of IF2 binding to the 30S subunit is 10-fold higher with GTP than with GDP (Pon *et al.*, 1985). Also the affinity of GTP (but not of GDP) to IF2 increased about 10-fold upon binding to the 30S IC, especially in the presence of mRNA and fMet-tRNA<sup>fMet</sup> (Antoun *et al.*,

2003). It was argued that if fMet-tRNA<sup>fMet</sup> stabilizes GTP binding to IF2 in the 30S IC, then the binding of initiator tRNA must be stabilized as well (Antoun *et al.*, 2003); hence the important role of the GTP-bound form of IF2 in fMet-tRNA<sup>fMet</sup> recruitment. IF2 in complex with GTP or a non-cleavable GTP analogue was shown to promote rapid association of the 50S subunit (Antoun *et al.*, 2003; Grigoriadou *et al.*, 2007a), whereas with GDP subunit joining was slower by about 50-fold (Antoun *et al.*, 2003). Notably, the sensitivity to GTP is relaxed in IF2 mutants that have a reduced selectivity against unformylated Met-tRNA<sup>fMet</sup> (Zorzet *et al.*, 2010; Pavlov *et al.*, 2011). This finding suggests that both the nucleotide bound to IF2 and fMet-tRNA<sup>fMet</sup> contribute to conformational remodeling of the structural landscape that is required for 50S subunit docking. The transition from the GTP- to the GDP-bound form of the factor after Pi release promotes conformational rearrangements of IF2 and fMet-tRNA<sup>fMet</sup> (see above), the dissociation of IF2 (Myasnikov *et al.*, 2005; Grigoriadou *et al.*, 2007a), and the rearrangement of the reverse rotation of the ribosomal subunits, thereby enabling the complex to progress towards elongation (Marshall *et al.*, 2009).

In addition to GTP and GDP, IF2 can also bind guanosine 3',5'-(bis) diphosphate (ppGpp), an alarmone involved in the stringent response in bacteria (Milon *et al.*, 2006; Mitkevich *et al.*, 2010). In cells growing under optimal conditions, the GTP concentration is high, and that of ppGpp very low. However, under stress conditions, the GTP concentration may decline by as much as 50%, and that of ppGpp can attain levels comparable to those of GTP. Binding of ppGpp to IF2 impairs all IF2 functions and blocks initiation of translation. Thus, IF2 has the properties of a cellular metabolic sensor that oscillates between an active GTP-bound form under conditions that allow active protein synthesis and an inactive

ppGpp-bound form when shortage of nutrients would be detrimental if not accompanied by a global decrease in translation initiation (Milon *et al.*, 2006).

### Kinetic partitioning model for the mRNA recruitment in translation

Recent kinetic and structural work provides a detailed framework for understanding the mechanisms of mRNA and fMet-tRNA<sup>Met</sup> selection during initiation. Initial mRNA docking to the 30S ribosomal subunit depends on the abundance of the mRNA in the cell. Furthermore, for different mRNAs the rates of the initial encounter with 30S subunit seem to vary up to 20-fold, mainly determined by the presence of secondary structure elements in the mRNA ( $k_1$  in Figure 8; (Studer & Joseph, 2006; Milon *et al.*, 2012)). Notably, other features of the TIR, such as the strength of the SD-aSD interaction or the presence of AUG as start codon are not monitored at this stage (Figure 8, checkpoint 1). Recruitment of structured mRNAs to the stand-by site at the platform of the 30S subunit (Yusupova *et al.*, 2006; Marzi *et al.*, 2007) is followed by unfolding of secondary structure elements and accommodation of the mRNA in the mRNA channel of the 30S subunit. The fate of an mRNA at this point depends on kinetic partitioning between mRNA dissociation ( $k_{-1}$  in Figure 8) and unfolding ( $k_2$ ; checkpoint 2): an mRNA with strong secondary structure elements which unfold slowly is more likely to dissociate from the 30S PIC and be replaced by a different mRNA than to enter the 30S IC (Studer & Joseph, 2006). The recruitment of the correct initiator tRNA, rather than of an incorrect elongator tRNA or non-formylated Met-tRNA<sup>Met</sup>, to the 30S PIC is mostly based on specific interactions between fMet-tRNA<sup>Met</sup> and IF2. The following start codon selection operates the affinity switch that leads to locking of the mRNA and fMet-tRNA<sup>Met</sup> on the 30S subunit. This comprises the next checkpoint for mRNA selection which favors the mRNAs that have a canonical start codon efficiently recognized by fMet-tRNA<sup>Met</sup> (high  $k_3$  value) and disfavors the mRNAs

with non-initiation codons (low  $k_3$  value) by delaying the transition towards the active 70S IC (checkpoint 3). IF1 and IF3 play a crucial role at this step by stabilizing IF2 (IF1), destabilizing the binding of tRNA (IF3), and modulating the conformation of the 30S subunit (IF1 and IF3). Finally, the transition from the 30S IC to the 70S IC provides the final checkpoint for the mRNA and tRNA selection which monitors the details of the SD-aSD interaction and the presence of the cognate mRNA-tRNA pairs (checkpoint 4) (Antoun *et al.*, 2006a; Milon *et al.*, 2008). Expression of reporter genes was affected by selected IF1 mutants in a TIR-dependent manner, underscoring the importance of IF1 in modulating the initiation efficiency (Surkov *et al.*, 2010). The 30S IC with an optimal mRNA and correctly bound tRNA provides a structural landscape that is favorable for 50S subunit docking (Grigoriadou *et al.*, 2007a; Milon *et al.*, 2008; Simonetti *et al.*, 2008; Julian *et al.*, 2011) (high  $k_4$  value) while the presence of an unfavorable mRNA delays 70S IC formation, thereby decreasing the translation efficiency of that mRNA (Milon *et al.*, 2008). Again, the conformation of the 30S subunit is crucial, as mutations in the 30S subunit that were isolated in IF1-dependent screens and do not involve intersubunit bridges affect the 50S subunit association (Belotserkovsky *et al.*, 2011). In addition, those 30S complexes on which codon-anticodon interaction is not perfect may dissociate faster (high  $k_{-3}$  value). Thus, the selection of mRNA and tRNA is achieved at several consecutive kinetic partitioning checkpoints which favor the forward steps for good substrates and disfavor or delay the entry of poor substrates further into initiation.

The selection against non-initiator tRNA provides a particularly good example for the importance of kinetic partitioning in initiation. The rate of the 50S subunit docking to the 30S IC is influenced by the nature of the tRNA: when the 30S IC is formed with Met-tRNA<sup>Met</sup> or Phe-tRNA<sup>Phe</sup>, the rate of 50S subunit joining is 60–400 times lower than with the complex containing fMet-tRNA<sup>Met</sup> (Antoun *et al.*, 2006a). This remarkable difference is observed only in the presence of IF3, which appears to

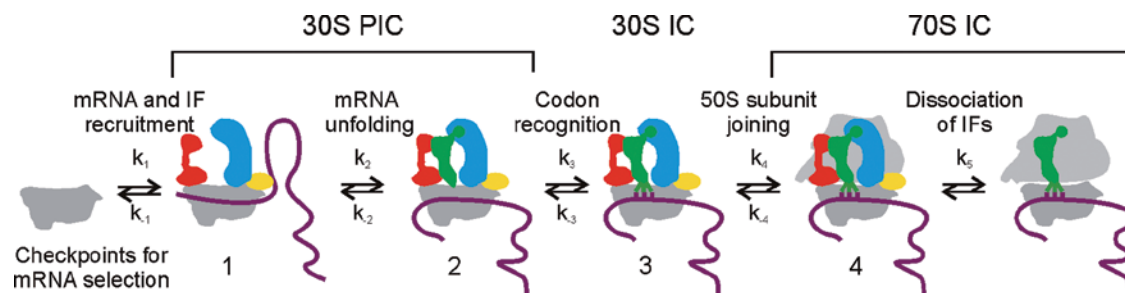


Figure 8. Kinetic partitioning mechanism of mRNA selection. Checkpoint 1, initial docking complex with mRNA bound to the platform of the 30S subunit (Marzi *et al.*, 2007). Checkpoint 2, mature 30S PIC. The step indicated as mRNA unfolding may entail a number or further intermediates, e.g. the formation of a stable SD-aSD interaction, rearrangements of the 30S subunit, and possibly the initial start codon sampling. As this checkpoint, mostly the secondary structure of the TIR is monitored. Recruitment of fMet-tRNA<sup>Met</sup> to 30S PIC, which is not shown as a separate step, constitutes a control checkpoint for the selection of fMet-tRNA<sup>Met</sup> against all other aminoacyl-tRNA due to the specific interactions with IF2. Checkpoint 3, 30S IC. Codon recognition is probably a composite step that triggers the stabilization of fMet-tRNA<sup>Met</sup> and destabilization of IF3 binding and promotes further conformational changes in the 30S IC; checkpoint 3 selects against mismatches in the codon-anticodon complex. Checkpoint 4, early 70S IC. Here, the fine properties of the TIR are sensed (Milon *et al.*, 2008). (See colour version of this figure online at [www.informahealthcare.com/bmg](http://www.informahealthcare.com/bmg))



destabilize tRNA binding and prevent 50S joining to a similar extent with any tRNA. However, the dissociation of fMet-tRNA<sup>fMet</sup> from the 30S IC remains significantly slower than the rate of 50S subunit joining, such that the tRNA is retained and the 30S IC can rearrange to the 70S IC. In contrast, the dissociation of Met-tRNA<sup>fMet</sup> or Phe-tRNA<sup>Phe</sup> is much faster than 50S subunit docking; as a result, incorrect tRNAs are discarded before the 70S IC can form and enter translation. An analogous selection mechanism applies when fMet-tRNA<sup>fMet</sup> in the 30S complex is bound to a near-cognate codon: the dissociation of the near-cognate 30S IC is faster, and the docking of the 50S subunit much slower, compared to the cognate complex (Milon *et al.*, 2008). As in the selection of the TIR described above, conformational rearrangements operated by the codon-dependent affinity switch alter the structural landscape of the 30S IC which is sensed by the 50S subunit, thereby affecting the rate of the transition to the 70S IC.

### Perspectives

Our understanding of fundamental aspects of *de novo* translation initiation has been greatly enriched recently and the future challenge will be to study other types of initiation, including the initiation on non-SD-led mRNAs and leaderless mRNAs. It will be important to work with full-length natural mRNAs in order to understand the level of complexity that is brought about by mRNA folding and unfolding on the ribosome. The less characterized mechanisms of coupled transcription/translation initiation and re-initiation should be further studied using the full richness of modern *in vitro* techniques. The major challenge for structural studies is the structure and dynamics of the 30S PIC, which has not been solved so far. Finally, the most exciting goal will be to follow how the ribosome transfers the environmental signals into changes in the proteome by modulating the kinetic checkpoints of initiation. Due to the progress in genomics, proteomics, biophysical techniques and structural methods, these questions are now within reach.

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### Declaration of interest

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