Study on the Function of Translation Initiation Factor IF1

by

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All truths are easy to understand once they are discovered; the point is to discover them.

Galileo Galilei (1564 - 1642)

Dedicated to my wife, Julieta
Abstract

Initiation is the first step in protein biosynthesis representing a fundamental event in cell life which determines fidelity, efficiency and regulation of gene expression. In addition to the ribosome and mRNA, three protein factors IF1, IF2 and IF3 are involved in the initiation of translation in prokaryotes. Several minor functions have been attributed to the smallest of these factors, IF1. However, the main function of IF1 remains to be elucidated.

In order to investigate the role of this protein in the initiation process we have mutated the corresponding gene infA. Using a high-copy plasmid and site-directed mutagenesis, the six arginine residues of IF1 were separately altered to leucine or aspartate. Another set of plasmid-encoded IF1 mutants with a cold-sensitive phenotype was collected using localized random mutagenesis. This strategy was followed by deletion of the chromosomal infA gene. All variants with a mutated infA gene on a plasmid and a deletion of the chromosomal infA copy were viable, except for an R65D alteration. Several of the mutated infA genes were successfully recombined into the chromosome thereby replacing the wild-type allele. Some of these mutants displayed reduced growth rates and a partial cold-sensitive phenotype.

The influence of the leucine group of mutants in IF1 on the expression of two reporter genes with different initiation and/or +2 codons has been investigated. Our results do not indicate any involvement of IF1 in recognition of the +2 codon immediately following the start codon, thus representing the A-site. In addition, this group of mutants has no changed efficiency of decoding at the near-cognate initiation codons UUG and GUG. However, one cold-sensitive IF1 mutant shows a general overexpression of both reporter genes, in particular at low temperatures. Overall, the results do not support the hypothesis that IF1 could possess codon discriminatory functions while blocking the A-site of the ribosome.

In this study we also identify that IF1 has RNA chaperone activity both in vitro and in vivo. The chaperone assays are based on splicing of the group I intron in the thymidylate synthase gene (td) from phage T4. Some of the IF1 mutant variants are more active as RNA chaperones than the wild-type. Both wild-type IF1 and mutant variants bind with high affinity to RNA in a band-shift assay. It is suggested that the RNA chaperone activity of IF1 contributes to RNA rearrangements during the early phase of translation initiation.
List of publications

This thesis is based on the following publications:

   Generation and characterization of functional mutants in the translation initiation factor IF1 of *Escherichia coli*.

    *In vivo* involvement of mutated initiation factor IF1 in gene expression control at the translational level.

    RNA chaperone activity of translation initiation factor IF1.
    Submitted manuscript.

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Abbreviations

ASD anti Shine-Dalgarno sequence
ASL anticodon stem-loop
A-site aminoacyl-tRNA acceptor site
DB downstream box
EF-Tu elongation factor Tu
E-site transfer RNA exit site
fMet-tRNA$_{f}^{Met}$ formylated initiator tRNA
GDP guanosine diphosphate
GTP guanosine triphosphate
IF1 initiation factor IF1
IF2 initiation factor IF2
IF3 initiation factor IF3
MTF methionyl-tRNA transformylase
mRNA messenger RNA
NMR nuclear magnetic resonance
OB oligomer-binding
PTC peptidyl transferase center
PTH peptidyl-tRNA hydrolase
P-site peptidyl-tRNA acceptor site
RBS ribosomal binding site
RNA ribonucleic acid
RRF ribosome recycling factor
rRNA ribosomal RNA
SD Shine-Dalgarno sequence
TIR translation initiation region
tRNA transfer RNA
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1. Introduction

The biosynthesis of proteins takes place on ribosomes - large macromolecular ribonucleoprotein complexes, in a process termed translation. The ribosomes are enzymatic complexes that catalyze peptide bond formation and synthesize polypeptides by reading the genetic code within the messenger RNA. Protein synthesis can be divided into four main steps: initiation, elongation, termination and ribosome recycling. Each ribosome consists of a small and large subunit, which are coupled on the translation initiation region (TIR) of the mRNA during the initiation phase of translation (Laursen et al., 2005 and references therein). Initiation is the rate-limiting step (Kozak, 1999) where the ribosomal subunits are associated on the mRNA together with the initiator tRNA$_{f}^{Met}$ assisted by the initiation factors (Gualerzi and Pon, 1990). Elongation is the process where the ribosome slides over the mRNA, one codon at a time, translating the genetic information and adding one amino acid to the growing peptide after each codon being read. When the ribosome reaches one of the three stop codons the nascent peptide chain is released upon interaction with the release factors (Nakamura et al., 1996), thus terminating the peptide chain elongation. During the recycling phase the termination factors and the tRNA are released from the ribosome, followed by dissociation of the two subunits from the mRNA (Janosi et al., 1996; Janosi et al., 1998).

1.1 Bacterial translation initiation

Initiation of protein synthesis is a fundamental event in bacteria from which efficiency, fidelity and regulation of gene expression can be determined (reviewed in Laursen et al., 2005). The early events in initiation entail the selection of the mRNA initiation codon by the 30S ribosomal subunit with the help of initiator fMet-tRNA$_{f}^{Met}$ and initiation factors IF1, IF2 and IF3, which all together form a “30S pre-initiation complex”. After, the choice of the start site is defined by the binding of the 50S ribosomal subunit to the 30S initiation complex resulting into a “70S initiation complex” (Gualerzi et al., 2001). The initiation factors IF1 and IF3 are ejected from the 30S subunit during “70S initiation complex” formation. This process activates the GTPase activity of IF2 and results in the adjustment of the initiator fMet-tRNA in the ribosomal P-site (Figure 1). Subsequently, the ribosomes translate the mRNA at a rate of approximately 12 amino acids per second (Kennell and Riezman, 1977).
Figure 1. Translation initiation pathway in prokaryotes. The 30S and 50S ribosomal subunits are shown in light and dark grey, respectively. Translation initiation factors IF1, IF2, and IF3, the mRNA, and the fMet-tRNA_{f}^{Met} are shown in red, blue, green, yellow, and magenta, respectively. Picture re-printed with permission from Laursen et al. 2005. Copyright © 2005, the American Society for Microbiology.
1.2 The machinery of translation initiation

1.2.1 The ribosome and its subunits

Each ribosome is composed of a small and a large subunit. The large subunit creates the peptide bond and provides the path for emerging proteins. The small subunit has key roles in initiating the process and controlling its fidelity. The small ribosomal subunit has a relative sedimentation rate of 30S and a mass of 0.8 mega Daltons (MDa), whereas the large ribosomal subunit has a relative sedimentation rate of 50S and a mass of 1.5 MDa. Assembled bacterial ribosomes have a relative sedimentation rate of 70S and a mass of 2.4 MDa. One third of the ribosome consists of proteins and two thirds consist of rRNA (Steitz and Moore, 2003). Thus, the ribosome is a large polyanion. The tertiary structure of the rRNA is mainly stabilized by three types of interactions: Mg\(^{2+}\) bridges, RNA-RNA interactions and RNA-protein interactions. The magnesium ions form neutralizing bridges between two or more phosphate groups from rRNA secondary structure elements distant in sequence. RNA-RNA interactions are formed via base pairing between nucleotides associated with secondary structure elements remote in sequence. RNA-protein interactions occur mainly via the sugar-phosphate backbone of the rRNA. The ribosomal proteins recognize the distinctive shape of the rRNA rather than the bases (Laursen et al., 2005 and references therein).

The first visualization of ribosomal shapes came in the early 1970s (Lake, 1976). Nowadays, the resolution of ribosomal particles made by cryo-electron microscopy has reached 7Å for the best reconstitutions (Frank, 2003; Wilson et al., 2002). However, atomic resolution structures of ribosomes can only be obtained by X-ray crystallography. During the 1980s Yonath started to explore the possibility of obtaining three-dimensional crystals of ribosomes or its subunits (Yonath et al., 1982). A number of thermophilic bacterial species like Thermus thermophilus and Bacillus stearothermophilus was used. However, the crystals were thin and fragile, not isomorphous, and sensitive to radiation damage (Harms et al., 1999; Yonath and Franceschi, 1998). During the years improvements in ribosomal treatment, crystal growth conditions and handling (Ban et al., 1998; Clemons et al., 2001; Gluehmann et al., 2001), crystal freezing (Hope et al., 1989), synchrotron equipment and methods (Helliwell, 1998; Hendrickson, 1991) have lead to the success we benefit today. Currently, high resolution structures of the 30S ribosomal subunit (Schluenzen et al., 2000; Wimberly et al., 2000), the 50S ribosomal subunit (Ban et al., 2000;
Harms et al., 2001) and the intact 70S ribosome (Vila-Sanjurjo et al., 2003; Yusupov et al., 2001) are available.

The ribosome has three binding sites for tRNA, shared between the two subunits. The aminoacyl (A) site has high affinity for aminoacyl-tRNA (aa-tRNA); the peptidyl (P) site has high affinity for peptidyl-tRNA; and the exit (E) site has high affinity for deacylated tRNA. During translation the tRNA is moved from the A-site through the P-site and then E-site before it exits the ribosome (Ramakrishnan, 2002).

The small ribosomal subunit

The small ribosomal subunit is composed of 21 proteins (S1-S21) and an rRNA of 1542 nucleotides (in E. coli) sedimenting at 16S. Two independent research groups determined the structure of the Thermus thermophilus 30S subunit at 3Å and 3.3Å resolution (Schluenzen et al., 2000; Wimberly et al., 2000). The structure of the small subunit can be subdivided into head, neck, platform, and body, which have obvious relationships to the structural domains of the 16S rRNA: 5’-domain, central domain, 3’-major domain, and 3’-minor domain (Yusupov et al., 2001). The 5’-domain corresponds to the body; the central domain – to most of the platform; and the 3’-major – to the head. The neck provides a relatively flexible connection between the head and the rest of the small subunit. The 3’-minor domain consists of the last two helices (44 and 45) and the 3’-end of the rRNA. The side of the 30S subunit facing the 50S subunit is called the front, whereas the solvent-exposed side is called the back. A comprehensive description of the domains and the location of proteins and their interactions with rRNA is available (Brodersen et al., 2002).

During translation of mRNA into protein by the ribosome, accurate selection of aminoacyl-tRNA (aa-tRNA) depends upon the correct pairing of three bases between the mRNA codon and the tRNA anticodon. This process is called decoding and it sets the translational fidelity (Rodnina and Wintermeyer, 2001), which is controlled by the 30S subunit. The decoding centre consists entirely of rRNA containing the upper part of helix 44 and the 3’ and 5’ ends of 16S rRNA (Schluenzen et al., 2000). Nucleotides G530 from the shoulder domain, and A1492 and A1493 in helix 44 come together to span the minor groove of the codon–anticodon duplex at the first two codon positions. This results in a closed conformation of the 30S subunit, in which the shoulder and the head domains are rotated towards the subunit centre, compared to a more open structure when the A site is unoccupied (Wimberly et al., 2000). To study the mechanism of decoding,
fragments of the anticodon stem-loop (ASL) of tRNA have been investigated when bound to the small subunit (Ogle et al., 2001; Ogle et al., 2002). Cognate ASL induces a closure of the 30S subunit around the A site, which is enhanced in the additional presence of paromomycin. This movement involves rotations of the head towards the shoulder and the subunit interface, while the shoulder (S4, G530 loop with surrounding regions of 16S RNA and S12) moves towards the intersubunit space and the h44/h27/platform region (Ogle et al., 2002). During decoding it is essential that high fidelity is maintained. The error rate of tRNA selection in the decoding process is $10^{-3} - 10^{-4}$ (Kurland, 1992). However, the difference in affinity between the cognate and near-cognate codon-anticodon pairs is not enough to explain the low error frequency. Two different mechanisms that can explain the high fidelity of translation are suggested: geometrical recognition and kinetic proofreading. During the geometrical recognition the decoding site of the ribosome screens the base-pairing between the codon and the anti-codon. This is possible if the correct codon-anticodon pairing leads to conformational changes of the decoding site (Ogle et al., 2001; Ogle et al., 2003). In the kinetic proofreading mechanism, the rates of binding and dissociation favor cognate tRNAs over non-cognate or near-cognate tRNAs. Here, the tRNA recognition is done in two steps: the initial recognition step and the proofreading step. They are separated by the irreversible hydrolysis of GTP by EF-Tu (Hopfield, 1974; Ogle et al., 2003).

The large ribosomal subunit

The large ribosomal subunit is composed of 34 proteins (L1-L34) and two rRNAs containing about 120 and 2900 nucleotides sedimenting at 5S and 23S respectively. Within the 23S rRNA six secondary structure domains are defined (Noller et al., 1981), while the 5S rRNA is considered as the seventh domain of the large subunit (Steitz and Moore, 2003). The 50S subunit is composed of a rounded base with three protuberances named the L1 protuberance, the central protuberance and the L7/L12 stalk (Wilson and Nierhaus, 2003). The protuberances are highly mobile, which is functionally important.

The central function of the large subunit is to perform peptidyl transfer. This is done in the peptidyl transfer center (PTC). A tunnel starts at the PTC being approximately 100Å long and 15Å in diameter (Nissen et al., 2000). The nascent polypeptide is supposed to exit through this tunnel at the cytoplasmatic side of the 50S subunit. The polypeptide exit tunnel is
largely formed by RNA, but has significant contributions from proteins L4 and L22, and its exit is encircled by proteins L19, L22, L23, L24 and L29 (Nissen et al., 2000).

The substrates of the reaction catalyzed by the large subunit are an aminoacyl-tRNA (aa-tRNA) and a peptidyl-tRNA. The former binds in the ribosome's A-site and the latter in its P-site. The \(\alpha\)-amino group of the aa-tRNA attacks the carbon of the carbonyl acylating the 3' hydroxyl group of the peptidyl-tRNA, and a tetrahedral intermediate is formed at the carbonyl carbon. The tetrahedral intermediate resolves to yield a peptide extended by one amino acid esterified to the A site-bound tRNA and a deacylated tRNA in the P-site (Nissen et al., 2000). Surprisingly, there are no protein side-chain atoms closer than about 18 angstroms to the peptide bond being synthesized. Thus, the ribosome is a ribozyme. Details about the mechanism of the peptidyl transferase reaction are presented in several works (Green and Lorsch, 2002; Steitz and Moore, 2003; Wilson and Nierhaus, 2003).

During elongation the deacylated tRNA from the P-site has to move into the E-site and the peptidyl-tRNA has to move from the A-site to the P-site. This process is called translocation, accompanied by the movement of mRNA by three nucleotides exposing the next codon in the A-site. A comprehensive review on the translocation process is available (Noller et al., 2002).

The relative orientation of the two subunits is elucidated (Yusupov et al., 2001), the location of mRNA (Yusupova et al., 2001) and the tRNA molecules in the A-, P- and E-sites are identified (Yusupov et al., 2001). The length and shape of the tRNA molecules correlate well with the location of the decoding site on the small subunit and the PTC on the large subunit. In fact, the main activities of the ribosome take place at the subunit interface. Intersubunit bridges hold the subunits together and the dynamic properties of the ribosome depend to a large extent on these bridges. The bridges were analyzed in the first cryo-EM maps (Frank et al., 1995; Gabashvili et al., 2000) and in the low-resolution crystal structure of the 70S ribosome (Cate et al., 1999). However, the components that are involved in formation of these bridges could not be identified in that period. The recent set of bridges counts 12 and they are named B1-B8 with B1, B2 and B7 being composed of several neighboring contacts (Yusupov et al., 2001). One main component at the subunit interface is helix 44, which is the penultimate stem of the 16S rRNA. It spans along the body of the small subunit and makes several contacts with the large subunit (B2a, B3, B5 and B6) (Yusupov et al., 2001).
1.2.2 The messenger RNA

The messenger RNA (mRNA) is a central molecule in the translation of a genetic message into protein. During initiation, the translation initiation region (TIR) of mRNA and the initiator tRNA (fMet-tRNA$^{\text{Met}}$) are selected by the 30S subunit to form a ternary complex (Gualerzi and Pon, 1990). This selection is kinetically controlled by initiation factors IF1, IF2 and IF3 (Laursen et al., 2005 and references therein). The mRNA covered by the ribosome in the initiation phase is called the ribosome binding site (RBS) and extends over about 30 nucleotides (Steitz, 1969). The recognition and binding of the 30S ribosomal subunit to the correct start site of the mRNA depends to various degrees on structural elements of the translational initiation region (TIR) of the mRNA. The main elements of a canonical TIR include the initiation triplet (most frequently AUG), the purine-rich Shine-Dalgarno (SD) sequence complementary to the 3´ end region of the 16S rRNA (Shine and Dalgarno, 1974), and a spacer of variable length separating the SD sequence and the initiation triplet (Gualerzi and Pon, 1990; McCarthy and Brimacombe, 1994). The sequence upstream of the initiation codon is called the 5´ untranslated region (5´UTR) and it includes the SD sequence. A direct consequence of the SD interaction is the adjustment of the initiation codon to the ribosomal P-site where it interacts with the initiator tRNA. In *E. coli* the mRNAs usually have the SD sequence AGGAGG located 7-9 nucleotides upstream from the initiation codon. In general, the spacer between the SD and the start codon varies between 3 and 12 nucleotides and has been shown to be crucial for initiation efficiency (Gold, 1988; Ringquist et al., 1992). The ribosomal machinery does not require a perfect spacing, but when it drops below 4 or is longer than 14 nucleotides, the expression decreases more than 10-fold (Gold, 1988; Ringquist et al., 1992).

In the canonical initiation complex the start codon can be AUG, GUG or UUG (McCarthy and Brimacombe, 1994). These initiation codons occur in *E. coli* at a frequency of 90% (AUG), 8% (GUG) and 1% (UUG) (Blattner et al., 1997; Schneider et al., 1986). The exceptional AUU initiation codon is used in the genes *infC* and *penB*, encoding IF3 (Sacerdot et al., 1996) and poly(A)polymerase (Binns and Masters, 2002) respectively.

The ribosomal protein S1 has been shown to influence translation of all mRNAs and was found to be indispensable for translation initiation of mRNAs lacking a SD sequence (Sorensen et al., 1998; Tzareva et al., 1994). Protein S1 displays a strong characteristic binding towards a pyrimidine-rich region upstream of the SD sequence of mRNAs (Boni et
al., 1991; Subramanian, 1983). The protein is located on the back of the 30S and binds to the ribosome by its N-terminus (Giorginis and Subramanian, 1980), while the C-terminus interacts with mRNA (Schnier and Isono, 1982). S1 consists of six repeats of the oligonucleotide-binding fold (OB-fold), each similar in sequence to translation initiation factor IF1 and several other RNA-binding proteins (Bycroft et al., 1997). It has been shown that a strong SD-rRNA interaction can overtake the requirements for protein S1 (Farwell et al., 1992), and that protein S1 deficient ribosomes can form initiation complexes on leaderless mRNA (Tedin et al., 1997).

Most of Gram-positive bacilli mRNA contain a strong SD region upstream of the initiation codon. At the same time the protein S1 is absent in *Bacillus subtilis* and other related Gram-positive bacteria (Roberts and Rabinowitz, 1989). These facts indicate that the protein S1 can act as an enhancer in mRNAs containing a weak SD sequence, providing non-specific ribosome-mRNA binding. A direct interaction has been confirmed by cryo-EM studies of S1 on the 30S ribosomal subunit with a bound mRNA (Sengupta et al., 2001).

Another translational cis-acting element which is often held responsible for the translation of leaderless mRNAs is the so-called “downstream box” (DB). The DB was originally described as one of many translational enhancers present in several highly expressed *E. coli* and bacteriophage mRNAs (Sprengart et al., 1990; Sprengart and Porter, 1997). These authors suggested that the relevant sequence element is located between nucleotides +15 to +26 downstream of the initiation codon. In addition, Sprengart and co-workers proposed a complimentary base pairing between the DB and the nucleotides 1469 to 1483 (so-called “anti-DB”) within the helix 44 of the 16S rRNA (Sprengart et al., 1990; Sprengart et al., 1996). It has been proposed that base pairing between DB and anti-DB sequences places the start codon of the mRNA in close contact with the decoding region of 16S rRNA, thereby mediating independent and efficient initiation of translation (Sprengart et al., 1996). However, the evidence in favor of a participation of the DB sequence in 30S-mRNA interaction is indirect, and stems primarily from genetic manipulations which cause an increased or decreased level of translation, depending on whether DB is removed, added, shifted or its complimentarity to 16S rRNA is weakened/strengthened (Etchegaray and Inouye, 1999; Mitta et al., 1997; Sprengart et al., 1996).

Crosslinking studies of the region downstream of the initiation codon revealed several bases that crosslink to the 16S rRNA (Sergiev et al., 1997). However, no crosslinks were found between helix 44 of the rRNA and the nucleotides situated in the downstream region of the mRNA.
Chemical protection studies also failed to support the DB-anti-DB interaction. Thus, chemical footprinting of the T4 gene 32 mRNA demonstrated no protection from modifications of the DB area, while the SD region of this mRNA was protected (Huttenhofer and Noller, 1994). In toeprinting studies using three different leaderless transcripts (λ cl, phage P2 gene V and Tn1721 tetR mRNA) and after mutagenic inactivation of the DB element of λ cl mRNA, Resch and coworkers (Resch et al., 1996) reached the conclusion that the DB element does not influence translation efficiency.

The presence of a DB-anti-DB interaction has been a matter of debate until the creation of a multiple mutation of the complete putative anti-DB in helix 44, reversing all 12 base pairs within the helix, while still possessing the same stability as the wild type (O’Connor et al., 1999). In this study, reporter constructs containing different DBs were completely unaffected by the radically altered mRNA base pairing compared to the wild-type. Moreover, there is no biochemical or genetic evidence in support of the proposed role of the DB-anti-DB interaction in ribosomal recruitment of mRNA (Moll et al., 2002; Moll et al., 2001). All these works clearly demonstrate that the enhancing effect of the DB does not involve complementary base pairing between DB and the anti-DB.

The dispensable nature of the Shine-Dalgarno sequence is evidenced by existence of leaderless mRNAs which begin directly with an AUG initiation triplet (Janssen, 1993). The only essential cis-acting recognition element identified with certainty in these mRNAs, which are sometimes very efficiently translated, is the 5'-AUG triplet. The occurrence of leaderless mRNAs in all domains of life suggested that the ability to translate this class of mRNAs is evolutionarily conserved (Janssen, 1993), and several studies demonstrated that leaderless mRNAs of different origin are faithfully translated in heterologous bacterial systems as well as in both archaeal and eukaryal in vitro translation systems (Grill et al., 2000; Moll et al., 2001; Tedin et al., 1997; Wu and Janssen, 1996; Wu and Janssen, 1997). Binding of leaderless mRNAs is dependent on the presence of the initiator tRNA, whereas canonical mRNAs bind independently of the initiator tRNA (Benelli et al., 2003). Studies in E. coli revealed that the IF2:IF3 molar ratio plays a decisive role in translation initiation of a leaderless mRNA both in vitro and in vivo (Grill et al., 2001). It was suggested that leaderless mRNA is recognized by a 30S-IF2-fMet-tRNA\textsubscript{Met} complex (Grill et al., 2000; Grill et al., 2001). This was based on the finding that an increase in the concentration of IF2 enhances the efficiency of leaderless mRNA translation, possibly by recruitment of fMet-tRNA\textsubscript{Met} to 30S ribosomal subunits, thus enabling codon-anticodon interaction.
Using a highly reconstituted cell-free translation system from *Escherichia coli* it was found that leaderless mRNAs bind preferentially to 70S ribosomes and that the leaderless mRNA-70S-fMet-tRNA complex can transit from the initiation to the elongation phase even in the absence of initiation factors (Udagawa et al., 2004).

1.2.3 The initiator tRNA

*Escherichia coli* contains a main and a minor form of initiator tRNA, namely tRNA<sub>f1</sub><sup>Met</sup> (~75%) and tRNA<sub>f2</sub><sup>Met</sup> (~25%). Both tRNAs contain 76 nucleotides and differ in the presence of either 7-methyl-G or A at position 46 (Gualerzi and Pon, 1990). The major fraction of cellular initiator tRNA (tRNA<sub>f1</sub><sup>Met</sup>) is encoded by the *metZ* gene. Three identical copies of the gene occur in tandem repeats within the operon known as the *metZ* operon (Kenri et al., 1994). A relatively small fraction of tRNA<sub>f2</sub><sup>Met</sup> (tRNA<sub>f2</sub><sup>Met</sup>) is encoded by the *metY* gene, located at the beginning of the *nusA/infB* operon (Ikemura and Ozeki, 1977).

Initiator tRNAs bind directly to the P-site of the small subunit of the ribosome, whereas elongator tRNAs enter the ribosome at the A-site and subsequently translocate to the P-site (Laursen et al., 2005 and references therein). The initiator tRNA is endowed with unique structural features connected with its special role in protein synthesis (Wakao et al., 1989). One of this features is the presence of three consecutive G:C base pairs in the anticodon stem that confer rigidity and regularity to the helix resulting in a particular conformation of the anticodon loop, which targets the initiator tRNA to the ribosomal P-site (Schweisguth and Moore, 1997; Seong and RajBhandary, 1987). The second attribute is the absence of a Watson-Crick base pair between positions 1 and 72 in the acceptor stem. This feature is responsible for the resistance of fMet-tRNA to the action of peptidyl-tRNA hydrolase (PTH), which is a 21kDa monomeric enzyme that recycles all N-blocked aminoacyl-tRNA molecules accumulating from abortive translation (Thanedar et al., 2000). In addition, the amino acid attached to the initiator tRNA is also important in conferring protection against PTH. The third feature of initiator tRNA is the presence of a purine-1:pyrimidine-24 in contrast to the pyrimidine-1:purine-24 base pair found in other tRNAs (Varshney et al., 1993).

The initiator tRNA has the same CAU anticodon as the tRNA<sup>Met</sup> used in elongation and is aminoacylated by the same synthetase, which recognizes primarily the bases of the anticodon (RajBhandary, 1994). Recognition of the anticodon by methionyl tRNA synthetase occurs
through the helical C-terminal region of the synthetase (Mechulam et al., 1999). Later, the aminoacylated initiator tRNA is formylated by methionyl tRNA transformylase (MTF). The most important determinant for formylation of the initiator tRNA is the absence of a 1:72 base pair. Formylation favors selection of the fMet-tRNA<sub>f</sub><sup>Met</sup> by IF2 (Sundari et al., 1976), and blocks binding to the elongation factor EF-Tu and thus the function as elongator tRNA (Hansen et al., 1986; Nissen et al., 1995). The nature of the amino acid attached to the tRNA is less important for IF2 binding than is formylation. However, a binary complex can be formed between fMet-tRNA<sub>f</sub><sup>Met</sup> and IF2 in vitro, most evidence suggest that IF2 interacts with fMet-tRNA<sub>f</sub><sup>Met</sup> on the 30S ribosomal subunit (Wu and RajBhandary, 1997).

It is known that binding of initiator tRNA to the P-site is kinetically controlled by initiation factors. Thus, the initiation factor IF3 appears to inspect the anticodon stem of the P-site bound tRNA through indirect interactions (Hartz et al., 1990). At the same time, initiation factor IF1 blocks the A-site (Dahlquist and Puglisi, 2000) and helps to direct the initiator tRNA solely into the P-site.

1.2.4 Initiation factor IF1

Initiation factor IF1 has a molecular weight of 8.2 kDa and is the smallest of the three protein factors required for optimal translation initiation in *Escherichia coli* (Hershey, 1987). In *E. coli* IF1 contains 71 amino acid residues of known sequence (Pon et al., 1979) and its structural gene *infA* has been cloned and mapped at 19.9 min on the *E. coli* chromosome (Sands et al., 1987). Transcription of *infA* is not physically linked to any other genes as are *infB* and *infC* (the genes encoding IF2 and IF3) (Cummings et al., 1991). Two promoters (P1 and P2) control the transcription of the *E. coli* gene as monocistronic mRNAs, both ending at one ρ-independent transcriptional terminator (Cummings et al., 1991). The 5’-untranslated region of P1 transcripts is approximately 200 nucleotides longer compared to the P2 transcripts. At normal growth temperatures the P2 promoter is more active than P1, and P2 transcripts are roughly two-fold more abundant than the P1 transcripts. In contrast, transcription from the *infA* P1 promoter is highly activated during cold shock (Ko et al., 2006). In *E. coli* the expression of *infA* from the P2 promoter is under growth-rate control (Cummings et al., 1991) and its inactivation cannot be tolerated by the cell without loss of viability (Cummings and Hershey, 1994). Since cold shock solely activates P1, while P2 is exclusively under the metabolic
control, it was suggested that differential activation of the *infA* promoters is a response to diverse environmental changes (Ko *et al.*, 2006).

The structure of IF1 in solution has been determined by NMR spectroscopy (Sette *et al.*, 1997). This structure is characterized by a remarkable rigid five-stranded β-barrel. The N- and C-termini outside the β-barrel are disordered and highly flexible. The loop connecting strands β3 and β4 of the β-barrel contains a short 3_10 helix (residues 38-44). This internally well ordered region shows substantial plasticity as a whole and moves probably as a rigid entity with respect to the β-barrel. The fold of IF1 reveals a striking structural similarity to proteins belonging to the oligomer-binding (OB) family, a class of proteins that interact with oligonucleotides and oligosaccharides (Sette *et al.*, 1997). The OB fold family includes RNA binding proteins such as ribosomal protein S1 (Gribskov, 1992), the cold shock proteins cspA and cspB (Bycroft *et al.*, 1997), eukaryotic initiation factors eIF1A (Battiste *et al.*, 2000), eIF2α (Gribskov, 1992) and N-terminal domain of aspartyl tRNA synthetase (Ruff *et al.*, 1991). Structures of the archaeal and eukaryotic IF1 homologues (aIF1A and eIF1A respectively) have also been determined (Battiste *et al.*, 2000; Li and Hoffman, 2001). These structures are highly similar regarding the OB fold (Figure 2).

**Figure 2. Initiation factor IF1 and its structural homologues.** IF1 from *E. coli*, human eIF1A (residues 40 to 125), aIF1A from *Methanococcus jannaschii* and cold shock protein A (CspA) from *E. coli* are shown. Picture adapted with permission from Laursen *et al.*, 2005. Copyright © 2005, the American Society for Microbiology.

The considerable similarity of the OB folds of eIF1A and IF1 supports the view of the universal role of IF1, which is amplified by a functional resemblance. However, the C-terminus of eIF1A contains α-helical structures that are important for the eukaryotic scanning mechanism.
and interactions with the small ribosomal subunit (Pestova and Hellen, 2001).

A recent study has shown that heterologous expression of the translation initiation factor IF1 from *E. coli* is able to cure cellular defects observed in a *B. subtilis cspB cspC* double deletion strain (Weber *et al.*, 2001), suggesting that IF1 and cold shock proteins have at least in part overlapping cellular function(s) (Sommerville, 1999).

Early topographical studies carried out by protein-protein crosslinking identified S12, S18 and IF2 as IF1 neighbors on the ribosome (Boileau *et al.*, 1983). Later, chemical probing with dimethyl sulfate and kethoxal showed that IF1 protects the bases G530, A1492 and A1493 in the 16S rRNA and thus indicating that the factor binds in the A-site of the 30S ribosomal subunit (Moazed *et al.*, 1995). This set of nucleotides is also protected by the A-site bound tRNA (Moazed and Noller, 1986; Moazed and Noller, 1990) strongly suggesting that IF1 is located at an overlapping binding site. Cleavage of 16S rRNA with cloacin DF13 between A1493 and A1494, two bases located in the A-site of the 30S subunit, specifically disrupts the function of IF1 (Baan *et al.*, 1976).

To characterize the IF1-16S rRNA interaction, several ribosomal mutants in the decoding region of 16S rRNA were created (Dahlquist and Puglisi, 2000). Mutation of any of the three adenosines in the A-site, A1408, A1492 or A1493 to a guanosine has an extremely detrimental effect on IF1 binding. A mutation in the 530 loop does not influence IF1 binding in the A-site. Mutational analysis also demonstrated that the C1407:G1494 base pair is required for optimal IF1 binding. This binding is sensitive to the conformation of the internal loop formed by A1408, A1492 and A1493 (Dahlquist and Puglisi, 2000).

More recently, the A-site localization of IF1 was confirmed by X-ray crystallography, which demonstrated that the factor binds in the cleft between ribosomal protein S12, helix 44 and the 530 loop of the 16S rRNA (Carter *et al.*, 2001) (Figure 3). This structure of the 30S ribosomal subunit from *Thermus thermophilus* in complex with IF1 agrees well with most mutagenesis and biochemical data. The side of IF1 that interacts with the ribosome is rich in basic residues, whereas most of the acidic residues are on the solvent-exposed surface. A loop from IF1 inserts into the minor groove of helix 44, forms contacts with the backbone of several nucleotides, and flips out bases A1492 and A1493. This insertion determines a 5Å lateral displacement of C1411 and C1412 which affects helix 44 conformation over a long distance (~70Å). In turn, these changes induce a rotation of the head, platform and shoulder of the 30S subunit towards the A-site (Carter *et al.*, 2001). This conformational change may
represent a transition state in the equilibrium between subunit association and dissociation (Ramakrishnan, 2002). In the crystal, A1493 is hidden in a pocket on the surface of IF1, whereas A1492 is located in a cavity formed at the interface between IF1 and S12 (Carter et al., 2001). The sequestering of bases A1492 and A1493 into protein pockets explains why IF1 totally protects them from chemical modification (Moazed et al., 1995). The crystallographic data confirmed the interaction of IF1 with the ribosomal A-site, but did not confirm the proposal that IF1 mimics A-site bound tRNA (Brock et al., 1998).

**Figure 3. Interaction of IF1 with the 30S subunit.**
(A) View of the IF1 binding site, with IF1 in purple, helix 44 in cyan, the G530 loop in green, and protein S12 in orange.
(B) Overview showing the position of IF1 (purple) with respect to the 30S subunit (gray). H44, G530 loop, and S12 are coloured as in (A). H, head; Bo, body; N, neck; Sh, shoulder; P, platform. Picture adapted with permission from Carter et al., 2001. Copyright © 2001, AAAS.

A number of amino acids crucial for IF1 function were identified by site directed mutagenesis (Croitoru et al., 2004; Gualerzi et al., 1989; Spurio et al., 1991) and NMR spectroscopy (Paci et al., 1983; Sette et al., 1997). In particular, specific changes of NMR signals of IF1 upon titration with 30S ribosomal subunits have identified several residues that are involved in or affected by the binding of IF1 to the 30S ribosomal subunit: Leu13, Arg22, Asn27, His29, His34, Ile35, Asn42, Ile44, Thr53, Val67 and
Arg71 (Sette et al., 1997). For His29 and His34 this corresponds to the results previously described by (Paci et al., 1983). These two histidines were also investigated using site-directed mutagenesis (Gualerzi et al., 1989). It was observed that replacement of His34 with Asp (but not with Tyr) severely impaired the binding of IF1 to the 30S ribosomal subunit, while His29 to Asp mutation allowed substantial binding to the small subunit, but the produced complexes were essentially inactive. Furthermore, His29 mutants proved to be defective in the ejection of IF1 from the ribosome during 30S-50S association (Gualerzi et al., 1989). In the same study it was found that a short form of IF1 lacking the C-terminal residues Arg69-Arg71 has lost the ability to form a complex with 30S ribosomal subunits and to stimulate the binding of fMet-tRNA to the ribosome. Recently, all six Arg residues of IF1 were separately mutated to Leu or Asp resulting in various degrees of cold-sensitivity for some of these IF1 variants (e.g. R40D, R45D, R69D, R69L+R71L) (Croitoru et al., 2004). Moreover, the Arg65 to Asp alteration was lethal. These data provided in vivo evidence for assigning the functional sites of IF1 to the flexible N-terminus, C-terminus and the 3₁₀ helix of the initiation factor (Croitoru et al., 2004). According to our interpretation, conserved residues in IF1 make tight electrostatic bonding interactions with the phosphate backbone of the 530 loop and of the helix 44 of the 16S rRNA (discussed in Paper I). In addition, the amino acids V40 and W42 of ribosomal protein S12 are important for the interaction of IF1 with the 30S subunit (Carter et al., 2001).

Several functions have been attributed to bacterial IF1. It affects the association/dissociation rate of ribosomal subunits (Dottavio-Martin et al., 1979), thereby favouring the ribosome dissociation activity of IF3 (Grunberg-Manago et al., 1975). An important function of IF1 is that of modulating the affinity of IF2 for the ribosome by favouring its binding to the 30S subunit. And vice-versa, the release of IF2 is indirectly promoted when IF1 is ejected (Celano et al., 1988; Moreno et al., 1999; Stringer et al., 1977). The influence of IF1 on the affinity of IF2 for the 30S ribosomal subunit may result from a physical contact between these two factors on the ribosome as suggested by cross-linking studies (Boileau et al., 1983) and phylogenetic comparisons (Choi et al., 2000) or may be indirectly caused by the IF1-induced conformational change described above. IF1 cooperates with IF2 to ensure that only the initiator tRNA binds to the P-site and that it interacts with the initiation codon of the mRNA (Canonaco et al., 1986; Hartz et al., 1990). In addition, the synergistic action of IF1 and IF2 promotes dissociation of peptidyl-tRNAs with polypeptides of different length from the P-site of translating E. coli ribosomes (Karimi et al., 1998).
In the same study it was also found that simultaneous overexpression of plasmid-encoded IF1 and IF2 inhibits growth of bacterial strains deficient in peptidyl-tRNA hydrolase (pth) activity, suggesting that initiation factor dependent drop-off of peptidyl-tRNA may occur quite frequently in the bacterial cell.

The binding of IF1 to the A-site of the 30S subunit suggests that this factor may participate in conferring specificity to the formation of the 30S initiation complex by occluding the access of elongator tRNA to the A-site until a 70S initiation complex is formed. Subsequently, ejection of IF1 opens the A-site for incoming aminoacyl-tRNAs (Moazed et al., 1995).

By binding to the A-site, IF1 may play an “initiation fidelity function” by recognising the +2 codon immediately following the initiation codon, thus representing the ribosomal A-site. Based on our recent results using several IF1 mutants and expression of two model genes with various +2 codons in mRNA, we suggested that IF1 has no codon discriminatory functions, while occluding the A-site (Croitoru et al., 2005).

1.2.5 Initiation factor IF2

IF2 is an essential protein (Cole et al., 1987) encoded by the infB gene being a part of the polycistronic nusA operon. Three isoforms of the initiation factor, named IF2-1 (97.3 kDa), IF2-2 (79.7 kDa), and IF2-3 (78.8 kDa), exist in E. coli and other members of the family Enterobacteriaceae (Laursen et al., 2002). The three isoforms are translated from three independent, but in frame, translational start sites of the infB mRNA. This attribute has been referred to as tandem translation (Nyengaard et al., 1991). Hence, IF2-2 and IF2-3 differ from IF2-1 only by the absence of the first 157 and 164 amino acid residues, respectively (Mortensen et al., 1995) (Figure 4).
Bacterial initiation factor IF2 consists of three major segments, a variable N-terminal region, a highly conserved 40 kDa part containing in its center a G-domain (GTP binding) and a 25 kDa C-terminal part which contains the fMet-tRNA binding site (Boelens and Gualerzi, 2002). Additionally, the factor can be divided in six domains based on interspecies homology (Mortensen et al., 1998) (Figure 4).

Homologues of IF2 have been found in archaea and eukaryotes, where the factor is referred to as aIF5B and eIF5B, respectively (Kyripides and Woese, 1998). Overall homology among bacterial IF2, eukaryotic eIF5B and archaeabacterial aIF5B is highest in the G-domain area of the factor (Laursen et al., 2005 and references therein). Significant sequence homology to other proteins is found for this domain. The GTP binding motif is shared with at least four other proteins involved in translation, namely, EF-Tu, EF-G, RF3, and SelB (Rodnina et al., 2000; Vetter and Wittinghofer, 2001). These factors bind in a site located on the 50S ribosomal subunit termed the factor binding site, which is composed of the α-sarcin loop, the L11-binding region and the L7/L12 stalk (Cameron et al., 2002; Moazed et al., 1988).
The crystal structures of IF2 from archaeal bacterium *Methanobacterium thermoautotrophicum* have been determined in three different states: free enzyme, “inactive” IF2-GDP and with GDPNP mimicking the “active” IF2-GTP (Roll-Mecak *et al*., 2000). Recently, the cryo-EM structure of a translation initiation complex from *E. coli* was presented (Allen *et al*., 2005). In this reconstruction IF2-GDPNP binds at the intersubunit cleft of the 70S ribosome. The IF2-GDPNP contacts the 30S and 50S subunits as well as fMet-tRNA^f_{Met}. Here IF2 adopts a conformation radically different from that seen in the crystal structures of IF2 reported above. The C-terminal domain of IF2 binds to the single-stranded portion of fMet-tRNA^f_{Met} (Spurio *et al*., 2000), thereby forcing the initiator tRNA into a novel orientation at the P site. The cryo-EM structure confirmed that the GTP binding domain of IF2 binds to the GTPase-associated center of the 50S subunit in a manner similar to EF-G and EF-Tu.

The role of the GTPase activity of IF2 has been a matter of debate for decades. Early studies suggested that GTP hydrolysis is important for the release of IF2 from the 70S initiation complex (Lelong *et al*., 1970; Lockwood *et al*., 1972) and for adjustment of initiator tRNA in the ribosomal P-site (La Teana *et al*., 1996). However, other groups concluded that neither the ejection of IF2 from the ribosome nor its recycling requires GTP hydrolysis (La Teana *et al*., 2001; Tomsic *et al*., 2000). A recent study of initiation complex formation by using stopped-flow experiments with light scattering concluded that the GTP-bound form of IF2 accelerates association of the ribosomal subunits and that GTP hydrolysis accelerates ejection of IF2 from the 70S ribosome (Antoun *et al*., 2003). The essential importance of GTP hydrolysis in translation initiation and its direct relation to cell viability have been confirmed by several studies of G-domain mutants of IF2 (Larigauderie *et al*., 2000; Laursen *et al*., 2003; Luchin *et al*., 1999). Additionally, it was suggested that IF2 could be a sensor of the metabolic state of the cell using the GDP/GTP binding site as a receptor where either GTP (under optimal growth conditions) or ppGpp (during nutritional stress) could bind (Gualerzi *et al*., 2001). Thus, when cells are starved for an essential amino acid or for a carbon source, ppGpp binds in place of GTP and induces an inactive conformation of IF2, which is now impaired in the formation of the 30S initiation complex and initiation dipeptide (Boelens and Gualerzi, 2002). However, further studies are needed to accomplish a complete understanding of the role of GTP hydrolysis in the translation initiation event.

IF2 has been cross-linked to S13, L7/L12, IF1, and IF3 (Boileau *et al*., 1983), as well as S1, S2, S11, S12, and S19 on the ribosome (Bollen *et
Chemical probing experiments with 23S rRNA indicated that IF2 protects A2476 and A2478 in helix 89 of domain V as well as G2655, A2660, G2661, and A2665 of the sarcin-ricin domain positioned in domain VI of 23S rRNA (La Teana et al., 2001).

The main function of IF2 is stimulation of fMet-tRNA binding to the ribosome. In spite of the common opinion that IF2 is the carrier of initiator tRNA to the ribosome, as EF-Tu is the carrier of the elongator aminoacyl-tRNAs, some data suggest that IF2 is already bound to 30S when it performs its activities (Canonaco et al., 1986). Additional functions reported for IF2 are that of promoting subunit association (Grunberg-Manago et al., 1975; La Teana et al., 2001) and, upon formation of the 70S initiation complex, that of favoring the first transpeptidation by inducing the correct positioning of fMet-tRNA in the ribosomal P-site (La Teana et al., 1996; Tomsic et al., 2000).

Besides the function as a translation factor, IF2 possess the properties of a protein chaperone. It forms stable complexes with unfolded proteins and promotes functional folding of proteins (Caldas et al., 2000). In addition, the expression of IF2 is upregulated during the cold shock response (Gualerzi et al., 2003), and the factor is important for the translation of leaderless transcripts (Grill et al., 2000).

1.2.6 Initiation factor IF3

Bacterial initiation factor IF3 consists of 180 amino acids in E. coli and is encoded by the essential infC gene mapped at 37.5 min (Sacerdot et al., 1982). The gene encoding IF3 is a member of the infC-rpmI-rplT operon encoding IF3 and the two ribosomal proteins L35 and L20 (Chiaruttini et al., 1996; Lesage et al., 1990). IF3 is a 20.5 kDa protein containing two domains of approximately equal size (Fortier et al., 1994; Kycia et al., 1995; Moreau et al., 1997) that are connected via ~45 Å long, lysine-rich and flexible linker (Hua and Raleigh, 1998; Moreau et al., 1997). The linker can be readily cleaved by proteases to obtain two stable domains (Kycia et al., 1995; Lammi et al., 1987). The linker is essential for IF3 function, but variation of its length and composition does not considerably change the activity of the factor (de Cock et al., 1999). The two domains are called the IF3N and IF3C, which move (Moreau et al., 1997) and bind to the 30S subunit (Sette et al., 1999) independently. The crystallographic structures of these separate domains of B. stearothermophilus IF3 have been determined with a resolution of 1.8 Å for the IF3N domain (residues 3-78) and 2.0 Å for the IF3C domain.
The structures of the same domains of the *E. coli* IF3 were analyzed by NMR spectroscopy (Garcia *et al*., 1995a; Garcia *et al*., 1995b). The IF3C domain possesses a two-layered α/β sandwich fold, comprising a four-stranded β-sheet that is packed against two parallel α-helices (α3 and α4) in a βαβαβ topology. The IF3N domain contains a globular α/β fold consisting of a single α-helix packed against a mixed four-stranded β-sheet that superficially resembles that of IF3C (Laursen *et al*., 2005) and (Figure 5).

Figure 5. Structures of the IF3N domain from *B. stearothermophilus* and the IF3C domain from *E. coli*. The side chains of arginine residues in IF3C involved in binding to the 30S subunit (99, 112, 116, 147 and 168) or mRNA (129, 131 and 133) are shown. Picture adapted with permission from Laursen *et al*., 2005. Copyright © 2005, the American Society for Microbiology.

The localization of IF3 on the 30S ribosomal subunit has been extensively investigated by protein-protein (Boileau *et al*., 1983) and protein-RNA crosslinking (Ehresmann *et al*., 1987), by chemical probing (Moazed *et al*., 1995; Muralikrishna and Wickstrom, 1989), immunoelectron microscopy (Stoffler and Stoffler-Meilicke, 1984), by cryo-electron microscopy (McCUTCHEON *et al*., 1999) and X-ray crystallography (PIOLETTI *et al*., 2001). As a result of these studies there is a general agreement that IF3 binds in the vicinity of ribosomal proteins S1, S7, S11, S13, S18, S19, S21 and of nucleotides 674-713 (helix 23), 783-799 (helix 24), 819-859 (helices 25 and 26), and 1506-1529 (helix 45). Nevertheless, the precise location of IF3 on the ribosome remains hard to pin down since the cryo-EM studies place IF3 on the 50S side of the 30S subunit.
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(McCutcheon et al., 1999), while the X-ray crystallography data place it on the opposite side of the platform, in contact with proteins S18, S2, S7 and S11 as well as with rRNA helices 23, 26 and 45 (Pioletti et al., 2001).

Translation initiation factor IF3 performs several functions, at least one of which is essential for cell viability (Olsson et al., 1996). In particular, IF3 promotes the dissociation of the 70S ribosome by antagonizing association of the 30S subunit to the 50S subunit (Grunberg-Manago et al., 1975). Through this activity the factor supplies the pool of free 30S subunits required for translation initiation.

IF3 monitors the codon-anticodon interaction by stimulating the on and off rates of “30S initiation complex” formation (Gualerzi et al., 1977), ensuring translational efficiency and fidelity by stimulating rapid formation of codon-anticodon interaction at the ribosomal P-site (Wintermeyer and Gualerzi, 1983). In fact, IF3 promotes the dissociation of coded or non-coded, charged or uncharged elongator tRNAs from the 30S subunits (Gualerzi et al., 1971; Gualerzi et al., 1979; Hartz et al., 1989) and the dissociation of fMet-tRNA bound to 30S subunits in response to non-canonical triplets other than AUG, GUG and UUG located in the P-site (Haggerty and Lovett, 1997; Meinnel et al., 1999; Sussman et al., 1996). In addition, IF3 promotes dissociation of fMet-tRNA from initiation complexes formed at the 5’ initiation codon (AUG) of leaderless mRNAs (La Teana et al., 1993; Tedin et al., 1999). Another property of IF3 is that of inducing a repositioning of 30S-bound mRNA, which is shifted from the “standby site” to the “P-decoding site” of the subunit as evidenced by the different pattern of site-directed cross-linking between mRNA and different ribosomal components obtained in the presence and absence of IF3 (La Teana et al., 1995). The functional significance of this shift is not entirely clear, but it has been suggested that it involves the kinetic selection of the correct initiation triplet by IF3 (Boelens and Gualerzi, 2002). Finally, a role for IF3 in recycling of ribosomal subunits has been investigated (Singh et al., 2005). IF3 was observed to enhance the dissociation of deacylated tRNAs from post-termination complexes and to dissociate 70S ribosomes into subunits after post-termination complex release by ribosome recycling factor RRF (Hirokawa et al., 2002; Karimi et al., 1999).

To explain the existence of such a large number of functions with a single mechanism, early studies suggested that the “translation fidelity” and the other functions of IF3 are a result of an IF3-induced conformational change of the 30S subunit (Pon and Gualerzi, 1974; Pon et al., 1982). After the subsequent elucidation of the 3D structure of IF3 identifying two discrete domains, it was suggested that the domains might carry out different IF3 functions independently. For instance, it was suggested that
IF3C is responsible for ribosomal binding and ribosome dissociation activity, while IF3N carries out the “initiation fidelity function” of the protein (de Cock et al., 1999). However, this prediction was found to be incorrect when it was demonstrated that isolated IF3C binds to 30S subunit being capable of performing all the functions of the intact molecule, provided that its concentration is high enough to compensate for the reduced affinity for the 30S subunit (Petrelli et al., 2001). On the other hand, the isolated IF3N was found to have no autonomous function, but to serve the purpose of modulating the thermodynamic stability of the IF3-30S complexes.

Site-directed mutagenesis of the eight arginine residues in the IF3C domain has been used to map the active sites of IF3 (Petrelli et al., 2003). The arginines at positions 99, 112, 116, 147, and 168 are important for the binding to the 30S ribosomal subunit. The ability of IF3 to dissociate the ribosome into subunits was affected mainly by mutations of R112 and R147 (and less extensively by mutations of R99 and R116). The stimulation of the pseudo-initiation complex dissociation (with a non-initiator tRNA bound) was affected by mutations of R99 and R112 (and less extensively of the arginine residues at positions 116, 129, 133, and 147). Dissociation of non-canonical initiation complexes (initiation codons other than AUG, GUG, and UUG) was not affected in any of the mutants. Stimulation of translation was affected by mutations of R116 and R129 (and less extensively of the arginine residues at positions 99, 112, and 131), whereas inhibition of non-canonical mRNA translation was affected by mutations of R99, R112, and R168 (and less extensively of the arginine residues at positions 116, 129, and 131). Finally, the repositioning of the mRNA from the “standby site” to the “P-decoding site” was weakly affected by mutations of the arginine residues at positions 129, 131, 133, 147, and 168 (reviewed in Laursen et al., 2005). The data indicate that IF3C contains at least two active surfaces, one embedded in the 30S subunit and the other facing the mRNA (Petrelli et al., 2003). Overall, mapping of IF3 residues implicated in binding to the 30S ribosomal subunit by NMR (Sette et al., 1999) and site-directed mutagenesis are in excellent agreement.

Two alternative mechanisms have been suggested to explain the function of IF3 in determining initiation fidelity. According to the “direct inspection” model, IF3 establishes a physical contact with at least some bases in mRNA and tRNA involved in codon-anticodon interaction (Meinnel et al., 1999) and/or with the anticodon stem-loop of initiator tRNA (Hartz et al., 1990). The alternative model, originally proposed by Pon and Gualerzi in 1974, postulates an indirect mechanism in which IF3 affects the conformational dynamics of the 30S ribosomal subunits,
whereby the dissociation rates of canonical, non-canonical and pseudo-30S initiation complexes are differentially increased, resulting in a kinetic discrimination against the latter two complexes (reviewed in Boelens and Gualerzi, 2002). Strong support for the indirect mechanism of initiation fidelity comes from the localization of IF3 on the 30S ribosomal subunit that has been determined by cryo-electron microscopy (McCutcheon et al., 1999) and X-ray crystallography (Pioletti et al., 2001). Although in sharp contrast to one another, both localizations place IF3C too far away from P-site decoding as well as from the anticodon stem-loop of P-site-bound tRNA (Cate et al., 1999; Schluenzen et al., 2000; Wimberly et al., 2000) to allow the C-domain of IF3 to establish a physical contact with either structure.

Since the two domains of IF3 were shown to be on opposite sides of the fMet-tRNA\textsubscript{f} Met (Dallas and Noller, 2001), IF3 has been thought to interact with the anticodon stem and loop of fMet-tRNA\textsubscript{f} Met (Hartz et al., 1990). However, IF3 is unable to reach the three conserved discriminator G:C base pairs in the anticodon stem of fMet-tRNA\textsubscript{f} Met in the current model. Therefore, discrimination against elongator tRNAs promoted by IF3 is probably indirect (Dallas and Noller, 2001).

1.3 Regulation of translation initiation

All organisms contain stress response systems that allow them to survive rapidly changing environmental conditions. The ribosome plays a central role in adaptation to environmental stress as a checkpoint for sensing shifts in temperature (Van Bogelen and Neidhardt, 1990) and nutrient levels (Ashe et al., 2000). Although transcriptional regulation is the primary mechanism in stress responses, regulation of translation is faster and consequently important. Post-transcriptional regulation occurs at different stages including mRNA stability and translation initiation (Laursen et al., 2005). Translational regulation can involve cis-acting elements of the mRNA that form secondary or tertiary structures which sequester the ribosomal binding site (RBS). trans-Acting elements include protein, antisense RNA, and other factors that control the alternative structures of the RBS and thus affect the efficiency of initiation complex formation (reviewed in Laursen et al., 2005).

The initiation of translation of a given gene relays on use of the same components (ribosomal subunits, translation factors, initiator tRNA). The only variable component is the mRNA, where different nucleotide composition encodes the specificity for each particular gene. The sequence
and structure of the mRNA determine its interaction with the translational machinery, thus influencing the efficiency of translation. The sequence requirements include a proper Shine-Dalgarno sequence, a genuine start codon and a favourable downstream region (Stenstrom et al., 2001a). Accessibility of the start site is of prime importance for the formation of the initiation complex. Secondary structures in the translational initiation region can influence the rate of translation in vivo (de Smit and van Duin, 1994; Hall et al., 1982).

Bacterial translation initiation can occur at multiple sites on a polycistronic mRNA. Translation of the individual cistrons is often coupled by base pairing between the SD/AUG region of a downstream cistron and a part of the preceding coding sequence. The base pairing is disrupted as ribosomes advance through the upstream cistron, thus activating the downstream start site (Lesage et al., 1992). Translational coupling also occurs by a reinitiation mechanism in which a ribosome reinitiates at the next cistron when a stop codon is reached (Govantes et al., 1998; Saito et al., 1994). Overlapping between the end of the first cistron and the beginning of the second cistron is often involved in reinitiation (e.g. AUGA). When the terminating ribosome loosens, it may slide forward or backward to locate a reinitiation site (Adhin and van Duin, 1990). Several studies suggest the potential role of termination factors in modulating reinitiation (Crawford et al., 1999; Janosi et al., 1998).

Other features including the initiation and even termination codons are involved in translational regulation. For instance, the initiation codon of the infC gene is the unusual AUU codon. As previously mentioned, IF3 discriminates against non-cognate initiation codons and hence represses the expression of its own gene (Butler et al., 1986). A recent study has suggested that an inefficient termination codon causes ribosomal pausing and queuing along the upstream mRNA region, thus blocking the translation initiation of short genes (Jin et al., 2002).

The codon usage in the early coding region of mRNA frequently varies from the rest of gene sequence (Bulmer, 1988) and could influence translation initiation efficiency. For the +2 codon immediately following the start codon, a bias in codon usage has been correlated with the level of gene expression at the translational level (Looman et al., 1987; Stenstrom et al., 2001b). For instance, G-rich codons at position +2 cause low expression and they are under-represented in natural genes. In contrast, A-rich codons give high expression. Furthermore, rare codons, such as AGA and AGG, appear to be preferentially used within the first 25 codons in E. coli (Chen and Inouye, 1990) and could be important in translation regulation. Several consecutive AGG codons can decrease expression.
leading to dissociation of ribosomes and mRNA (Gao et al., 1997). These codons can form an SD-like sequence (e.g. AGGAGG) located downstream of an initiation codon. Analysis of these SD-like sequences indicated a negative effect on upstream initiation, resulting from the binding of these sequences to the ASD region of 16S rRNA (Alexandrova et al., 1995; Jin et al., 2006). A recent study shows that NGG codons (AGG, CGG, UGG and GGG) located at positions +2 to +5 strongly decrease gene expression (Gonzalez de Valdivia and Isaksson, 2004). In order to obtain this effect, the NGG codon must be in the correct reading frame. Another study shows that NGG codons trigger peptidyl-tRNA drop-off if located at the early coding positions in the mRNA, thereby explaining the strongly reduced gene expression observed for these codons (Gonzalez de Valdivia and Isaksson, 2005).

The SD sequence can also be active at a later stage during translation of an mRNA, regulating translation by signalling for a frameshifting event. In case of the prfB gene encoding RF2, the stop codon UGA that is exclusively identified by RF2 occurs early in the gene in the correct reading frame. In consequence, if there is enough RF2, further synthesis will be stopped. If there is a deficiency of RF2, an internal SD-like sequence will assist a +1 frameshifting, giving a functional release factor (Baranov et al., 2002; Craigen et al., 1985).

1.4 RNA chaperones

RNA molecules have remarkable structural and functional flexibility. This structural adaptability could lead to complications in defining a distinct native structure. The multitude of possible structures that RNA can adopt is known as the RNA-folding problem (Herschlag, 1995). Only a single or a small number of possible structures usually lead to function, thus the RNA must avoid folding into alternative, non-functional structures. RNAs can easily misfold and become trapped in inactive conformations that can be very stable and persistent (Schroeder et al., 2004). RNA-binding proteins can help solving these folding problems by acting as RNA chaperones (Herschlag, 1995). The activity of a protein that unfolds RNAs non-specifically or impedes the formation of misfolded structures is identified as an RNA-chaperone activity (Schroeder et al., 2004). Once the RNA is correctly folded, the chaperones are no longer required.

Recent advances, such as the identification of the ribosome as a ribozyme (Yusupov et al., 2001) and the revealing of how the ribosome senses cognate tRNAs (Ogle et al., 2001) have drawn attention to
fundamental questions regarding the structural conformation of RNA. During the ribosome assembly the rRNA folds sequentially through various intermediates and ribosomal protein-dependent conformational changes. Recently, nearly one third of the 34 large ribosomal subunit proteins have been found to exhibit RNA chaperone activity (Semrad et al., 2004). In addition, early studies identified the small subunit ribosomal protein S12 having RNA chaperone activity \textit{in vitro} (Coetzee et al., 1994) and \textit{in vivo} (Clodi et al., 1999).

Several assays have been used to measure RNA chaperone activity (Rajkowitsch et al., 2005). In some assays, this activity is related to the monomolecular reactions such as group I intron \textit{cis}-splicing or anti-termination of transcription. In other assays, bimolecular reactions are monitored, including acceleration of annealing of two complementary RNAs and group I intron \textit{trans}-splicing. In addition, two assays have been reported that detect RNA chaperone activity \textit{in vivo} (Clodi et al., 1999; Phadtare et al., 2003). The number of proteins for which RNA chaperone activity has been reported is growing rapidly. A wide collection of proteins ranging from bacterial proteins (Hfq, StpA, CspA, CspC, CspE, S12), viral proteins (NCp7) and human proteins (La and Ro60) were evaluated in these assays (Schroeder et al., 2004 and references therein).
2. Results and Discussion

2.1 Generation and characterization of functional mutants in the translation initiation factor IF1 of *Escherichia coli* (Paper I)

Starting from the concept that IF1 is a small protein and essential for cell viability in *E. coli* (Cummings and Hershey, 1994), we were challenged to investigate the function of this factor *in vivo*, taking into account that most of the available data on IF1 have been reported from *in vitro* research. Thus, at the beginning of this project we were driven by the idea to create functional mutants in *infA*(IF1), since a selection of mutants in this factor was missing at that time. In parallel, a chromosomal deletion of the *infA* gene was desired in order to study the effect of plasmid-encoded mutated *infA* genes. After analysis of the literature data (section 1.2.4) and finding that basic aminoacids of IF1 are involved in the binding to RNA, we have decided to independently mutate all six arginine residues of IF1 as a first choice to leucine, and as an alternative to aspartate. Additionally, two lysine residues in position 2 and 51 were mutated to alanine and leucine respectively. Thus, after site-directed mutagenesis of the plasmid encoded *infA* gene the following alterations in the basic residues of IF1 were generated: R22L, R40L, R45L, R65L, R69L, R71L, R22D, R40D, R45D, R65D, R69D, R71D, R69L+R71L, K2A and K51L. Later, four mutants with a cold-sensitive phenotype were obtained after random mutagenesis: G28D, V12A, S36P and D50G. Consequently, nineteen plasmid-borne variants of *infA*(IF1) constituted the first group of mutants in our collection.

The second set of mutants was generated by site-directed mutagenesis followed by gene replacement of the chromosomal *infA* gene. This group consisted of nine chromosomal mutant strains: CVR22L, CVR40L, CVR45L, CVR65L, CVR69L, CVR71L, CVR22D, CVR40D and CVR71D. Initiation factor IF1 is a highly conserved component of the prokaryotic translational apparatus (Sorensen *et al.*, 2001) and the mutant variants analyzed in this study are altered in highly conserved residues.

Overall, paper I describes the generation of these mutants, followed by characterization of their growth behaviour at different conditions and a study of the stability of these altered initiation factors. In particular, it is worth mentioning that the R65D alteration was considered lethal, since it could not be constructed in a strain with a deleted chromosomal *infA* gene. Moreover, this alteration confers a dominant inhibitory effect when overexpressed in the presence of a chromosomal *infA* gene, but is not dominant lethal. Another interesting feature is grouping of several mutants
into the cold-sensitive category. This group included R45L, R69L, R69L+R71L, R40D, R45D, R69D, S36P and D50G plasmid-encoded alterations of IF1 (Table 3, Paper I). The mutants in this group displayed a reduction in growth rate up to two-fold when cultivated at 30 °C. The lower growth rate found for these IF1 mutants could be explained either by a defect in activity or by a lower stability of the factor. To show that the activity is defective, we have checked the IF1 levels produced by the mutant plasmids using the maxi-cell system. In cases of R69L, R69L+R71L, R40D, R45D, R69D and S36P the lowered level of mutated IF1 (Fig. 2, Paper I) is correlated with decreased growth rate (Table 3, Paper I), suggesting that the activity of IF1 is affected.

A further particularity was revealed by cultivation of mutants at high (1mM) induction by IPTG (Table 3, Paper I). Thus, the reduced growth rates upon induction suggested that the alterations R45L, R69L, R69L+R71L, R45D, R69D and R71D are toxic to the cell. In contrast, three variants (R40L, R65L and R40D) were less detrimental than wild-type IF1 if overexpressed. The toxic effect by IF1 overexpression could be explained by the synergistic action of IF1 and IF2. It is known, that an increased level of wild-type IF1 and IF2 is toxic to the cell, since it promotes drop-off at the P-site by removing peptidyl-tRNAs charged with short polypeptides (Karimi et al., 1998). One could guess that an increase in the steady-state concentration of a defective IF1 with increasing IPTG concentrations should cause a loss of the defective phenotype. However, this is not true for the mutants that display reduced growth rates upon induction, suggesting that these variants are toxic at high concentrations.

The studies with plasmid encoded variants of IF1 were carried in the PFM1A strain, which contains a deletion of the infA gene. Since bacteria without this gene are not viable, the PFM1A strain must always contain a plasmid with a functional infA allele. In order to obtain such a strain, a gene replacement procedure was applied. Later, the infA deletion was transferred via P1 transduction into individual strains containing plasmids encoding either one of the IF1 variants described above.

Analysis of the chromosomal mutants in IF1 focused our attention on two cold-sensitive strains: CVR69L and CVR40D (Table 4). Notably, these mutant strains showed growth rates of half that of wild-type E. coli at 30 °C in LB medium. In addition, both strains displayed very slow growth on LB plates at 20 °C. In fact, these two strains were chosen for further investigation in the cold environment. It should also be mentioned that mutations giving the R45D, R69D and R69L+R71L variants of IF1 as a single chromosomal infA allele were not viable.
In general, the decreased growth rates observed for several IF1 mutants reported in this study could be explained by a decreased level of functional IF1 in these cells. This assumption is supported by previous studies that declared reduced cellular levels of IF1 as held responsible for the slowing of cell growth (Cummings and Hershey, 1994). However, the mechanism of growth rate decrease could alternatively be related to variations in the dissociation constant (Kd) of mutated IF1s from the 30S ribosomal subunits. Since some altered IF1s might have better RNA binding efficiencies than wild type, this could lead to a slower dissociation of these IF1 variants from the 30S subunit upon 50S binding and subsequently slow down the speed of initiation. In context of this discussion, it should be mentioned that IF1 acts at the level of the 30S ribosomal subunit as a kinetic effector of the rate-limiting step of 30S initiation complex formation (Pon and Gualerzi, 1984).

According to their phenotype the IF1 mutants presented in this study can be grouped into three categories: N-terminal, C-terminal and 310 helix mutants. Our mutagenesis data emphasize the importance of these three flexible regions of IF1, as the mutations in the N-terminus, C-terminus and 310 helix give the most significant phenotypes. Particularly relevant in this context are mutations K2A in the N-terminus, S36P, R40D, R45D in the 310 helix, and R65D, R69D, R69L+R71L in the C-terminus of IF1. These alterations confer various degrees of cold-sensitivity or even lethality (R65D).

In summary, paper I presents a collection of IF1 mutants designed for in vivo and in vitro studies on the function of IF1.

2.2 In vivo involvement of mutated initiation factor IF1 in gene expression control at the translational level (Paper II)

Previous studies in our group have shown that the nature of the +2 codon, that follows the initiation codon, noticeably influences gene expression (Stenstrom et al., 2001b). However, no explanation for this phenomenon was formulated at that time. Since IF1 is situated near the +2 codon during translation initiation, it appeared possible that IF1 might interact with this codon, thus contributing to the observed effect. In order to investigate this possibility, we have used lacZ and 3A′ reporter gene constructs containing various codons at the +2 position. For this study we have selected six chromosomal infA(IF1) mutants, containing specific arginine to leucine changes at different positions (22, 40, 45, 65, 69 and 71). Afterwards, we have measured expression of the lacZ or 3A′ reporter
gene constructs with various +2 codon alterations, or near-cognate initiation codons, in these mutant strains. No significant effects of the mutationally altered IF1 on the expression pattern associated with the +2 codons were observed when compared to the wild-type strain. Nevertheless, a slight upregulation of the lacZ reporter gene expression was indicated in the cases of the CVR65L and CVR69L mutant strains grown at 37 °C. In contrast, a slight downregulation of lacZ gene expression was observed in the case of the CVR71L mutant. This general up- or downregulation found for all tested codons could be explained by higher or lower affinity of the mutated protein for the ribosome or other factors taking part in the initiation step. Moreover, most reporter lacZ gene variants in the cold sensitive mutant CVR69L were increased in expression, compared to gene expression in the wild-type strain at 30 °C, being almost twofold increased in LB medium. Using the 3A' reporter system, we observed a similar overexpression pattern also for this test gene when monitored in the CVR69L mutant in LB medium at 30 °C, even though this effect was slightly less pronounced when compared to the lacZ test system.

The possibility that different expression levels in the CVR69L and CVR71L mutants were the result of different 3A' mRNA pools was analyzed. The 3A'/2A' mRNA ratios for different +2 codon constructs were similar for these two mutants, as compared to the infA+ control strain. On the contrary, 3A' protein levels, relative to the internal control 2A', were increased in the cases of analyzed +2 codons in CVR69L. Since the 3A' protein, but not mRNA level was increased, this suggests that overexpression of the 3A' test gene in CVR69L takes place at the translational level. Thus, the general overexpression observed in this mutant occurs post-transcriptionally and most probably involves IF1 as a trans-acting factor contributing to the translational stimulation of the reporter mRNAs. In a recent study it was found that an important source of cold-selective stimulation of some mRNA translation is an increased level of initiation factors IF1, IF2 and IF3 (Gualerzi et al., 2003). In the same study, it was established that at low temperatures the cold shock protein CspA stimulates all tested mRNAs about twofold, without a clear preference for one in particular. Since IF1 and CspA share a common structural fold (Sette et al., 1997) and IF1 is able to complement the loss of a CspA homolog in Bacillus subtilis (Weber et al., 2001), the stimulatory effect of CspA on translation of mRNAs could be extended to IF1 in light of the fact that CspA and IF1 might share common cellular functions.

Additionally, to investigate for any influence of the altered IF1 on initiation at different initiation codons, lacZ constructs with AUG, GUG or UUG initiation codons were tested in the context of some different A-site
(+2) codons. If IF1 is involved in discrimination of the initiation codon, a changed expression from various initiation codons could possibly be expected in the case of some IF1 mutants. Notably, the CVR69L mutant gave twofold increased expression levels for all tested initiation codons, when compared to the wild-type strain, in line with results discussed above. This increase was independent of the initiation codon context. No significant effect on gene expression was observed for any of the five other IF1 mutants, also having Arg to Leu alterations.

In conclusion, based on our results using several infA(IF1) mutants and expression of two different model genes with various +2 codons in the mRNA, we suggest that IF1 has no codon discriminatory functions with respect to the +2 codon, or to the nature of the initiation codon (AUG, UUG or GUG), while occluding the A-site. Our finding that the R69L alteration in IF1 causes a twofold increase in gene expression at low temperature, as well as cold-sensitivity, suggests that IF1 is an active player in the initiation complex. Since the mRNA levels for the 3A′ test gene are unaffected by the infA mutations, this strongly suggests that the increased reporter gene expression seen in the case of the cold-sensitive CVR69L mutant arises at the translational level. If many E. coli genes are similarly increased in expression, this could result in higher demands from the translational apparatus. This could lead to a slow growth at low temperatures, thus explaining the slow growth observed for the CVR69L mutant. Stimulation of gene expression in this mutant could be explained by kinetic variations in the 30S initiation complex formation. It is known that Arg 69 has an important role in binding of IF1 to the 30S subunit and deletion of this residue results in substantially lower affinity of the factor for the small subunit (Gualerzi et al., 1989). The reduced affinity is most probably responsible for a faster dissociation of the mutated factor from the initiation complex and subsequently decreases the rate-limiting step in 30S complex formation. In turn, this possibly will result in more rapid initiation in the CVR69L variant and overall increase in reporter protein production.

2.3 RNA chaperone activity of translation initiation factor IF1 (Paper III)

Since IF1 is an RNA binding protein being a member of the OB-fold family of proteins, which include some RNA chaperones, we wanted to investigate if IF1 could possibly posses RNA chaperoning activity. For this purpose we have selected several His-tagged IF1 mutant variants and tested them in vitro in the RNA band-shift assay, in the trans-splicing assay and...
in the RNA annealing assay. According to the results of the band-shift assay the IF1 mutant variants can be grouped in two categories. One group contains the R69L+R71L, R22D, R69D and R71D mutants which display better binding to the RNA compared to the wild-type IF1. Another group contains the R65D, S36P, R40D and R45D variants which show a decrease in gel-shifting demonstrating a weaker affinity for RNA. Among the analysed mutants the R65D is the weakest binder.

In the trans-splicing assay we investigate the ability of IF1 to unwind and open RNA duplicates. The assay revealed that all tested IF1 mutant variants possess RNA chaperone activity in vitro. Remarkably, there is a stronger increase in RNA chaperone activity in the cases of R71D and R69L+R71L variants compared to wild-type or any other IF1 mutants. These two mutant variants also show stronger RNA binding than wild type IF1 according to the band-shift assay mentioned above.

In the RNA annealing assay the coefficients for the IF1 mutants were higher than that of the wild-type IF1, except for the R40D variant. These coefficients confirm the in vitro chaperone activity of IF1. Both annealing and unwinding activities have been attributed to RNA chaperones and recent observations show that indeed some RNA chaperones do have both activities. IF1 does show both activities in vitro with the annealing activity being more pronounced than unwinding.

Since IF1 shows RNA chaperone activity in vitro we also tested IF1 for chaperone activity using an in vivo folding trap assay based on splicing of the thymidylate synthase gene (td) from phage T4. In contrast to the td wild-type, the td SH1 mutant variant is significantly impaired in splicing due to an introduced exonic stop codon mutation and an additional intronic mutation C865U. Over-expression of RNA chaperones is known to alleviate the splicing deficiency of the td SH1 mutant. Here, splicing was monitored using a reverse transcriptase assay and indicated that over-expression of IF1 increases splicing of the mutant SH1 pre-mRNA at least 5-6 fold. The results from the folding trap assay show that IF1 over-expression rescues splicing of the SH1 mutant pre-mRNA suggesting that IF1 has RNA chaperone activity also in vivo.

We suppose that the RNA chaperone activity of IF1 contributes to RNA rearrangements during the early phase of translation initiation. A chaperoning function might be crucial for IF1’s action in the A-site during initiation and could contribute to the previously reported changes at the ribosomal A-site leading to large-scale alterations in the conformation of the 30S subunit. The question to what extent such chaperoning activity involves specific structures of mRNA and/or rRNA is a challenging issue for future investigations.
3. Concluding remarks and perspectives

This thesis is focused on better understanding of IF1 function, since the role of this essential factor for cell viability remains unclear. The efforts to generate and characterize the functional mutants in IF1 have brought to the scientific community a collection of IF1 mutants designed for in vivo and in vitro studies on the function of this factor. Characterization of these variants has revealed a cold-sensitive group of mutants, which can be used in further gene expression studies. Our observations suggest that IF1 interacts with the A-site in an electrostatic manner, involving the positively charged surface of this protein and the backbone phosphates of the neighboring rRNA. In addition, our mutagenesis data provide in vivo evidence for assigning the functional sites of IF1 to the flexible N-terminus, C-terminus and the 3₁₀ helix of the factor. In the future, using the mutated infA genes presented in this thesis, the interactions of IF1 with specific structures of the 16S rRNA or mRNA can be investigated. Moreover, the interaction of IF1 with IF2 can be elucidated using mutants located at the possible sites of interaction between the factors.

The involvement of IF1 in the fidelity of initiation has been addressed in the presented work. These data are important since they provide experimental facts in opposition to the longstanding hypothesis that IF1 could perform codon discriminatory functions while blocking the aminoacyl-tRNA acceptor site (A-site) of the ribosome. Our results also suggest that IF1 is not involved in any recognition of the initiation codon context. The analysis reveals one cold-sensitive IF1 mutant which shows a general overexpression of the reporter genes used in this study. This is particularly relevant at low temperatures and the overexpression is due to higher reporter protein levels, but not mRNA level. The increased expression during growth in the cold relates IF1 to the cold shock protein CspA, since both proteins stimulate translation at low temperatures without mRNA selectivity. Since IF1 can complement the loss of a CspA homologue in Bacillus subtilis, it would be interesting to investigate if CspA can complement the lack of IF1 in the deletion strain designed in our laboratory.

One key result described in the thesis is the elucidation of IF1 function as an RNA chaperone. We showed that IF1 possesses in vitro and in vivo chaperone activity, as well as significant RNA binding. During initiation complex formation IF1 could resolve misfolded mRNAs and facilitate resolution of intermediate conformations of rRNA in the A-site. As for the future, it would be interesting to investigate if IF1 binds to the 5’UTR of cellular mRNAs and destabilizes secondary structures of these
mRNAs. The implication of IF1 as an RNA chaperone in translation of cellular mRNAs at low temperatures is another appealing direction. Further studies can also be extended to IF2 and IF3. Perhaps they possess RNA chaperone activity in analogy to IF1.

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