Chapter 1

INTRODUCTION

More than 100 different molecules participate in the translation of the genetic message. The way this plethora of molecules is orchestrated for protein synthesis is studied mainly by *in vitro* systems. The most global one is the coupled transcription/translation system, where the input is a gene and the output the corresponding protein. It is a homogenous system, which means that it has components from one and the same organisms. One essential component is the cell free extract, also called lysate and/or S30-extract, which contains most of the cellular cytoplasmic compounds necessary for protein synthesis itself, *i.e.* ribosomes, translational factors for initiation, elongation and termination (IFs, EFs, and RFs), all tRNA synthetases (RS), and tRNAs as well. Of course, it also contains the components important for transcription, but here not an *E. coli* RNA polymerase (RNAP), is utilized, but RNAP from the T7 bacteriophage. The gene of interest is introduced into the system on a plasmid or as a linearised double-stranded DNA flanked by the T7 RNAP promoter and terminator, in order to get a transcript – messenger RNA. Of course, the presence of the building blocks for both, mRNA and protein, are also present. These are nucleotides and amino acids. As any other process in cells, also in cell-free systems the energy supplying pathways are utilized e.g. regeneration of ATP, (Kim and Swartz, 2000).

Historically, these kind of cell-free systems have been studied for about 50 years. Several groups independently demonstrated that disruptured cells or their isolated fractions were capable of synthesizing proteins (Borsook *et al.*, 1950; Gale and Folkes, 1954; Winnick, 1950). Meanwhile, in cells ribonucleoprotein particles were observed and identified (Palade, 1955) whose ability to synthesise proteins was experimentally proven (Keller and Littlefield, 1957; Littlefield *et al.*, 1955). The word “ribosome” was coined by Horward Dintzis in 1958 (Rheinberger, 2004, p. 15) to designate their ribonucleoprotein entity. Programmed with endogenous mRNA, this particles were simply reading this

molecules to which they had been already attached at the time of cell disruption. Still the significance of these investigations was great, since they opened the way for reseach of protein biosynthesis on the molecular level, including all the large and small molecules involved into this process, as well as the mechanisms of their interaction with each other (Zamecnik, 1969).

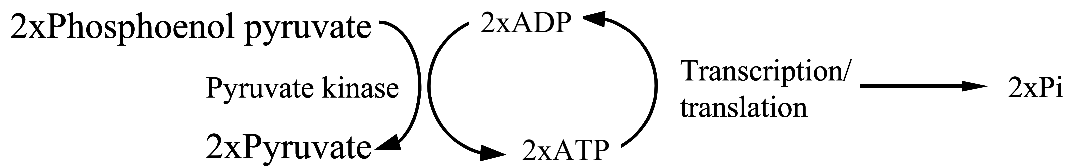
A revolutionary step in the development of cell-free translational systems was the introduction of exogenous messangers. This was first done by Nirenberg and Matthaei in 1961 with a bacterial system (Nirenberg and Matthaei, 1961). They also seem to be the first ones to report dependence of the bacterial cell-free system on the presence of DNA (Matthaei and Nirenberg, 1961). A couped transcription-translation systems came into wide use after some major improvements made by two groups (Gold and Schweiger, 1969; Lederman and Zubay, 1967). In the case of prokariotic systems addition of a proper DNA (plasmid, isolated gene or a synthetic DNA fragment) to the DNA-free extract instead of mRNA results in synthesis of a corresponding mRNA. In this case, ribosomes start to translate the nascent chains of mRNA even prior to the completion of the RNA synthesis. Thus, translation is going on while mRNA is still synthesized and the rates of transcription and translation are coordinated: such systems are called coupled transcritpion-translation systems.

Several types of cell-free systems can be distinguished, the most often applied ones are (i) the so called batch system (Kim and Swartz, 1999; Kim and Swartz, 2000), where all the components are present in one and the same reaction vial, and (ii) a continuous-exchange cell-free (CECF) system (Alakhov *et al.*, 1995). The latter is characterized by a reaction chamber that contains molecules for the synthesis itself, and a supplementary chamber with building blocks for the synthesised product and regeneration of energy. A semi permeable membrane, thus allowing the concentration of the final product, separates these two.

The composition and energy supply of the bacterial batch systems was significantly improved during past 50 years. Kim and Swartz discovered that in

a conventional cell-free synthesis system derived from *E. coli,* phosphoenolpyruvate (PEP), the secondary energy source for ATP regeneration, and several amino acids are rapidly degraded during incubation of the reaction mixture (Kim and Swartz, 1999; Kim and Swartz, 2000). The degradation of such compounds takes place even in the absence of protein synthesis, thus reducing the capacity for it to take place. However, the lost potency is completely recovered when the reaction mixture is supplied with an additional PEP and amino acids, suggesting that catalytic activity is relatively stable.

In several papers Kim and Swartz have published their study of factors that cause early cessation of protein synthesis in cell-free system from *E. coli* (Kim and Swartz, 1999; Kim and Swartz, 2000). They have reported, that PEP, the secondary energy source for ATP regeneration, and several amino acids are rapidly degraded during the cell-free protein synthesis reaction, which severely reduces the capacity for protein synthesis. Of twenty amino acids, only arginine, cysteine, and tryptophan were required to restore system activity. Through coordinated addition of PEP, arginine, cysteine, tryptophan, and magnesium, the final concentration of cell-free synthesised chloramphenicol acetyltransferase (CAT) increased more than 4-fold. The figure 1.0 below schematically describes the conventional ATP regeneration system (A) and that one proposed by Kim and Swartz, 1999 (B).



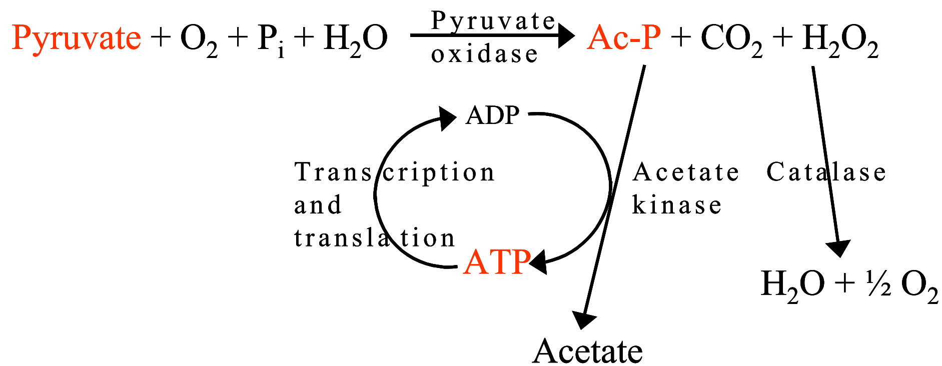


Figure 1.0 Regeneration of ATP in an in vitro *protein synthesis systems.* (**A**) Conventional scheme for energy regeneration using phosphoenol pyruvate and pyruvate kinase. (**B**) A new scheme proposed by (Kim and Swartz, 1999), using pyruvate, pyruvate oxidase and endogenous acetate kinase.

A system like this has a number of advantages compared to *in vivo* over-expression of a target protein. These are:

1. Direct expression from linear or circular DNA template and no need for purification of an mRNA after *in vitro* transcription, when a significant loss of a template can occur, as well as partial or complete degradation of mRNA due to RNases, present in the cell lysate. After all, when transcription occurs in such a coupled system, partial decay of mRNA is always restored by newly transcribed molecules.

2. Expression of outer membrane proteins, study of their assembly and processing in the *in vivo* near conditions can be allowed.

3. Proteins expressed at very low levels *in vivo*, toxic and alike, may be expressed at higher levels *in vitro*, also allowing a closer look on their function.

4. The incorporation of the added labelled or unnatural amino acids, modification of active centre and different protein chains may allow a deeper

understanding of a protein function, may also allow synthesis of novel proteins with a desired function. (Only, application of last is questionable)

5. Relative purity of a product allows studying proteins as they are when (if) not involved into macro-molecular complexes.

6. Studying processes of synthesis and factors involved into the regulation of transcription and translation still remains an up to date subject of interest.

7. Studies of co-translational folding pathways during protein synthesis.

8. Formation of truncated proteins during/after synthesis and means to suppress these unwanted products.

In order to understand all this it's necessary to understand the working principles of the protein synthesis machinery – the ribosome itself. At the moment quiet a lot is known about prokaryotic ribosome, and analysing this knowledge, (putting pieces into a puzzle), allows us to make some of the features of the ribosome to work for us in order to improve what already is achieved on the field of *in vitro* systems for protein synthesis. Because, there are still possibilities to go further in the "*in vivo* near" conditions, to increase protein yield. Besides that, not all proteins can be expressed *in vitro* at high levels, there are several difficulties which have been investigated during time-frame of this work and attempts to improve the last were taken.

First I will focus on the protein synthesis machinery – the ribosome.

1.1 Protein synthesis

The ribosome is a macromolecular complex which catalyses peptide bond formation – a process vital to all organisms. Information is transported from the genome via mediator molecules called messenger RNAs (mRNAs), to the ribosome and it translates the sequence of the codons on the mRNA into the corresponding sequence of amino acids, using adaptor molecules – transfer RNAs (tRNAs). A single ribosome can incorporate 10 to 20 amino acids per second (Bremer and Dennis, 1996) with an accuracy of about one misincorporation per 3000 amino acids incorporations (Bouadloun *et al.*, 1983).

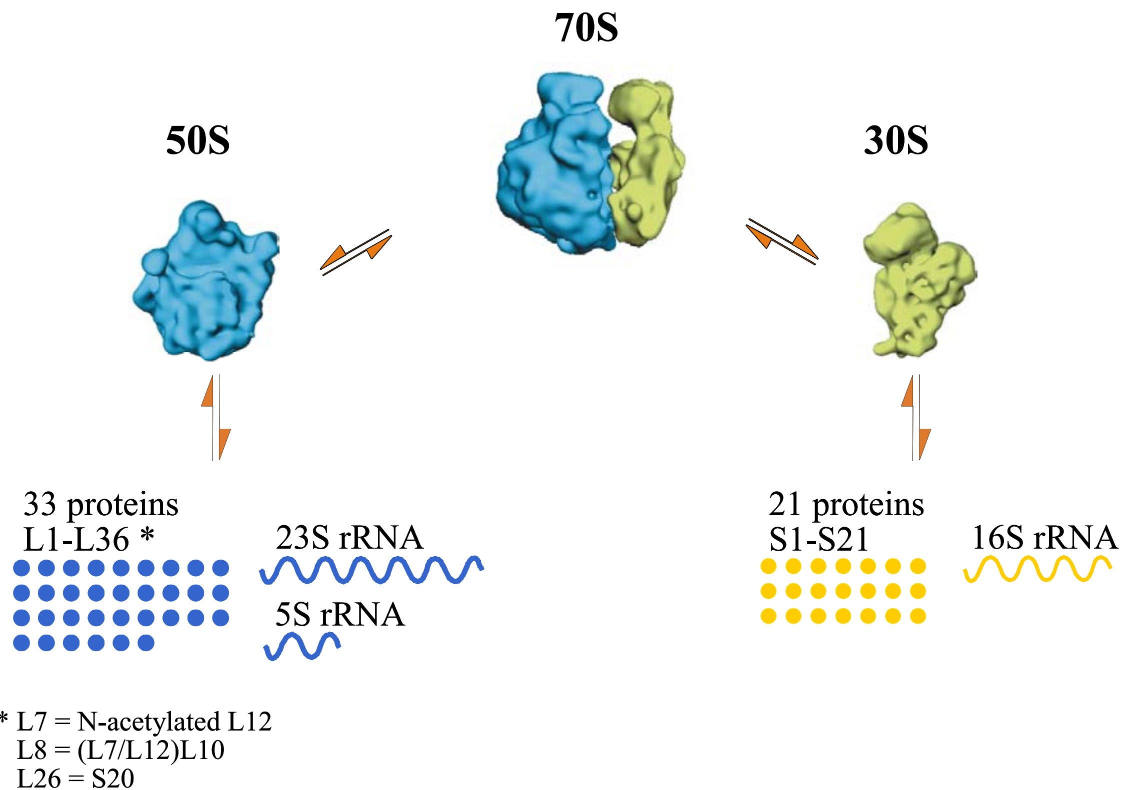


Figure 1.1-1 Composition of the E. coli *ribosome.*

The bacterial ribosome has a mass of approximately 2.6-2.8 MDa, a relative sedimentation of 70 S and a diameter of 200-250 Å. Under certain functional states the 70S ribosome falls apart into two unequal subunits: a large 50S subunit and a small 30S subunit. Each subunit is a ribonucleoprotein particle with one third of the mass consisting of protein and the other two thirds (Moore and Steitz, 2002; Ramakrishnan and Moore, 2001) of ribosomal RNA (rRNA): 33 proteins and two rRNA molecules – 5S (120 nts), and 23S (~2900 nts) rRNA in large subunit, and 20 proteins and a single 16S (~ 1500 nts) rRNA in the 30S small subunit (Figure 1.1-1).

Both subunits differ in functions as well. On the 30S subunit the codon-anticodon interaction between the mRNA and tRNA substrates, namely, process of decoding occurs. The large 50S subunit performs the central catalytic function of protein synthesis, in its active center for peptide bond formation between the nascent polipeptide chain and the incoming aminoacylated tRNA. One of the special features of the 50S subunit is the tunnel for peptide exit. It

runs from the peptidyl-transferase (PTF) centre at the foot of the central protuberance through the subunit down to the base of the cytoplasmic side of it with a length of about 100 Å and a width of 10 to 20 Å (Ban *et al.*, 2000; Stark *et al.*, 1995). Additionally, the 50S subunit has a factor-binding centre and all of the G-protein factors involved in protein synthesis interact with it during at least part of their duty cycles. Both subunits are involved in translocation of the mRNA by one codon in each cycle. It was mentioned above, that 2/3 of each subunit consists of rRNA, which in fact plays the main role in protein biosynthesis.

Structurally, the 50S subunit is spherical with three almost cylindrical extensions (Figure 1.1-2). These extensions are called L1 protuberance, the central protuberance and the L7/L12 stalk. A striking difference between the two subunits has to do with the relationship between the secondary structures of their RNAs and their overall morphology. The six secondary structure domains of 23S rRNA are intricately interwoven in the 50S subunit to form a monolithic structure. On the other hand, the 30S subunit is divided into three domains (head, body, and platform). Each of these domains contains one of the principal secondary structure domains of 16S rRNA: The 5' major domain represents the body, the central domain the platform and the 3' major domain the head of the small subunit. The 3' minor domain of the 16S rRNA forms an extended helix (h44 in *E. coli* helix numbering) and runs down the long axis of the 30S subunit surface that interacts with the 50S subunit. All four domains of the 30S particle join at a narrow neck region. The two active sites (the decoding centre and the peptidyl transferase centre) face each other across the subunit interface and are functionally linked by both the two ends of A-site tRNA and a prominent inter-subunit bridge (bridge 2B).

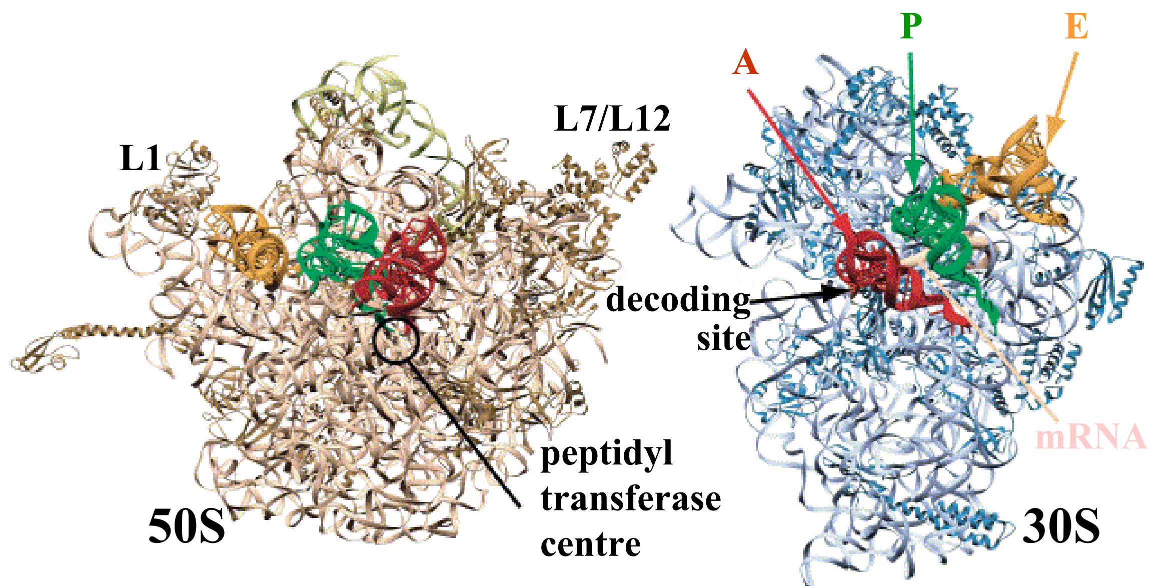


Figure 1.1-2 Crystal structure of the 50S and 30S subunits in the 70S ribosome. The location of the A-, P-, E-site tRNAs are indicated by arrows, respectively. The passage on the mRNA is indicated in pink. Essential landmarks of the 50S subunit are prescribed (Ramakrishnan, 2002).

The architectural difference between the subunits may reflect a greater functional need for flexibility on the part of the small subunit and, not necessarily alternative, a different evolutionary age of the subunits, where the large one is probably the older one (Sardesai *et al.*, 1999).

Cryo-EM and X-ray maps of the 70S ribosome show a number of highly conserved bridges connecting the subunits (Cate *et al.*, 1999; Frank *et al.*, 1995; Gabashvili *et al.*, 2000). The inter-subunit interface of both subunits, especially the part that binds mRNA and tRNAs, is largely free of protein.

Protein synthesis would not be able without participation of the adaptor molecules that ensure amino acids transfer to the ribosome – tRNAs. These molecules are on the border from RNA to protein world. Initially, two binding sites for tRNAs were proposed for the ribosome (Lipmann, 1963; Watson, 1963; Watson, 1964). The two sites of this model are the “A” site (for aminoacyl-tRNA or acceptor site) and the “P” site (for peptidyl-tRNA). However, functional studies at the beginning of 80’s (Grajevskaja *et al.*, 1982; Lill *et al.*, 1984; Rheinberger and Nierhaus, 1980; Rheinberger *et al.*, 1981); have demonstrated a third tRNA binding site, the “E” site (E for the exit), from which

deacylated tRNA leaves the ribosome. Neutron scattering, cryo-electron microscopy and X-rays diffraction studies have confirmed the existence of the E-site on the ribosome (Agrawal *et al.*, 2000; Nierhaus *et al.*, 1998; Wadzack *et al.*, 1997; Yusupov *et al.*, 2001). This third ribosomal binding site has been found on ribosomes of all kingdoms and seems to be a universal feature of ribosomes (for review see Blaha and Nierhaus, 2001). However; disagreement exists on several points concerning the importance of the E site (for more details see Burkhardt *et al.*, 1998; Wilson and Nierhaus, 2003).

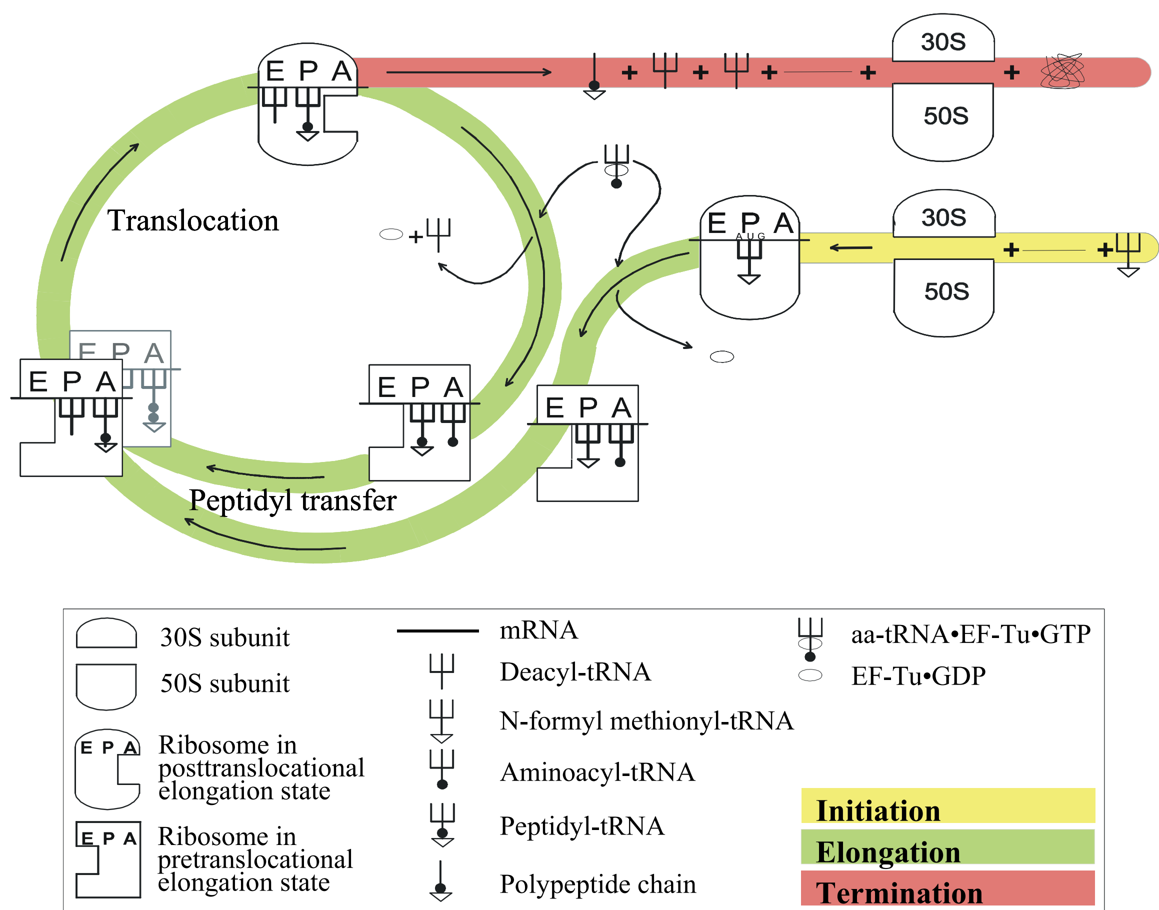


Figure 1.1-3 The functional phases of the ribosome during protein synthesis.

The whole process of protein synthesis is conventionally divided into three consecutive phases: initiation, elongation and termination (Figure 1.1-3). Briefly, during initiation mRNA is positioned with the AUG start-codon at the P-site on a small subunit, and large subunit associates. Within an elongation stage ribosome goes “codon-by-codon” along the mRNA synthesising the given

peptide until a stop-codon enters the A-site. This is a signal for translation termination followed by peptide release and subunits dissociation. Each stage is assisted by translational factors of initiation (IFs), elongation (EFs), and termination or release factors (RFs), respectively.

1.2 Initiation

The initiation phase of protein synthesis is one of the rate-limiting steps of translation. There are significant differences between translation-initiation events in eukaryotes, archaea and eubacteria; however, the final state of the ribosome following initiation is principally the same, namely, a ribosome programmed with an initiator tRNA and mRNA, such that the start codon and tRNA are both positioned at the P-site. Indeed, the production of functionally active proteins necessitates that translation initiates at the start codon within the mRNA. As well as the use of the correct codon as the start codon, the placement at the P-site of the ribosome must also be precise; since codons are composed of three bases, incorrect placement by one or two bases will result in a complete loss of the correct reading frame. There are two major contributors to ensure the fidelity of this process: (i) the mRNA itself and (ii) a subset of translation factors termed the initiation factors (IFs). Unlike eukaryotic protein synthesis the mechanism of translation initiation in prokaryotes is relatively simple and requires at least three factors for initiation, namely IF1, IF2 and IF3, which form an intermediate initiation complex consisting of an these factors, mRNA, initiator tRNA and the 30S subunit.

The exact role of the factors and the chronology of events surrounding initiation are still controversial. IF1 accelerates IF3-dependent 70S dissociation and stimulates IF2 and IF3 to form an initiation complex (Figure 1.2-1). Protection studies suggest that IF1 binds to the A-site, preventing binding of the elongator tRNA to the ribosomal A-site (or decoding site). The presence of IF1 increases the affinity of IF2 for the ribosome. IF2, as a binary complex with GTP, binds the 30S subunit and directs the initiator tRNA into the prospective

P-site on the 30S subunit. IF2 specifically recognises the blocked α-amino group of the initiator tRNA, thus excluding aminoacylated elongator tRNAs in the initiation step. IF3 participates in both the first and last steps of translational initiation, being involved in selection of the initiator tRNA as well as dissociation of the 70S ribosome into subunits. IF3 binds to the stem of the initiator tRNA and prevents the binding of the elongator tRNA to the P-site of the 30S initiation complex.

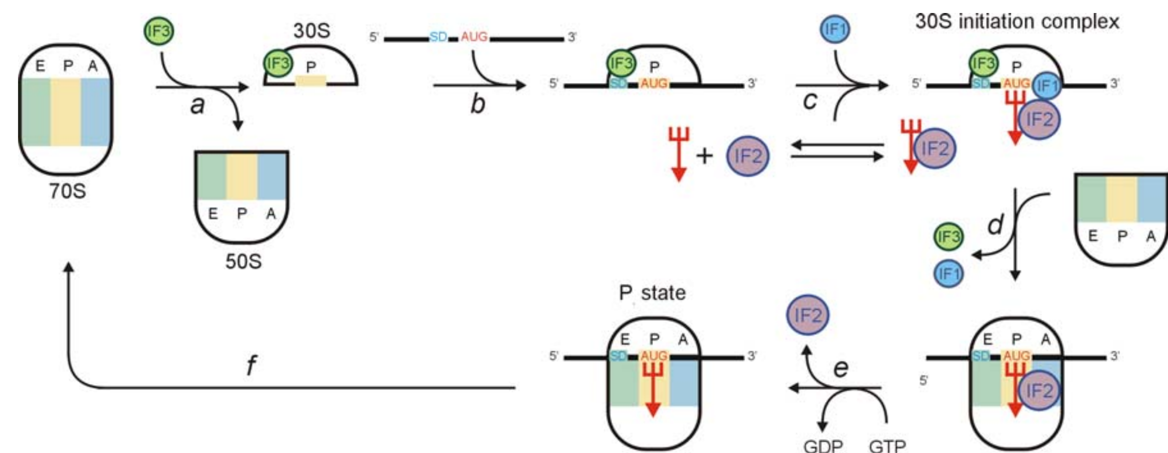


Figure 1.2-1 Schematic representation of the initiation of protein.

(**a**) Binding of IF3 to the 30S subunit dissociates empty 70S ribosomes into composing 30S and 50S subunits. (**b**) IF3 aids in positioning of the mRNA in a way that the AUG start codon is located at the P-site of the 30S subunit. The Shine–Dalgarno (SD) sequence of the mRNA is located in the vicinity of the binding position of IF3, and interacts with the anti-SD sequence of the 16S rRNA of the 30S subunit. (**c**) Binding of the initiator fMet-$tRNA_f^{Met}$ (red) can occur directly or in the form of a ternary complex with IF2 (purple) and GTP is stimulated by the presence of IF1 (blue) and results in the formation of the 30S initiation complex. (**d**) Association of the 50S subunit with 30S initiation complex results in the release of IF3 and IF1, but IF2 remains bound at the A-site. (**e**) The GTPase activity of IF2 is stimulated by 50S subunit and ultimately leads to the release of IF2·GDP from the ribosome, allowing full accommodation of the initiator tRNA at the P-site on the 50S subunit. This complex is termed the Pi state, i.e., P-site is occupied and the A- and E-sites are free. (**f**) Following the translation elongation, termination and ribosome recycling empty 70S ribosomes are ready to renter into translation-initiation phase.

The conventional mechanism of translation in bacteria includes complementary interactions between a purine rich sequence at the 5’-nontranslated region of mRNA (known as Shine-Dalgarno, SD), and the 3’ end of 16S rRNA (anti-SD sequence) (Gold, 1988), facilitating the positioning of the correct AUG start codon at the P-site. There are data in the literature indicating

that the interaction between mRNA and the 30S ribosomal subunit is much more complex than thought before (Kozak, 1999; Mccarthy and Brimacombe, 1994; Sprengart and Porter, 1997). It has been found that besides the SD sequence other nucleotides located upstream of the initiation codon, are also involved in this interaction (Mccarthy and Brimacombe, 1994). The complex type of mRNA-ribosome interaction is illustrated as well by the existance of nucleotide sequences enhancing translation. Such sequences were found in several phage, viral and bacterial genes. The first enhancer of translation that was active in both prokaryotic and eukaryotic cells (Gallie and Kado, 1989) was identified at the 5'-nontranslated region of the tobacco mosaic virus (TMV) RNA. Enhancers of translation were also found in the genome of bacteriophages T7 (Olins *et al.*, 1988), λ (Wu and Janssen, 1996), Qβ (Ugarov *et al.*, 1994), and *Mycoplasma genitalium* (Loechel *et al.*, 1991). It is noteworthy that unlike the purine-rich SD sequence all bacterial enhancers of translation were poor in purines, and particularly guanine (G). Several studies show that besides their enhancing activity, some nucleotide sequences were also capable of independently (in the absence of SD sequence), initiating translation in *E. coli* cells (Ivanov *et al.*, 1992; Walz *et al.*, 1976).

1.2.1 70S initiation type

In bacteria most mRNAs result from the simultaneous transcription of a row of adjacent genes (operon) and therefore carry the information for the synthesis of several proteins, often functionally related. Such mRNAs are called polycistronic: they contain several translation initiation sites, one for each cistron. In *E. coli* polycistronic mRNAs contain four cistrons on average.

The recognition of the translation start sites on the mRNA is performed by an initiation complex including the small ribosomal subunit (30S), the initiator tRNA carrying the amino acid formylmethionine (fMet-tRNA$_f^{Met}$), and initiation factors. Theoretically, the various initiation codons of a bacterial polycistronic mRNA can be recognized independently of one another. Aided by the SD

sequences, the 30S initiation complexes can land on any of the available translation initiation sites (Figure 1.2-2).

In practice, and it is true for ribosomal proteins (r-proteins, it has been observed that in order to be accessible to the initiation complex an initiation region of mRNA (including the start codon and the SD motif) must be in a single-stranded, nonhydrogen-bonded state. Once the initiation site is recognized a translating ribosome may unfold the secondary structure of the mRNA.

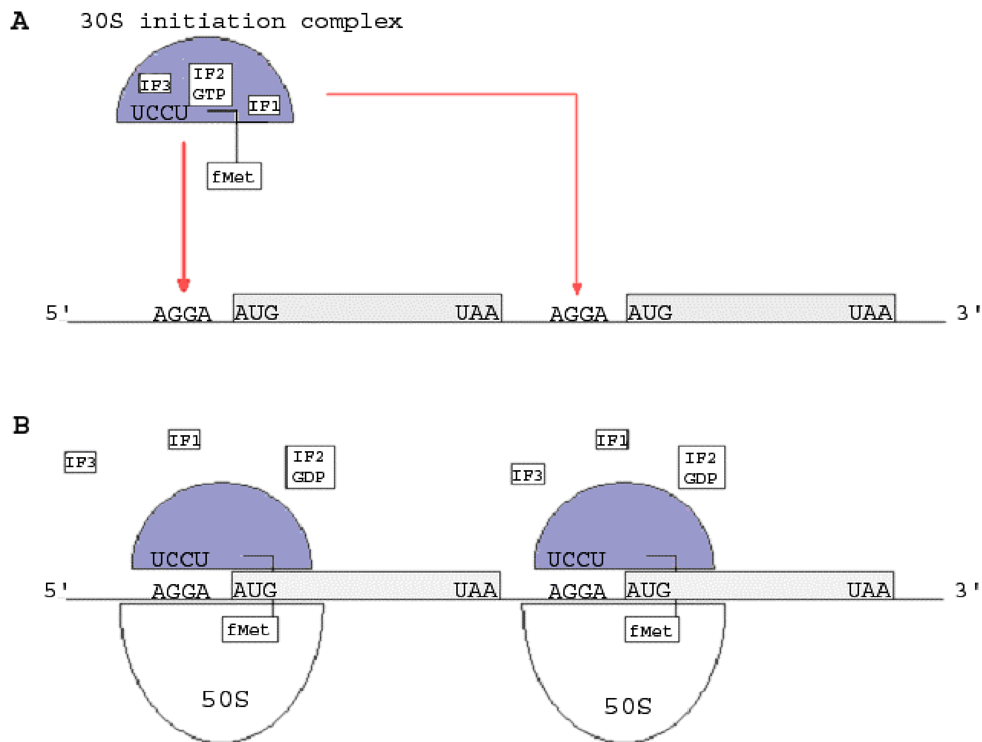


Figure 1.2-2 Translational initiation on a bacterial polycistronic mRNA. (**A**) The initiation complex, comprising the 30S ribosomal subunit, the indicated initiation factors (IF) and the initiator tRNA charged with the amino acid formylmethionine (fMet) recognizes two initiation codons on a bicistronic mRNA. The anti-Shine–Dalgarno sequence at the 3' end of the 16S ribosomal RNA (schematically indicated as UCCU) base-pairs with the Shine–Dalgarno sequence on the mRNA (AGGA), located a few nucleotides upstream of the initiation codons (AUG). The coding regions are illustrated as grey boxes, each terminating with a stop codon UUA. (**B**) Following recognition of the start codons by the initiation complex, the large ribosomal subunit (50S) joins the 30S subunit and the initiation factors dissociate from the ribosome.

This often creates a situation that renders the translation of the various cistrons in a polycistronic mRNA interdependent. In many cases, 'downstream' cistrons may not be expressed efficiently if those 'upstream' have not been

previously translated; for instance, when the initiation region of a downstream cistron is sequestered within a double-helical structure. Translational initiation at that site may take place only if a ribosome completing translation of the previous cistron unfolds the structured region thus unmasking the initiation codon. This phenomenon is termed *translational coupling*.

Sometimes, translational coupling is exploited to cause autogenous translational regulation; a repressor protein (usually a translation product of that same mRNA), will bind at a specific site on a polycistronic mRNA, thereby inhibiting the translation of most of the cistrons located downstream from the repressor-binding site. Presumably, the repressor hinders ribosome access to the initiation site of a critical upstream cistron whose translation is coupled to those downstream. Autogenous translational regulation is regularly observed with relatively stable bacterial mRNAs such as those encoding ribosomal proteins.

Often, the downstream cistron is translated by reinitiation, meaning that the ribosomes terminating translation of the upstream cistron do not dissociate from the mRNA but proceed directly to translate the next cistron, occasionally shifting the reading frame if this is required (Londei, 2001; http://www.els.net). What happens is that a 70S ribosome after termination on a stop codon of a preceding cistron and peptide release does not dissociate from such a polycistronic mRNA. An empty 70S (no peptide chain and tRNAs present) is capable of scanning in a one-dimensional manner upwards and downwards the mRNA up to 40 nucleotides, until it is “caught” by a nearby SD-sequence due to a base pairing of the last with a 3’-region of 16S rRNA anti-SD sequence. This helps to position a following AUG-start codon directly at the P-site. Petersen HU, Danchin A, Grunberg-Manago M., (Petersen *et al.*, 1976) had shown that the formylation of the methionyl initiator tRNA is only obligatory when polypeptide synthesis is initiated by non-dissociated 70S ribosomes. The site of IF2 binding on 70S ribosomes overlaps that of EF-Tu. Thus, the proper initiator tRNA, fMet-tRNA$_f^{Met}$, is delivered by the IF2 in the GTP form. GTP increases

the affinity of the IF2•fMet-tRNA$_f^{Met}$ complex to ribosome. This means that discrimination is on the factor and not on the ribosomal level.

1.3 Elongation

Elongation of the peptide chain (for reviews see Nierhaus *et al.*, 1998; Spahn and Nierhaus, 1998; Wilson and Noller, 1998) is the central event in protein synthesis. The elongation cycle directly involves two main protein elongation factors, EF-Tu and EF-G. EF-Tu forms a ternary complex with GTP and an aminoacyl-tRNA, directing the tRNA to the ribosomal A site. After peptidyl-transfer, EF-G is involved in translocating the mRNA•peptidyl-tRNA complex from the A site to the P site. This enables the next EF-Tu ternary complex access to the new codon present in the A site and thus the elongation cycle is repeated.

Three models for elongation have been proposed. The allosteric three-site model is based on functional experiments suggesting the A and E sites are allosterically linked – such that occupation of the E site by a deacylated tRNA (posttranslocation) creates a low affinity A site, and *vice versa* (Nierhaus, 1990). The hybrid-site model is based on structural experiments, which suggest that the tRNA movements during translocation occur in two steps, creating hybrid A/P and P/E sites (Moazed and Noller, 1989). The α-ε model is based on the observation that the ribosome contact patterns of the acylated tRNA before and after translocation in the A and P sites were similar, as were the patterns of the deacylated-tRNA in the P and E sites respectively (Nierhaus *et al.*, 1995). The main feature of this model is a ribosomal domain that is tightly bound to the tRNAs and moves with them during translocation.

The elongation cycle ends when translocation of a termination codon into the A site occurs. Simply, the termination of protein synthesis involves the hydrolytic release of the completed polypeptide from the peptidyl-tRNA in response to a stop codon appearing in the decoding site (A site) of the ribosome. It is this process which is mediated by the RFs.

1.4 Termination

The synthesis of the polypeptide chain continues until a stop codon (UAA, UAG or UGA), is invading the A-site. Protein factors, called release factors (RF), are in charge of the release of a nascent polypeptide chain from the ribosome and recycling of the ribosomes for the next initiation. Two classes of release factors are distinguished: *Class I* factors that do not consume energy and are specific decoding factors responsible for the hydrolysis of the peptidyl-tRNA. RF1 and RF2 belong to this group, and recognize UAG and UGA respectively; both RFs overlap in the recognition of the termination codon UAA. Class I RFs promote hydrolysis of the ester bond between the polypeptide and the P-site tRNA on the ribosome. RF1 and RF2 genes (*prfA* and *prfB*), have been shown to have a high similarity at the amino acid level (Caskey *et al.*, 1984; Craigen *et al.*, 1985; Weiss *et al.*, 1984). In eukaryotes and in archaea only a single class I factor, eRF1 (and aRF1) has been identified that recognizes all three-stop codons (reviewed by Wilson and Nierhaus, 2003).

The *Class II* release factors are non-decoding and energy consuming. The RF3 belongs to this class. It stimulates the termination process in a GTP dependent manner.

RF3 in *E. coli* is not essential; knockout strains of its *prfC* gene are viable. The main function of RF3 is to support removal of the class I RFs from the ribosome using GTP hydrolysis, once the peptide hydrolysis has taken place (Freistroffer *et al.*, 1997; Zavialov *et al.*, 2001). In other words, the post-termination complex is the substrate for RF3 that stimulates the recycling of RF1 and RF2 (Zavialov *et al.*, 2001).