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Review

A structural view of translation initiation in bacteria

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Abstract. The assembly of the protein synthesis machinery occurs during translation initiation. In bacteria, this process involves the binding of messenger RNA (mRNA) start site and fMet-tRNA^{fMet} to the ribosome, which results in the formation of the first codon-anticodon interaction and sets the reading frame for the decoding of the mRNA. This interaction takes place in the peptidyl site of the 30S ribosomal subunit and is controlled by the initiation factors IF1, IF2 and IF3 to form the 30S initiation complex. The binding of the 50S subunit and the ejection of the IFs mark the irreversible transition to the elongation phase. Visualization of these ligands on the ribosome has been achieved by cryo-electron microscopy and X-ray crystallography studies, which has helped to understand the mechanism of translation initiation at the molecular level. Conformational changes associated with different functional states provide a dynamic view of the initiation process and of its regulation.

Keywords. Initiation of translation, regulation of translation, ribosome structure, mRNA, initiation factor, initiator tRNA, functional ribosomal complexes.

Introduction

Bacteria have evolved numerous mechanisms to rapidly adapt their growth in response to environmental changes. Besides the transcriptional control, translation initiation is one of the key steps submitted to tight regulation. Due to the transcription-translation coupling, the ribosome is able to initiate translation already on a nascent mRNA. This has also the advantage of protecting the mRNA against degradation. Translation initiation is the rate-limiting step of the protein synthesis since the ribosomes assemble on the mRNA in the order of seconds, while elongation occurs at a faster rate of several amino acids per second [1–3]. In, *E. coli*, many *cis*- or *trans*-acting regulators act at this step by modulating the interaction of the mRNA onto the ribosome, leading either to activation or to repression of protein synthesis (for reviews [4–6]). In contrast to eukarya and archaea (for reviews [7, 8]), the initiation process in bacteria involves a rather low number of *trans*-acting

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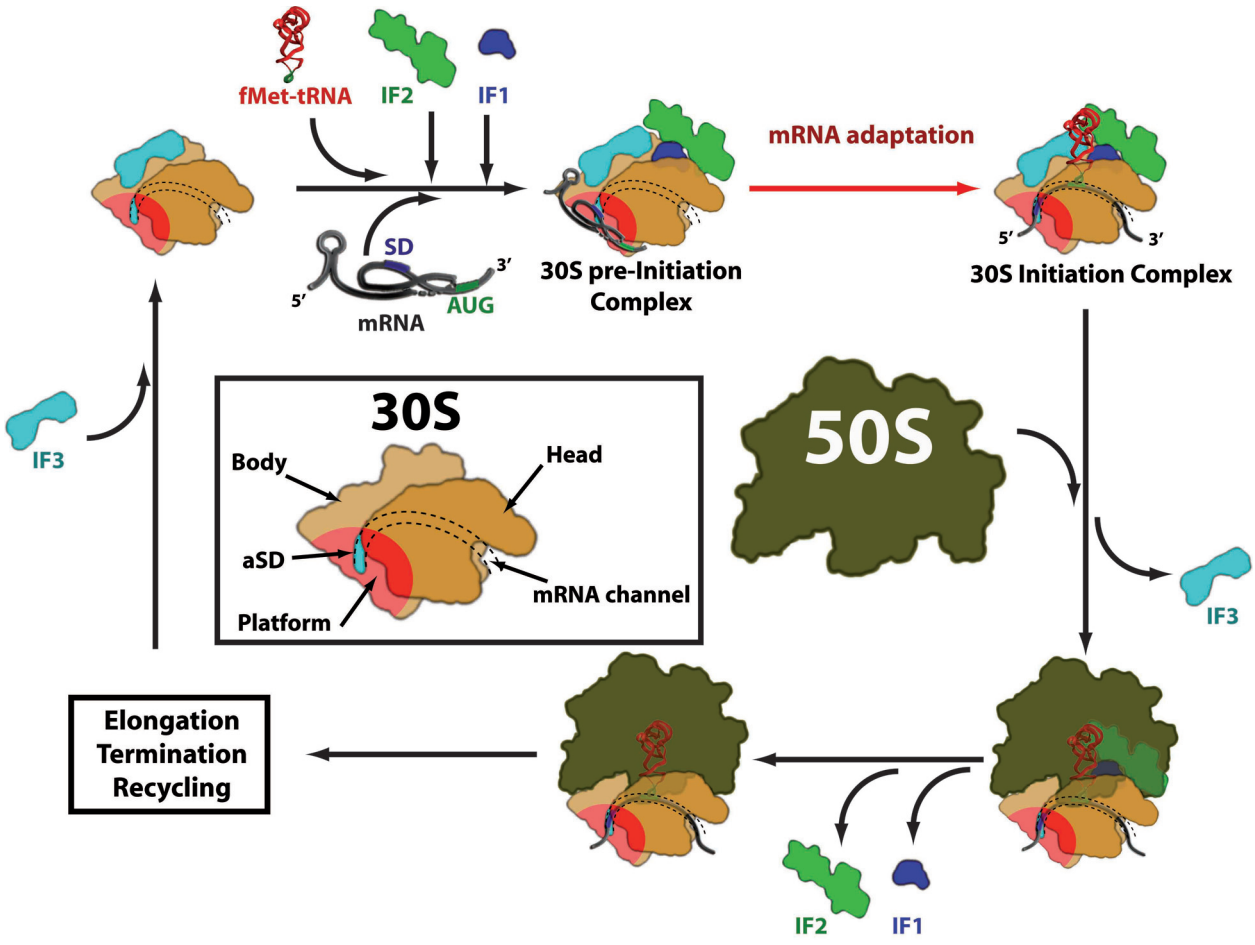

Figure 1. Schematic view of the initiation process. Formation of 30S (30SIC) and 70S (70SIC) translation initiation complexes, containing ribosomes (30S subunit in orange, 50S in brown), initiator fMet-tRNA^{fMet}, mRNA and initiation factors IF1 (in blue), IF2 (in green) and IF3 (in light blue). View of 30S ribosomal subunit and ribosome from the top. The platform of the 30S is in red with the anti-Shine-Dalgarno (aSD) sequence in cyan. Structured mRNA binds to 30S in two distinct steps: the docking of the mRNA on the platform of the 30S subunit forms the pre-initiation complex that is followed by the accommodation of the mRNA into the normal path to promote the codon-anticodon interaction in the P site [21]. The resulting 30SIC engages the 50S subunit to form the 70SIC from which the initiation factors are expelled and the synthesis of the encoded protein can proceed through the elongation, termination and ribosome recycling phases (adapted from [6]).

ligands. Indeed, the ribosome together with the aminoacylated and formylated initiator tRNA (fMet-tRNA^{fMet}), the mRNA, and the three initiation factors (IF1, IF2, IF3) assemble in a multi-step process to form an active initiation complex (Fig. 1).

A score of highly important breakthroughs have been carried out in the last few years yielding high-resolution structures of the ribosome associated with different ligands. These works have set the basis for understanding the mechanisms involved in protein synthesis at the molecular and atomic level. In 2000–2001, the elucidation of high-resolution crystal structures of the isolated prokaryotic ribosomal 30S and 50S subunits [9–12] and of the whole 70S ribosome [13, 14] showed detailed views of this large and intricate ribonucleoprotein complex. The path of an unstructured mRNA (gene 32 mRNA) with the Shine and Dalgarno (SD) sequence (a sequence specific to prokaryotes that base pairs with the 3' end of the 16S ribosomal RNA) and the AUG start and tRNA binding sites were determined on *Thermus thermophilus* ribosome [13, 15]. Later on, crystallographic studies on several initiation complexes provided the mechanism by which the ribosome accommodates regulatory elements of structured mRNAs [16], and visualized the movement of the SD-anti-SD helix during the initiation process [17, 18].

Moreover, cryo-electron microscopy (cryo-EM) reconstructions have provided important insights into the mechanism of translation initiation. For example, several translation factors and a folded mRNA have been localized on the ribosome and the conformational changes associated with different functional states of the complexes have been described [19–22].

This review summarizes recent knowledge acquired on structure-function relations of bacterial translation initiation mainly derived from X-ray and cryo-EM studies. Particular emphasis will be given to the mRNA accommodation step that is the site of many regulatory events.

A snapshot of the multi-step pathway of the initiation of translation

Eubacteria initiate translation *via* a multi-step process (translation initiation) in which several successive ribosomal complexes (Fig. 1), which differ in composition and conformations, lead to the formation of the active 70S ribosomal initiation complex (70SIC). In this form, peptide bond formation can then proceed. In the 70SIC, the start site and the reading frame of the mRNA are set and the fMet-initiator tRNA is correctly positioned into the P (peptidyl) site, thus connecting the decoding center (DC) of the 30S subunit with the peptidyl transferase center (PTC) of the 50S subunit.

The process starts at the level of the 30S subunit that is maintained dissociated from the 50S subunit by the action of initiation factor IF3. The other two factors (IF2 and IF1) subsequently bind the 30S together favoring the recruitment of the fMet-tRNA^{fMet} and the mRNA. Collectively, the three bacterial IFs kinetically control the process and assure its fidelity and accuracy (for a review [23]). The mRNA is then accommodated on the 30S *via* a process that comprises at least two steps (see section "mRNA binding and adaptation"), leading to the formation of an active 30S initiation complex (30SIC). In this complex, the first codon of the mRNA, usually AUG, physically interacts with the anticodon of the initiator tRNA in the P site. The last step of the initiation process involves the docking of the large ribosomal subunit (50S) to the 30SIC. This transition triggers the hydrolysis of the GTP molecule bound to IF2, makes the choice of the start site irreversible and dissociates spurious complexes [24]. The machinery then proceeds to the elongation phase during which rapid reading of the mRNA occurs [1–3].

The major components of the initiation complex

Structure of the 30S ribosomal subunit

The bacterial small ribosomal subunit, which has a sedimentation rate of 30 S (Svedberg constant), contains 21 proteins and an rRNA of around 1500 nucleotides (the length varies in different species) with a sedimentation coefficient of 16 S. During the initiation process, the 30S ribosomal subunit plays a key role in the selection of the correct start site on the mRNA, facilitating base pairings with the anticodon of the tRNA.

The first image of the 30S subunit, obtained by electron-microscopy (EM) investigation of negative stained particles, already showed its main morphological features [25–27]. With the development of cryo-EM reconstructions obtained from single particles, more precise information became available [28]. The 30S resembles a "chick" with its upper third constituted by a "head" with a tapered end (the "beak"), which is connected to the remaining lower two-third called "body" by a narrow "neck" (Figs 1 and 2). The main part of the 30S body presents three morphological domains characterized as "shoulder", "platform" and a lower protrusion called "spur" or "toe". The front is the side of the 30S subunit that faces the 50S subunit, whereas the back is the side exposed to the cytoplasm. This description allowed the construction of the first models for the 30S subunit based on cross-linking and probing data [29, 30]. In 2000, the Yonath group [9] and Ramakrishnan group [10] published the high-resolution structure of the *T. thermophilus* 30S subunit at 3.3 and 3.0 Å respectively, giving atomic insights into the 30S subunit structure. This was a major breakthrough in understanding the architecture and function of the ribosome. These data showed that the structure of 16S rRNA is largely responsible for the global shape and morphological features of the small subunit (Fig. 2). Distributed in a non-uniform way, the proteins are concentrated in the top, side and back of the 30S subunit. Notably, many proteins have long extensions that make intimate contacts with the RNA, but none of them bind entirely inside an RNA domain [31]. Almost entirely constructed of RNA and located between the head and the body of the small ribosomal subunit, there is the decoding center where the codon-anticodon interaction takes place during the initiation (fMet-tRNA^{fMet}) and elongation phases. This functional region of the 30S ribosomal subunit, among the other components, contains the 3' and 5' ends of the 16S rRNA and the upper part of helix 44 [9], a key element for ribosome function.

Beside the decoding center, at least two more regions have been described having specific activities in translation. These regions are the helicase center constituted by proteins S3, S4 and S5 devoted to the unfolding of structures at the 3' end of mRNAs during the elongation [14, 32], and the platform center which is important for the binding and adaptation of the mRNA during the initiation [16, 21].

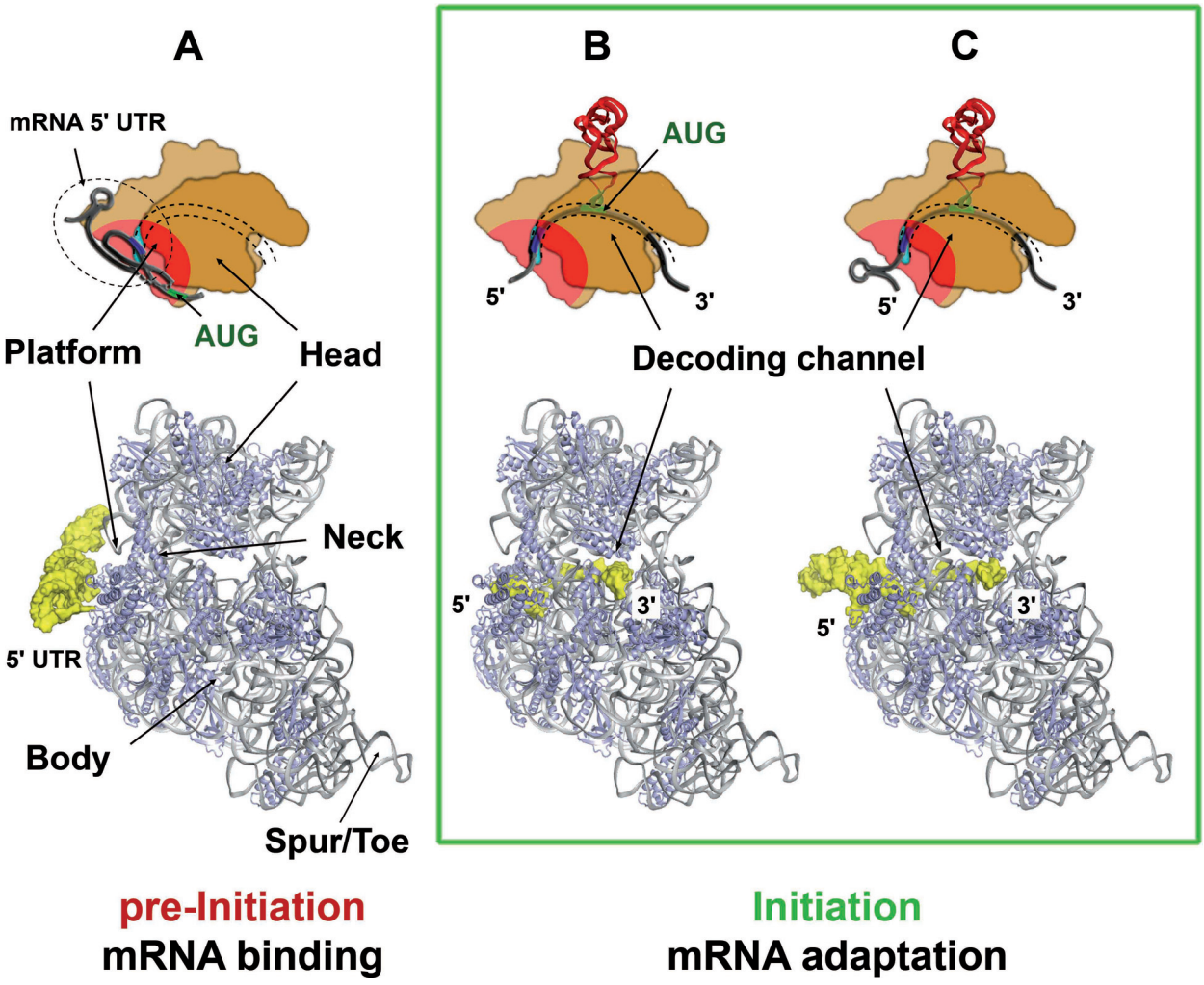

Figure 2. mRNA binding and adaptation into the mRNA channel of the 30S subunit. Three structures describe the mechanism through which a structured mRNA is first recruited on the platform of the 30S and then adapted into the 30S channel. (*A*) The first binding event of a structured mRNA on the platform has been described by Marzi et al. [21] using cryo-EM data. At this step the mRNA is anchored on the 30S *via* the SD-aSD interaction and the decoding channel is still empty. Stabilization of the mRNA at the docking step leads to translation repression through an entrapment mechanism. (*B*) The subsequent adaptation step is described by the crystal structure of the 70S initiation complex [14, 17] showing the path of the mRNA through the mRNA decoding channel encircling the neck of the 30S subunit. The transition from A to B demands unfolding of the mRNA structures involving the nucleotides from the SD to the +16 position. In this adapted state, the codon-anticodon interaction between the first codon and the initiator fMet-tRNA^{fMet} takes place in the ribosomal P site. (*C*) The crystal structure of *thrS* mRNA carrying a translational operator domain bound to the ribosome [16] shows how other nucleotides preceding the SD sequence can be maintained in a folded state on the platform even after the adaptation of the mRNA in the channel. These folded elements are used for translation regulation since they can be recognized by ligands able to compete with the ribosome. The landmarks of the 30S subunit (head, body, neck, platform and spur/toe) are indicated in (*A*).

mRNA binding and adaptation onto the 30S subunit

Binding of the mRNA to the 30S subunit is one of the most critical phases of the initiation process. For many bacterial mRNAs, selection of the appropriate initiation codon and of the translational reading frame depends largely on the formation of a short duplex between the SD sequence GGAGG, located in the 5' untranslated regions (UTR) of mRNAs, and the anti-SD (aSD), a conserved sequence present at the 3' end of 16S rRNA [33] (Fig. 2). A pre-initiation complex has been modeled by forming a complex of the *T. thermophilus* ribosome with a short RNA fragment containing the aSD sequence. The X-ray structure of this complex has shown the position of SD duplex on the "platform" of 30S subunit [17, 34].

Other mRNA sequence elements present in the translation initiation region (TIR) have been shown to affect the initiation complex formation: the initiation codon, the spacing region between initiation codon and SD, the non-random distribution of nucleotides upstream of the SD and downstream of the initiation codon, and secondary structure elements of the mRNA present in the whole TIR [6, 21, 35–37]. For example, in *E. coli,* mRNAs with a weak SD

sequence usually carry a pyrimidine-rich region 5' to the SD that acts as a recognition motif for the ribosomal protein S1 [38, 39]. Thus, ribosomal protein S1 overcomes a potential problem to anchor the mRNA on the 30S. Structured elements in the ribosome binding site of mRNAs play an important role in the control of translation initiation. Although the mRNA is almost unstructured within the mRNA channel onto the 30S subunit, recent studies indicate that the platform of the 30S subunit is able to recognize and bind structured mRNAs [16, 21, 36] (Fig. 2). It was shown that these structured mRNAs bind to the 30S in a two-step process [36, 40]. The first step, commonly referred to as the pre-initiation step is rapid and transient, and involves docking of the mRNA onto the platform of the 30S subunit. Recent work shows that the platform of the 30S is well appropriate for the docking of structured mRNAs [21]. The anchoring step may either involve the SD, when accessible to form the SD-aSD helix [21], or require an unpaired sequence upstream of the hairpin structure sequestering the SD sequence of the mRNA [36, 37, 40]. At this point, despite the simultaneous presence of mRNA and initiator tRNA on the 30S, the first codon-anticodon interaction is not yet formed. The resulting pre-initiation complex (Figs 1 and 2) is therefore not active and can be easily displaced. This step is followed by a slower one in which the mRNA is accommodated, with the help of the IFs and the fMet-tRNA^{fMet}, into the mRNA channel marking the formation of the active 30SIC.

The cryo-EM structure of the 30S pre-IC was recently determined [21] taking advantage of the fact that structured mRNAs can be stabilized on the 30S if the accommodation step is blocked. This mechanism, called entrapment, is used by *E. coli* ribosomal proteins S15 and S4 to repress the translation of their own mRNAs and consequently of their whole operons [41, 42]. The structure of the 30S pre-IC (*i.e.*, in the presence of the repressor protein S15) shows that the pre-accommodated mRNA is bound in its folded state on the platform of the ribosome (Fig. 2A). Several ribosomal proteins located at the platform (S2, S7, S11 and S18) are either in contact or in close proximity to the folded mRNA (Fig. 2A). A systematic structure and sequence analysis revealed that conserved residues of these proteins form patches of positive charges on the surface of the platform close to the trapped mRNA [2]. Strikingly, structured 5'UTR of several prokaryotic and eukaryotic mRNAs have been found to partially overlap with some of those conserved residues. This is the case for *E. coli rpsO* [21] (Fig. 2A) and *thrS* mRNAs [16] (Fig. 2C), and for the Internal Ribosome Entry Site (IRESs) of the hepatitis C (HCV) virus [43, 44] and the cricket paralysis virus (CrPV) [45]. Rather unexpectedly, 5' poly(A)- or poly(U)-rich extensions upstream of the SD sequence also can form stable stem-loop structure on the platform of the 30S subunit [17]. This suggests the existence of a common platform docking site for structured mRNA during pre-initiation, subsequently followed by the adaptation of the mRNA into the mRNA channel. The delay time between docking and adaptation reflects the stability of the mRNA structures that need to be eventually melted to promote the codon-anticodon interaction. Thus, ligands (proteins, metabolites, non-coding RNAs, etc.) that stabilize the folded state of the mRNA, can block the ribosome at the pre-initiation stage by preventing the initiator codon from reaching the decoding site inside the ribosome [21]. Moreover, the platform is a wide and accessible open space, providing the possibility to get an on and off binding of regulatory ligands directly on the platform (such as the repressor protein S15 to the *rpsO* mRNA). It thus underlines the importance of this ribosomal binding site in timing and regulating translation.

From the platform, the mRNA unfolds and adopts the classical path in the mRNA channel, leading to the formation of an active translation initiation complex (Fig. 2B, C). The adaptation process has been monitored by site-directed cross-linking [46] and fluorescence resonance energy transfer (FRET) experiments [40], showing that the final adjustment in the decoding center is promoted by the simultaneous presence of initiation factors and fMet-tRNA^{fMet}. Some of the ribosomal proteins of the platform may facilitate the unfolding of mRNA structure promoting the adaptation. At this regard, beside ribosomal proteins S7, S2, S11, S18 and S21, S1 is also expected to be in the vicinity of the platform binding site. Since it is known that this protein has some helicase properties [47], it is possible that its role in translation initiation could also concern the adaptation process. More experiments are necessary to specifically investigate this issue.

The X-ray structures of the 70S-mRNA-tRNA initiation complexes [14, 17] show that, in the adapted state, about 30 nucleotides encompassing the SD and the AUG codon are lying in a groove that encircles the neck of the 30S subunit, confirming previous biochemical and cross-linking indications [48–50] (Fig. 2B, C). Downstream of the SD interaction site (see below), the mRNA passes through a short tunnel, delimitated by the C-terminal α-helix of ribosomal protein S7, a loop connecting its two unique β-strand and the 16S rRNA helices h23 and h28. In the decoding tunnel, at the interface region, interactions between the mRNA and 30S are established almost exclusively through the 16S rRNA, except for some contacts with protein S12 in the A site. The phosphate

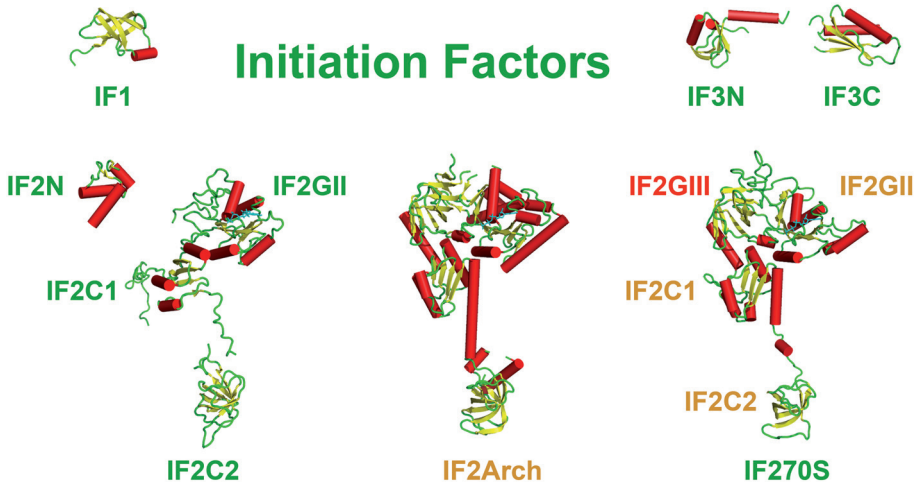

Figure 3. Structures of bacterial translation initiation factors. The structures of IF1 and of different individual domains of IF3 and IF2 are represented together with a model of bacterial IF2 based on the crystal structure of archaeal aIF5B (IF2Arch). The figure has been assembled using the following pdb files: *E. coli* IF1 NMR structure [73] (file 1AH9.pdb); *B. stearothermophilus* IF3 N domain crystal structure [61] (file 1TIF.pdb); *B. stearothermophilus* IF3 C domain crystal structure [61] (file 1TIG.pdb); *E. coli* IF2 N domain (fragment) NMR structure [87] (file 1ND9.pdb); *B. stearothermophilus* IF2 C1 domain NMR structure [89] (file 1Z9B.pdb); *B. stearothermophilus* IF2 C2 domain NMR structure [88] (file 1D1N.pdb); *B. stearothermophilus* IF2 GII domain NMR structure (Rolf Boelens and Claudio Gualerzi, personal communication); archaeal aIF5B-GDPNP crystal structure [77] (file 1G7T.pdb); model of *T. thermophilus* IF2-GDPCP obtained using the structures of the individual domain of IF2, the general architecture of aIF5B and the fitting in the 70SIC cryo-EM map [20].

group of nucleotide 1401, at the top of helix h44, is positioned directly in the path of the mRNA between the P and A codon, forcing the mRNA backbone to kink between the two codons. Downstream from the A codon, the mRNA passes through a larger tunnel, formed between the head and shoulder of the subunit composed by the proteins S3, S4 and S5. These ribosomal proteins form a ring able to unwind structured mRNA tracts during elongation [14, 32, 51].

Structure-function of the initiation protein factors

The three IFs exert distinct but coordinated functions that are required (1) to enhance the rate of the formation of the initiation complex, and (2) to insure fidelity.

Initiation factor IF3. IF3 dissociates the 70S during ribosome recycling. By binding strongly to the 30S subunit it maintains the cellular pool of the 30S subunit free to initiate translation [52]. It stimulates the P site codon-anticodon interaction between the fMet-tRNA and mRNA, thus promoting the formation of the correct 30SIC. Moreover, IF3 acts as a fidelity factor that destabilizes the interaction of an incorrectly bound near- or non-cognate aminoacyl-tRNA in the P site [53]. As a consequence, the dissociation rate of non-canonical and pseudo-30S initiation complexes are enhanced [54–56]. IF3 is structurally characterized by two domains [57, 58], the N- and C-terminal domains which are connected through a ~45-Å flexible lysine-rich linker [59, 60]. The structures of the separate domains were determined by X-ray crystallography [61] and nuclear magnetic resonance (NMR) [57, 62, 63]. While the N-terminal domain of IF3 is characterized by a globular α/β fold, the C-terminal part consists of a two-layer α/β sandwich (see Fig. 3). There are contradictory data concerning the localization of IF3 on the ribosome. The C-terminal domain was placed at the interface side of the platform based on different approaches such as immuno-EM [64], cryo-EM data [65], chemical probing [66, 67], NMR [68] and site-directed chemical probing [69]. In contrast, the X-ray structure of the 30S subunit crystals soaked with IF3C shows its localization on the solvent side of the platform [70]. For the N-terminal domain, the situation is even more complex since no high-resolution picture of IF3-Nter on the 30S is available and biochemical approaches such as chemical probing position this domain in different regions [69, 67].

Initiation factor IF1. Several functions have been attributed to the smallest initiation factor IF1 (8.2 kDa in *E. coli*). It promotes a more efficient binding of IF2 and IF3 to the 30S subunit, thus stimulating their activities [71, 72]. IF1 cooperates with IF2 to ensure the correct location of the initiator tRNA in the P site. The solution structure of *E. coli* IF1, determined by NMR [73], is characterized by a rigid five-stranded β-barrel flanked by flexible and disordered extremities. IF1 fold is similar to that of

proteins belonging to the family of oligonucleotide and oligosaccharide binding (OB) proteins. This motif is present in several RNA-binding proteins, notably in ribosomal proteins like S1, S17, L2 and L17. The high-resolution structure of the 30S-IF1 complex shows that IF1 binds the 30S subunit in proximity to the A site [74]. The protein lies in a niche created by the ribosomal protein S12, the penultimate stem loop (530 loop) and helix 44 of the 16S RNA. IF1 interacts specifically with the two adenines A1492 and A1493 of the 16S RNA, causing these nucleotides to flip out [74]. IF1 induces in turn conformational changes over a long distance within the 30S affecting the association-dissociation equilibrium of the ribosomal subunits [55, 75]. IF1 may also contribute to the binding and correct positioning of the initiator tRNA in the P site.

Initiation factor IF2. The most important contact occurring during translation initiation is the specific interaction of IF2 with the initiator tRNA. IF2 is the largest of the three factors with a molecular mass of around 97.3 kDa in *E. coli* and belongs to the family of the GTP-GDP binding protein like the elongation factors EF-Tu, EF-G [76] and the termination factor RF3. In this review, we used the nomenclature based on the functional characterization of the proteolytic fragments of *E. coli* IF2. At the extremities, a conserved C-terminal region consists of two subdomains (IF2C1 and IF2C2), while the less conserved N-terminal region encompasses domains N1 and N2. Finally, the G domain, containing the GTP binding site, is constituted by three separate subdomains (GI, GII and GIII). So far, the main source of structural information regarding IF2 comes from the high-resolution structures of the homologous protein IF2/eIF5B (aIF2) in *M. thermoautotrophicum* in either its free form, or bound to GDP and GDPNP [77]. The structure of aIF2 shows an extended shape, a characteristic "chalice" form that is derived from the arrangement of the four domains (Fig. 3). Structurally similar to $p21^{Ras}$ and to the N-terminal domain of EF-Tu and EF-G, the G domain (residues 1–225, corresponding to the GII-GIII bacterial domains) is organized in an eight-stranded β-sheet of mixed polarity. An α-helix (H8) of 17 residues connects the β barrel domain homologous of GIII (residues 231–327 in aIF2 adopting a structure similar to that of domain II of EF-Tu and EF-G) to a four-stranded β-barrel domain homologous of C1 (residues 344–445 in aIF2, structurally homologous to domain III of EF-Tu and EF-G). From the dorsal face of domain C1, helix H12 protrudes towards the homologous of domain C2, an eight-stranded antiparallel β-sheet followed by two α-helices (Fig. 3). Although aIF2 is structurally homologous to bacterial IF2 (40% of identity [78]), this factor has functional properties slightly different from those of IF2 [23, 54]. In fact, the factor that recruits the initiator tRNA on the small subunit of both archaeal and eukaryotic ribosome is the trimeric factor eIF2 [79–81]. In these organisms, it has been proposed that aIF2, like its eukaryal homologue eIF5B, could be solely implicated in promoting subunit joining in a late initiation step where it would interact with the Met-$tRNA_i$ [82–85]. Interestingly, recent data suggest that aIF2 can substitute the action of eIF2 under stress conditions to initiate translation at the hepatitis C virus IRES [86]. From the structural point of view, aIF2 lacks a large polypeptide segment at its N-terminal domain.

The NMR structures of a fragment of the N-terminal domain of *E. coli* IF2 [87] as well as the C-terminal domain of *B. stearothermophilus* IF2 [88, 89] have been characterized allowing the description of the specific features of bacterial IF2 (Fig. 3). In contrast to the C-terminal regions, the N-terminal region is less conserved in length and sequence among the species. Its function is to enhance the interaction of IF2 with the 30S and 50S ribosomal subunits [90–93]. The N-terminal domain of *E. coli* IF2 is connected to IF2GI *via* a long and flexible linker [94]. Highly conserved, the IF2GII domain contains the complete GDP/GTP binding motif characteristic of many GTPases, while the IF2GIII is structurally homologous to domain II of the elongation protein factors. Finally, the C-terminal part of IF2 consists of two separate modules, IF2C1 and IF2C2. The structural core region of IF2C1 shows a flattened disk shape due to a central four-stranded parallel β−sheet that is surrounded by three α-helices. The latter C2 module has a β−barrel structure and contains all molecular determinants that are necessary for the recognition of the formylated α-amino group of fMet-tRNA, specific of the bacterial IF2 protein. The structure of the entire bacterial IF2 is still lacking and its localization on the 30S has been an unresolved puzzle for many years.

Structure of initiator $tRNA^{fMet}$: an unusual structure of the anticodon arm

The X-ray structure of bacterial initiator $tRNA^{fMet}$ has been determined first at 3.5-Å resolution [95]. Later, the crystal structure of *E. coli* methionyl-$tRNA^{fMet}$ transformylase (MTF) complexed with formylmethionyl-$tRNA^{fMet}$ was solved at 2.8-Å resolution [96]. These structures, together with biochemical studies, provide functional explanations for several of the specific characteristics of the initiator tRNA in contrast to the elongator tRNA (for a review [97]). These differences include: a formyl group located on the methionine that is specifically recognized by bacterial

IF2; nucleotides C1 and A72 that are not base-paired in the initiator tRNA to facilitate the positioning of the methionine bound to the 3' end of the tRNA within the catalytic site of MTF; the A11-U24 base pair in the D-arm being specifically recognized by MTF; the three successive G-C pairs in the anticodon arm of the initiator tRNA that confers a specific IF3-dependent selection of the initiator tRNA to the P site [98]. It was also described that this particular G-C-rich motif found in all initiator tRNAs confers a specific structure to the anticodon arm [99]. Interestingly, A1339 and G1338 of 16S rRNA form type I and type II A-minor interactions with the G-C base pairs 30–40 and 29–41 [17, 100], while A790 makes contacts through its ribose phosphate with the other side of the anticodon arm of the tRNA. A genetic analysis shows that these minor groove interactions involving G1338-A1339 of 16S rRNA play a role in the discrimination of the initiator tRNA by IF3 [101]. Thus, these networks of interactions involving the G-C-rich anticodon stem may provide stabilization and selectivity for the initiator tRNA in the P site. Recently, another crystal structure of *E. coli* initiator $tRNA^{fMet}$ has been determined at 3.1-Å resolution [102]. This study reveals a unique structure of the anticodon domain. It involves (i) a Cm32-A38 base pair, thus extending the length of the anticodon arm by one base pair; (ii) a base triple between A37 and the G29-C41 pair in the major groove of the anticodon stem, and (iii) a modified stacking organization of the anticodon loop. Since this peculiar conformation of the anticodon loop is not observed on the 70S complexes [13, 17, 100, 103], it may be a conformation required during the first stages of protein synthesis when the tRNA needs to accommodate into the P site.

Towards the structure of the 30S initiation complex

Most recently, the structure of the 30S ribosomal subunit in complex with the fMet-$tRNA^{fMet}$, mRNA and the initiator factors IF1 and GTP-bound IF2 has been determined by cryo-EM [22]. This structure reveals the molecular interactions between the 30S and its partners at the initiation step. Electron density that fits with the size and positioning of IF1 is visible close to the decoding site, consistent with the 30SIF1 crystal structure [74]. The cryo-EM structure shows that IF2 is organized in two main modules corresponding in size to domains I/II (GII/GIII) and III/IV (C1 and C2), while the N-terminal and GI domains are not visible, possibly due to a higher flexibility on the 30SIC (Figs 3 and 4A). Notably, the topology of the domains of IF2 suggests that, when IF2 is bound to the 30S subunit, domain III (C1) appears to be shifted toward domain IV (C2). The front side of the 30S subunit presents a long density, attributable to IF2 and fMet-$tRNA^{fMet}$, stretched out on the surface of the 30S and connected with it *via* two anchor points. The contact of domain I/II of IF2 with helices h5 and h14 of the 16S RNA represents the first anchor point, while the second one is obtained through the interaction of the decoding stem of the fMet-$tRNA^{fMet}$ with the neck of the 30S subunit. This bridge is stabilized through a strong interaction that the β-barrel C-terminal domain of IF2 establishes with the conserved hexa-nucleotide CAACCA and part of the double-stranded region of the initiator tRNA acceptor stem. Induced by the interaction with IF2, the 3' tail of the acceptor stem is kinked around the position of residues C72-C73 and, as the main result of this conformational change, the interface between IF2 and fMet-$tRNA^{fMet}$ is enhanced.

Concerning the interaction of the initiator tRNA with the 30S subunit, the cryo-EM structure of the 30SIC complex [22] shows that the decoding stem of the initiator tRNA and the head of the 30S are similar to that observed in the crystal structures of *T. thermophilus* 70S ribosomes bound to mRNA and tRNAs [13, 17, 100, 103]. The anticodon stem is accommodated in a pocket of the 30S formed by helices h24, h29, h30, h31, h34 and h44 of the 16S RNA and ribosomal proteins S9 and S13. On the side oriented towards the A site, the tRNA anticodon stem interacts with three lysine residues of ribosomal protein S13, as seen in the 70S ribosome crystal structure [17, 100, 103]. On the other side, the anticodon stem interacts with helix h24 and the extended C-terminal tail of protein S9.

However, compared to 70S tRNA complexes, in the 30SIC the P-tRNA appears to be distorted [22]. The decoding loop of the fMet-$tRNA^{fMet}$ is bent towards the mRNA, apparently to promote the codon-anticodon interaction without further constraints. The conformational change of the initiator tRNA most likely occurs during the interaction with the 30S and IF2, thus accompanying the codon-anticodon recognition in a way very similar to the binding of the elongator tRNAs in the A site. Indeed a model has been proposed for the elongator tRNA interacting with EF-Tu (in the A/T state), in which the anticodon loop is kinked and the T and D loops of the elbow are more flexible [104–107]. These conformational changes of the tRNA that accompany GTP hydrolysis of both EF-Tu and IF2, illustrate the intrinsic flexibility of the tRNA as previously stated [108, 109]. These data highlight how the decoding process is facilitated by a dynamic interplay between the ribosome and the tRNA in which the tRNA acts like a molecular spring.

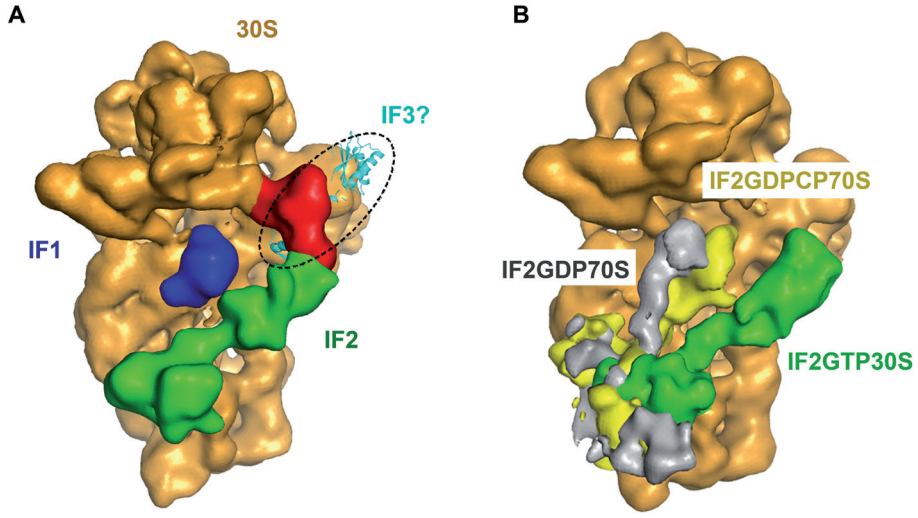

Figure 4. 30S initiation complex and IF2 positions during translation initiation. (*A*) Model for the 30S initiation complex. The model has been obtained using the structure of the 30S initiation complex with mRNA, fMet-tRNA^{fMet}, IF1 and IF2-GTP [22]. The localization of IF3 was derived from chemical probing [67, 69]. 30S subunits are represented in gold, IF2 in green, IF1 in blue, IF3 in cyan and fMet-tRNA^{fMet} in red. (*B*) IF2 positions before and after joining of the ribosomal subunits according to the 30S initiation complex structure [22] and the 70S initiation complex structures [20] representing the states before (IF2 GTP 30S, green), after GTP-hydrolysis (IF2 GDPCP 70S, yellow) and after P_i release (IF2 GDP 70S, gray); the 30S model in the back is shown for general orientation. Adapted from [22].

The specific structural features of the anticodon stem-loop of the initiator tRNA [102] might confer specificity toward the accommodation in the 30S P site and could play a role in the proofreading activity exerted by IF3 that assures the *bona fide* of the first codon-anticodon interaction. Since the cryo-EM structure of the 30SIC [22] has been obtained in the absence of IF3, one cannot completely rule out that the positioning of the tRNA might be slightly different in the presence of the three IFs. The role of IF3, in proof-reading and correct positioning of the initiator tRNA within the translation initiation complexes therefore remains to be addressed.

The 70S initiation complex structure: action of initiation factors IF1, 2 and 3

The classical view of the last step of the initiation of protein synthesis shows that a stable 30S initiation complex associates with the 50S ribosomal subunit to form a 70S initiation complex. This process comprises by the ejection of initiation factors IF1 and IF3 from the 30S subunit. Concomitantly, the adjustment of the fMet-tRNA^{fMet} in the ribosomal P site and the association between the subunits operate as the results of GTP hydrolysis and P_i release by IF2. After the release of IF2, the ribosome enters the elongation phase that allows A site binding of the elongator aminoacyl-tRNA and translocation. Clearly, the initiation of translation involves not only the interplay between the ribosomal subunits but also the binding and the dissociation of the initiation protein factors, and in the case of IF2, the hydrolysis of GTP. All these events are accompanied by conformational changes of the ribosome and the binding partners.

Single particle cryo-EM coupled to high-resolution X-ray structure determination has been an appropriate strategy to visualize the transition states (functional states) of the ribosome. In 2005, two cryo-EM studies described intermediate states of the process leading to the formation of a 70S initiation complex from two different organisms. Both the structure of the *E. coli* 70SIC stalled by GDPNP and containing the three initiation factors [19] and that of the *T. thermophilus* 70SIC with IF2 [20] shows that IF2 is located in the subunit cleft of the 70S and makes contacts with the two ribosomal subunits. The *E. coli* 70SIC structure mimics the state of the initiation complex after the binding of the 50S subunit, but preceding GTP hydrolysis on IF2, which is still bound to the fMet-tRNA. Myasnikov et al. [20] produced two different structures of *T. thermophilus* 70S ribosome complexed with the initiator tRNA, GDPCP- or GDP-bound IF2, in the absence of IF1 and IF3. Interestingly, these structures show no interaction between IF2 and fMet-tRNA.

The comparison between the three structures in combination with kinetics data [24, 110] illustrates in some way the movement of IF2 leaving the ribosome (Fig. 4). The GTP-bound state of IF2 is restricted to the 30SIC since GTP hydrolysis occurs immediately upon 50S subunit joining [24]. Indeed, the use of GDPNP and the presence of IF1 and IF3 [19] allowed

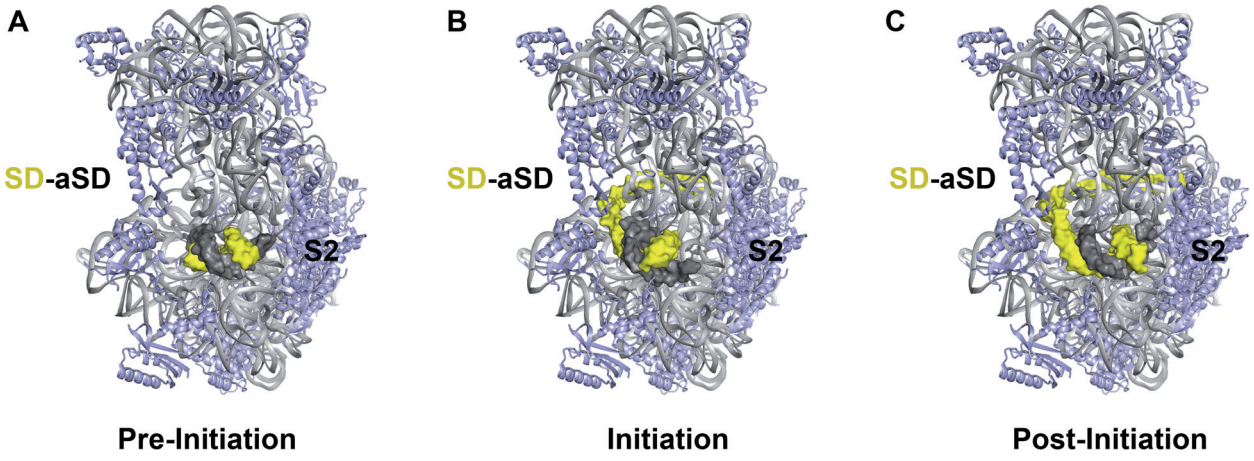

Figure 5. mRNA movement on the ribosome. (*A*) Pre-initiation state, (*B*) initiation state, (*C*) post-initiation state. The Shine and Dalgarno (SD) sequence of the mRNA is in yellow and the anti-SD sequence present at the 3' end of 16 rRNA is in dark gray.

the visualization of a transient complex leading to the 70SIC in which the bond between IF2 and fMet-tRNA^{fMet} is not yet resolved. When compared with the kinetics data on the transition from the 30SIC to the 70SIC [24, 110], the structure by Allen et al. [19] can be considered as an illustration of the state preceding GTP hydrolysis, whereas the structures by Myasnikov et al. [20] represent the states following GTP hydrolysis. In fact, they have been obtained without IF1 and IF3, and show IF2 released from its interaction with the initiator tRNA. Since a conformational change of IF2 and the ribosome seems to occur upon P_i release [24, 110], the GDPCP complex could represents the state before P_i release.

The late initiation phase and elongation state

During initiation of translation, the ribosomal complexes contains fMet-tRNA in the P site, whereas the A site carries no tRNA, ready to accept the first elongator aminoacyl-tRNA after IF1/IF3 release. In the initiation complex, first used for X-ray structure determination of the 70S ribosome [15], it was not possible to bind A site tRNA without serious degradation of crystal quality and effective diffracting resolution [13]. This observation shows that A site tRNA is not natural for initiation. In this initiation complex the mRNA is stretched tightly between the SD helix and the start AUG codon resulting in a distorted E site codon making codon-anticodon interaction in the E-site impossible [13, 17, 100] (Fig. 5). Recently, the X-ray structure of a post-initiation ribosome complex with all three tRNA binding sites occupied by tRNAs was reported [17]. In this post-initiation complex as well as in an elongation complex, the mRNA adopts a "relaxed" conformation with the mRNA codon in the E-site adopting a regular A-helical conformation similar to the codon-anticodon duplexes found in the P and A sites [18] (Fig. 5C). Comparison of the initiation and post-initiation ribosome complexes has revealed several intriguing structural differences. The mRNA starts moving linearly in the tunnel, sliding in the A site-E site direction (Fig. 5B, C) marking the beginning of the elongation process. Simultaneously, the SD helix moves with a clockwise rotation on the surface of the 30S platform, extending the SD-aSD duplex to 12 nucleotides, although classical Watson–Crick base pairing in the double helix occurs in only nine pairs. The movement and rotation of the post-initiation mRNA, compared with the initiation mRNA, brings the 5' end in direct contact with protein S2, where it fits into a cavity formed by highly conserved residues (Fig. 5C). The interactions between mRNA and protein S2 are unspecific and weak, which makes sense in terms of functionality, because the mRNA has to slide through the cavity during the first steps after initiation before release and melting of the SD duplex [17].

Conclusions

Great progress has been made by combining cryo-EM and X-ray structures of initiation complexes, addressing the positions of several initiation factors, mRNA and initiator tRNA on the 30S ribosomal subunit, and on the assembled 70S before and after GTP hydrolysis. Clearly, these studies show interplay of specific interactions between the 30S subunit and its different ligands accompanied by orchestrated conformational switches (tRNA, IF2 movement, etc.). Despite a large number of studies, several questions remain to be

addressed. The high-resolution structure of prokaryotic IF2 is still unknown. The localization of IF3 on the 30S subunit is also controversial, waiting for the resolution of the structure of the whole 30SIC in the presence of three IFs. This may provide the missing information to understand how the three IFs enhance fidelity, and select the correct initiator tRNA in the P site. Although the movement of the SD-aSD has been visualized during the initiation process, it remains to be clarified how the ribosome dissociates the SD-aSD helix after the first steps of elongation. To understand the molecular mechanism of how the ribosome starts translation and how this first ribosome action depends on GTPase reaction will require further high-resolution studies. These recent structural analyses also unravel the key role played by the platform of the 30S not only to promote the movement of the SD-aSD helix during the initiation process, but also to anchor any structured mRNAs. This pre-initiation binding of mRNA structural elements on the "platform" of the small ribosomal subunit could be similar for prokaryotes and eukaryotes. The obvious consequence is a direct implication of this ribosomal center on regulation and modulation of translation initiation. These concepts have nevertheless to be generalized by studying the structure of other regulatory ribosomal complexes, and analyzing more precisely the function of the ribosomal proteins that constitute the platform binding center.

We anticipate that a much deeper understanding of translation initiation and its regulation in bacteria will pave the way to designing novel strategies to inhibit specifically the initiation process in bacteria.

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