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KALLE KIPPER

Studies on the role of helix 69 of 23S rRNA in the factor-dependent stages of translation initiation, elongation, and termination

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Supervisors: Prof. Jaanus Remme, PhD
Department of Molecular Biology,
Institute of Molecular and Cell Biology,
University of Tartu, Estonia

Dr. Aivar Liiv
Department of Molecular Biology,
Institute of Molecular and Cell Biology,
University of Tartu, Estonia

Opponent: Prof. Claudio O. Gualerzi, PhD
Department of Animal and Cellular Molecular Biology,
University of Camerino, Italy

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LIST OF ORIGINAL PUBLICATIONS

The present dissertation is based on the following original publications which will be referred to in the text in the following order:

Ref.1 Kipper, K., Hetényi, C., Sild, S., Liiv, A. and Remme, J. (2009). Ribosomal Intersubunit Bridge B2a Is Involved in Factor-Dependent Translation Initiation and Translational Processivity. *J. Mol. Biol*. 385, 405–422.

Ref.2 Kipper, K., Sild, S., Hetényi, C., Remme, J. and Liiv, A. (2011). Pseudouridylation of 23S rRNA helix 69 promotes peptide release by release factor RF2 but not by release factor RF1. *Biochimie*, doi:10.1016/j.biochi.2010.12.018

Ref.3 Leppik, M., Peil, L., Kipper, K., Liiv, A. and Remme, J. (2007). Substrate specificity of the pseudouridine synthase RluD in *Escherichia coli*. *FEBS J*. 274, 5759–5766

My contribution to the publications is as follows:

Ref.1. Participated in performing the experiments (except for the MD simulations) and in writing the manuscript.

Ref.2. Participated in performing the experiments (except for the MD simulations) and in writing the manuscript

Ref.3. Provided the plasmid coding for the A1912U and A1919U variant 23S rRNA.

LIST OF ABBREVIATIONS

aa-tRNA	aminoacyl-tRNA
aSD	anti-Shine-Dalgarno
ASL	anticodon stem loop (in tRNA)
CMCT	1-cyclohexyl-3-(2-morpholinoethyl)carbo-diimidemetho-p-toluenesulfonate
Cryo-EM	cryoelectron microscopy
EF-G	elongation factor G
EF-Tu	elongation factor Tu
fMet	N-formylmethionine
fMet-PMN	N-formylmethionyl-puromycine
IF	initiation factor
k_{obs}	apparent rate constant
l-protein	large subunit protein
mΨ	methylpseudouridine
poly(Phe)	polyphenylalanine
PTC	peptidyl transferase centre
RF	release factor
r-protein	ribosomal protein
SD	Shine-Dalgarno
s-protein	small subunit protein
70SIC	70S initiation complex
30SIC	30S initiation complex
Ψ	pseudouridine

FOREWORD

It seems almost commonplace to say that the structure of a molecule – however big or small it is – determines its function. However, it is far from trivial to establish a link between structure and activity in a molecule as big as the ribosome that has not a single but many interrelated functions. First and foremost, one has to know what the structure looks like. Determining the structure of the ribosome has itself been a serious challenge for scientists. Fortunately, over the past ten years we have witnessed the accumulation of considerable amount of structural information about the ribosome, especially its prokaryotic variant. This knowledge has been useful in integrating into a coherent picture the biochemical and genetic information that had been gathered over the preceding decades. In addition, this newly gained knowledge about the structure of the ribosome has revealed previously unanticipated interactions and thereby generated new ideas about how parts of this large ribonucleoprotein enzyme carry out their function. Unfortunately, the mere knowledge of the structure of a molecule is not a proof of its function. In the words of Jeremy Knowles: "Looking at a picture of a racehorse does not tell us how fast it will run". Therefore, the structure-based hypotheses about the function of the ribosome and its substructures require biochemical and genetic testing. This testing has been the task of the present dissertation. Due to the size and complexity of the ribosome, the author and his colleagues narrowed their focus on a particular stem-loop structure called helix 69 in the 23S rRNA of the eubacterium *Escherichia coli*. Their interest in this seemingly small part of the ribosome was aroused by the observation – gleaned from the ribosomal structures – that this helix lies in the "functional" center of the ribosome and interacts with various components of the translational apparatus. Underlying its importance for ribosomal functioning helix 69 has been seen to form part of bridge B2a – one of the largest intersubunit contacts in the 70S ribosome. Another interesting feature of helix 69 is the presence of three modified nucleosides – two pseudouridines and one methylpseudouridine – in its structure. Beside helix 69, pseudouridines occur in only a limited number of locations in the 23S rRNA that lie close to the functional centers of the ribosome. Collectively, this structural information prompted us to form a hypothesis according to which helix 69 acts as a control element in factor-dependent reactions of the ribosomal working cycle. We reasoned that this hypothesis could be tested by modifying the structure of helix 69 and monitoring the ability of the variant ribosomes to carry out those factor-specific reactions. The modifying was done by introducing base replacements at specific positions in helix 69 using site-directed mutagenesis. Mutagenesis is a powerful technique in dissecting structure-activity relationships in biology. However, its results are not always unambiguous. One of the pitfalls of mutagenesis is that an effort to disrupt a specific interaction in a macromolecule may in unfortunate cases scramble the tertiary structure of the entire molecule. In such a case the

contribution of this specific interaction to the overall functioning of the molecule cannot be assessed. Carefully devised control experiments are therefore needed to ensure that the tinkering with one limited part of a macromolecule leave its overall architecture intact. Considering the various controls in the three publications under defense the author feels himself sufficiently confident to state that the mutations in helix 69 did not affect the large-scale structure of the ribosome and the effects observed are due to changes in the structure of helix 69.

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I. REVIEW OF THE LITERATURE

I.1. Structure of the ribosome

Translation of the genetic information encoded in DNA occurs exclusively on the ribosome. Although sometimes called an organelle, the ribosome is in fact a large enzyme that contains both RNA and proteins. The molecular weight of a ribosome is around 2.3 MDa in prokaryotes and 4 MDa in eukaryotes, making it one of the largest enzymes in the cell. Instead of molecular weight, the size of a ribosome is more often expressed by the sedimentation coefficient S ($1\ S = 10^{-13}$ s) that is a complex function of its shape and molecular weight. A functional ribosomal particle has a sedimentation coefficient of 70S in prokaryotes and 80S in eukaryotes. A universal feature of the ribosome is its composition of two unequally sized subunits that undergo repeated cycles of association and dissociation during protein synthesis. Due to its large size and the complexity of protein synthesis, a functional ribosome contains a number of active centres on both subunits where different part reactions of protein synthesis occur. Although many of the functional centres lie far from each other, structural and biochemical data indicate extensive communications between the centres (Chan *et al.* 2006; Jenner *et al.* 2010a). Those interactions are mediated by both the ribosomal RNA (rRNA) and ribosomal proteins.

The ribosome in prokaryotes contains three different species of rRNA and around fifty proteins (21 proteins in the small subunit and 34 proteins in the large subunit in the case of *E.coli*) (Kaltschmidt and Wittmann 1970; for excellent reviews on ribosomal proteins see Wilson and Nierhaus 2005 and Wilson *et al.* 2009). The somewhat larger eukaryotic ribosome is composed of four different rRNAs and around eighty r-proteins. The protein content per particle is higher in eukaryotic and mitochondrial ribosomes as compared to the prokaryotic ones (Sharma *et al.* 2003). Most of the ribosomal proteins are relatively small (M_n around 19 kDa for small subunit proteins and 16,3 kDa for large subunit proteins) (Dzionara *et al.* 1970) and basic (pI around 10) (Kaltschmidt and Wittmann 1970; Wilson and Nierhaus 2005) polypeptides that occupy the crevices and holes between regions of rRNA (Ban *et al.* 2000; Yusupov *et al.* 2001; Klein *et al.* 2004). They are either globular or contain extensions from the globular parts that penetrate into the rRNA scaffold (Figure 1 and 2). Not surprisingly therefore, basic amino acids of the r-proteins are overrepresented in the extended regions residing in the interior of the ribosomal particle (Klein *et al.* 2004). Notably, r-proteins from both subunits are largely excluded from the subunit interface (Ban *et al.* 2000; Yusupov *et al.* 2001; Klein *et al.* 2004; Schuwirth *et al.* 2005).

Differently from the relatively simple structure of the r-proteins, the structure of the rRNA seems to be dauntingly complex at first sight (Figure 1 and 2). On closer inspection, however, it is seen to possess a modular structure that can be decomposed into more or less regular elements at different levels of

structure. At the level of secondary structure ribosomal RNA is made up of short helical segments that are connected by (mostly structured) single-stranded regions. The helical regions regularly assume a standard A-form conformation. In fact, roughly 50% of rRNA nucleotides are located in helices (Moore 1999). The helical regions likely form first during rRNA folding, followed by the formation of tertiary interactions that are stabilized by divalent, especially Mg^{2+}, ions (Zarrinkar and Williamson 1994; Cate *et al.* 1996a; Ban *et al.* 2001). The folding of rRNA into a functional conformation is a complex process that can occur through multiple but still highly ordered pathways and requires the assistance of r-proteins as well as nonribosomal proteins (reviewed in Kaczanowska and Ryden-Aulin 2007). A number of recurring motifs can be discerned in rRNA at the secondary structure level (Moore 1999). Those motifs include terminal loop structures like U-turns and tetraloops at the ends of helices and various internal loops in the middle of an otherwise helical structure (Moore 1999; Holbrook 2008). The secondary structure elements are packed into an intricate three-dimensional rRNA scaffold with the help of various tertiary interactions where the distinguishing chemical group of RNA – the 2' hydroxyl – is an essential ingredient (Cate *et al.* 1996a; Holbrook 2008). Prominent among those interactions are tetraloop-tetraloop receptor interactions and ribose zippers (Cate *et al.* 1996a), A-minor motifs (Nissen *et al.* 2001; Battle and Doudna 2002) as well as different types of coaxial stacks (Kim *et al.* 1974) of the helices (Noller 2005; Holbrook 2008). Those interactions allow rRNA regions located far apart at the secondary structure level to be close in the three-dimensional structure. The prominent involvment of adenines in the secondary and tertiary interactions of rRNA – most notably in the A-A platforms (Cate *et al.* 1996b) and A-minor interactions (Nissen *et al.* 2001) – likely explains the overrepresentation of unpaired A-s relative to other unpaired bases in rRNA (Gutell *et al.* 1985; Noller 2005).

In prokaryotes the large or 50S subunit contains two species of rRNA: 5S and 23S rRNA that are densely packed with r-proteins (Ban *et al.* 2000). The small or 30S subunit contains the 16S rRNA that is similarly complexed with r-proteins (Schlünzen *et al.* 2000). The rRNAs have a characteristic secondary structure that is divided into different domains for both the 16S (Noller and Woese 1981) as well as the 23S rRNA (Noller et al 1981; Leffers *et al.* 1987). During the formation of a ribosomal particle the rRNA folds into a compact three-dimensional wireframe where residues from different domains are close to each other and contribute to the formation of the functional centres (Ban *et al.* 2000; Yusupov *et al.* 2001). Thus, in the 30S subunit the majority of the "body" is formed by the "5'" domain of the 16S rRNA while the "central" and "3' Major" domains form most of the "platform" and "head" regions, respectively (Schlünzen *et al.* 2000; Yusupov *et al.* 2001). The last of the four domains of the 16S rRNA – the "3' minor" – domain contains a long helical structure known as "helix 44" or "penultimate stem" that runs across the body of the 30S and is involved in both tRNA selection (Ogle *et al.* 2001) and formation of

important intersubunit contacts (Cate *et al.* 1999; Yusupov *et al.* 2001). The domains of the 16S rRNA within the 30S subunit are relatively autonomous as indicated by the x-ray analyses (Yusupov *et al.* 2001) and by the ability of the *in vitro* synthesized RNA fragments corresponding to the 5'-, central-, and 3'- domain of 16S rRNA to assemble into particles that resemble the domains in intact 16S rRNA by their morphology in electron micrographs and ability to bind specific sets of r-proteins (Weitzmann *et al.* 1993; Samaha *et al.* 1994; Agalarov *et al.* 1998). Due to their autonomous organization within the 30S subunit the domains of the 16S rRNA can move relative to each other during protein synthesis (Frank and Agrawal 2000; Schuwirth *et al.* 2005; Zhang *et al.* 2009). The higher intrinsic dynamism of the 30S subunit (in comparison with the 50S) is also observed in dielectric spectroscopy and microcalorimetry studies (Bonicontro and Risuleo 2005). The mobility of the 30S is conspicuously exemplified by the swivelling motion of the 30S head that undergoes a 12^{o} counterclockwise rotation (corresponding to a 20 Å shift at the interface) relative to the rest of the 30S (Schuwirth *et al.* 2005; Zhang *et al.* 2009). The "penultimate stem" in the "3'minor" domain is also conformationally flexible and shifts by 4 to 7 Å towards the 30S "shoulder" upon subunit association (Van Loock *et al.* 2000; Schuwirth *et al.* 2005).

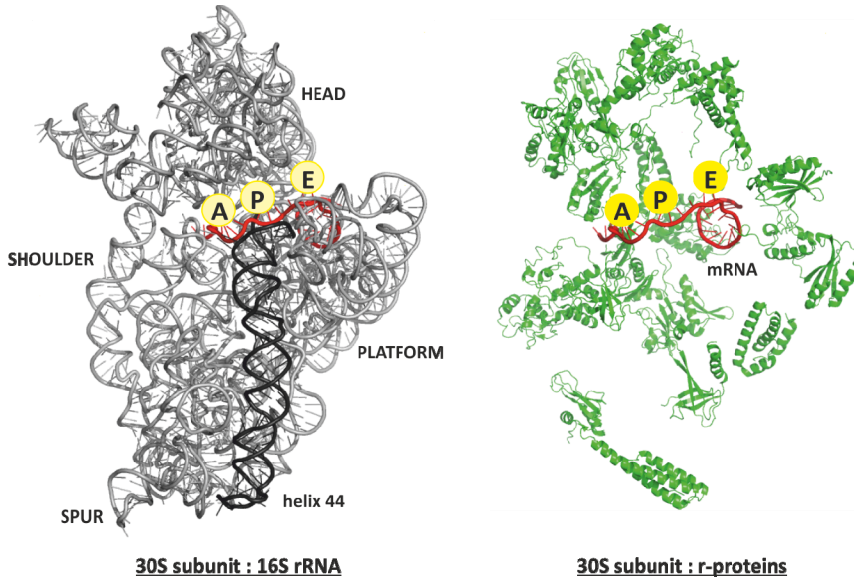

Figure 1. Structure of the small ribosomal subunit from *Thermus thermophilus*. The subunit is shown in such a fashion that the intersubunit contact area ("interface") is facing the viewer. Letters A, P, and E denote the approximate locations on the 30S of the binding sites for the aminoacyl-, peptidyl-, and deacylated-tRNA, respectively. mRNA is coloured in red and helix 44 of 16S rRNA in black. The structure was rendered with PyMol (www.pymol.org) using the coordinates from Jenner and coworkers (Jenner *et al.* 2010a; PDB ID **3I8G**).

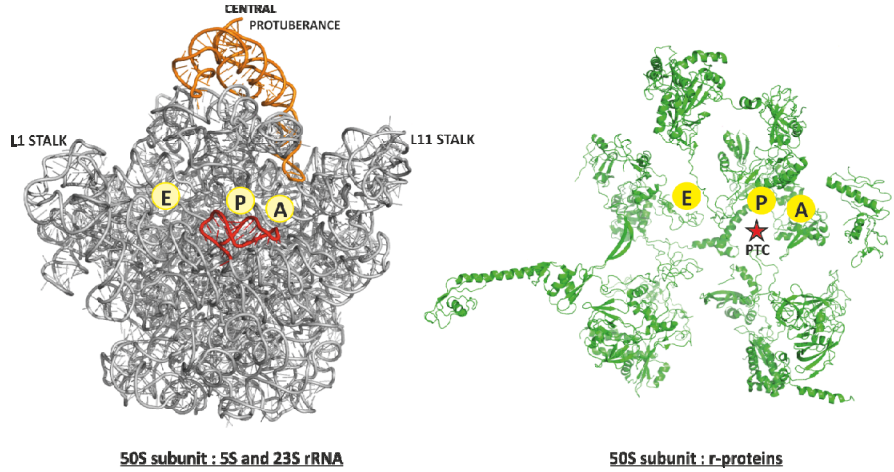

Figure 2. Structure of the large subunit from *Thermus thermophilus*. The subunit is shown with its interface side facing the viewer. Letters A, P, and E denote the approximate locations on the 50S of the binding sites for the aminoacyl-, peptidyl-, and deacylated-tRNA, respectively. 23S rRNA is colored in gray, 5S rRNA is colored in orange. Helix 69 of 23S rRNA is highlighted in red. The structure was rendered with PyMol using the coordinates from Jenner and coworkers (Jenner *et al.* 2010a; PDB ID **3I8F**).

In contrast to the relative independence of the domains of the 16S rRNA, the six domains (I–VI) of the 23S rRNA are densely interwoven and form a monolithic particle which is studded with proteins on the outer surface but is mostly composed of RNA at its core (Ban *et al.* 2000; Yusupov *et al.* 2001). Due to its specific domain organization the conformation of the 50S particle is less dynamic than that of the 30S (Ban *et al.* 2000; Yusupov *et al.* 2001; Bonicontro and Risuleo 2005). However, an exeption to this rule are the L1 and L11 stalk regions of the 23S rRNA that are involved in the exiting and binding of tRNA, respectively, during protein synthesis. Both of those regions have been shown to undergo large conformational changes by cryo-EM (Agirrezabala *et al.* 2008; Fischer *et al.* 2010) and FRET measurements (Cornish *et al.* 2009; Fei *et al.* 2009). Additional mobile elements in 23S rRNA are helices 38 (domain II) and 69 (domain IV) that protrude from the bulk of the 50S and are therefore capable of independent movements (Yusupov *et al.* 2001; Harms *et al.* 2001; Schuwirth *et al.* 2005). Although closely interconnected, the domains of 23S rRNA have different functional roles in the ribosome. Thus, a large fraction of the residues participating in intersubunit contacts is clustered in domain IV of 23S rRNA (helices 62, 64, 67, 69 and 71) (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). Additional contributions to intersubunit interactions are made by residues from helices 34 and 38 in domain II of 23S rRNA (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). Domain V that forms the innermost part of the 50S subunit contains

the peptidyl transferase region whose components participate in the binding of the 3'-CCA ends of tRNAs (helices 80 and 92) (Lieberman and Dahlberg 1994; Puglisi *et al.* 1997; Saarma *et al.* 1998; Kim and Green 1999; Nissen *et al.* 2000) and form part of the proton shuttle network which facilitates the peptide bond formation (residues C2063, A2451, U2584 and A2602 in the "central loop" of domain V) (Schmeing *et al.* 2005a; Schmeing *et al.* 2005b; Simonović and Steitz 2009). A characteristic of the peptidyl transferase centre is a dyad symmetry in its structure. Due to this symmetric structure one half of the petidyl transferase centre (a region comprising helices 74, 75 and 80) is related to the other half (helices 90, 92 and 93) by a two-fold rotation with the dyad axis lying close to the residue A2602 (Bashan *et al.* 2003; Agmon *et al.* 2005; Bashan and Yonath 2005). The symmetry of the peptidyl transferase centre may have arisen via a gene duplication early in evolution (Agmon *et al.* 2005). According to the current view, there are no residues from ribosomal proteins that would directly facilitate the catalysis of peptide bond formation in the sense of providing a group for the proton shuttle network (Simonović and Steitz 2009). However, ribosomal proteins may serve to fix the relative orientation of the acceptor ends of the A- and P-site tRNA in preparation of the peptidyl transferase reaction similarly to the 23S rRNA helices 80 ("P-loop") and 92 ("A-loop"). The N-terminus of the large subunit protein L27 and parts of protein L16 are located between the 3'-CCA ends of the A- and P-site tRNAs (Jenner *et al.* 2010a; Voorhees *et al.* 2010). Additionally, the C-terminal part of protein L25 has been seen to interact with the elbow of the A-site tRNA (Jenner *et al.* 2010a). As the proper positioning of the substrates in the peptidyl transferase centre is thought to substantially contribute to the lowering of the activation barrier for peptide bond formation (Sievers *et al.* 2004), ribosomal proteins L16, L25 and L27 have an important role in peptidyl transferase in addition to rRNA (Maguire *et al.* 2005; Voorhees *et al.* 2010). In fact, as for the role of ribosomal proteins in the polypeptide synthesis, one is reminded of the auguring words of James D. Watson in his Nobel Lecture in the year 1962: "We believe that all these proteins (i.e. r-proteins) have primarily a structural role. That is, they are not enzymes but largely function to hold the ribosomal RNA and necessary intermediates in the correct position for peptide bond formation."

Connected to the peptidyl transferase region via 23S rRNA helices 89 and 93 is the "L11 stalk". This structure is composed of 23S rRNA helices 42, 43 and 44 (residues 1030 – 1124 of 23S rRNA in *E.coli*) that form the binding platform for proteins L10 and L11 (Schmidt *et al.* 1981; Egebjerg *et al.* 1990; Diaconu *et al.* 2005). L10 interacts with helices 42 and 43 of 23S rRNA by its N-terminal region while L11 contacts helix 44 by its C-terminus (Wimberly *et al.* 1999; Diaconu *et al.* 2005). The C-terminal α-helix of L10 in turn serves as a binding site for the (L7/L12) tetramer (Diaconu *et al.* 2005). Together, helices 42 – 43 and L10, L11 and $(L7/L12)_4$ form a protruding structure that is important for translation factor recruitment and the activation of the GTPase activity of various translational GTPases (Beauclerk *et al.* 1984; Moazed *et al.* 1988;

Savelsbergh *et al.* 2000; Mohr *et al.* 2002; Diaconu *et al.* 2005). In addition, residues A1067 and U1068 in the 23S rRNA helix 43 of the "L11 stalk" are seen to interact with the "elbow" region of the tRNA in the A/T-state (i.e. tRNA bound to the ribosome prior to the accommodation), thus stabilizing the strained conformation of the tRNA (Schmeing *et al.* 2009). The "L11 stalk" is also the binding site for the antibiotics thiostrepton and micrococcin that either inhibit (thiostrepton) or stimulate (micrococcin) the GTPase activity of elongation factor EF-G (Cundliffe and Thompson 1981; Egebjerg *et al.* 1989). In addition to the "L11 stalk" helix 95 of 23S rRNA is important for the activation of the GTPase activity of the translational GTPases. As helix 95 is the target of ribotoxins ricin (cleaves a specific phosphodiester bond in H95) and α-sarcin (depurinates a specific A in H95) it is more commonly known under the name of "sarcin-ricin loop" or SRL (Endo and Wool 1982; Endo *et al.* 1987). SRL (residues 2653 – 2667 in *E.coli*) interacts with the G domains of elongation factors EF-Tu and EF-G (Hausner *et al.* 1987; Moazed *et al.* 1988; Chan *et al.* 2004; Schmeing *et al.* 2009; Villa *et al.* 2009; Gao *et al.* 2009). In EF-Tu, nucleotides A2660 and G2661 of SRL interact with residues His19 and His 84, respectively, that are involved in the hydrolysis of GTP on EF-Tu (Villa *et al.* 2009; Schmeing *et al.* 2009). Since the A2662 of SRL interacts with the A2531 of the peptidyl transferase centre region (Chan *et al.* 2006), this interaction may serve as a signal transmitter of the GTP hydrolysis on EF-Tu/EF-G to the PTC. Cleavage of the loop was demonstrated to block the binding of EF-Tu and EF-G to the 70S ribosome while leaving the EF-independent events (subunit association, tRNA binding, peptidyl transferase and factor-independent translocation) unaffected (Hausner *et al.* 1987).

Crucial to the ribosomal functioning is the presence of appropriate tRNAs in the three tRNA binding sites. Those sites are denoted as the A, P (Watson 1964; Leder 1973) and E-site (Rheinberger *et al.* 1981; Blaha and Nierhaus 2001). For recent reviews on the ribosomal sites see Màrquez *et al.* 2002 and Nierhaus (Nierhaus 2004). The sites correspond to the different functional states of the tRNA. Thus, at the beginning of the peptide bond formation the A-site is occupied by the aminoacyl-tRNA and the P-site by the peptidyl-tRNA. The E-site in turn contains the deacylated tRNA that is waiting for its expulsion from the ribosome. The A, P and E sites are formed by rRNA and r-proteins from both subunits that interact with different parts of the tRNA molecules (Yusupov *et al.* 2001; Selmer *et al.* 2006; Jenner *et al.* 2010a). The contacts to the tRNAs are made at conserved residues, thus allowing the ribosome to bind different tRNA species with an similar affinity (Yusupov *et al.* 2001; Schäfer *et al.* 2002). Residues from both subunits are involved in contacting the tRNAs (Rinke-Appel *et al.* 1995; Yusupov *et al.* 2001; Selmer *et al.* 2006; Jenner *et al.* 2010a). Overall, the tRNAs make more extensive contacts with the rRNA than with r-proteins that mostly interact with the tRNAs by the ends of their extensions (Yusupov *et al.* 2001; Jenner *et al.* 2010a). The anticodon regions of the tRNAs reside on the 30S subunit whereas the D-, T- and acceptor stems

interact with various rRNA and protein residues from the 50S subunit (Yusupov *et al.* 2001; Schäfer *et al.* 2002; Jenner *et al.* 2010a) (Figure 3). The mRNA molecule resides in the 30S-part of the A, P and E-sites (Yusupov *et al.* 2001; Yusupova *et al.* 2001; Yusupova *et al.* 2006; Jenner *et al.* 2010b). Within the A- and P-site the tRNAs are located in such a fashion that their 3'-CCA ends – carrying the aminoacyl and peptidyl group, respectively – are closest to each other and suitably positioned for the transfer of the peptidyl group (Yusupov *et al.* 2001) (Figure 3). In contrast, with the P- and E-site tRNAs the 3'-CCA ends are around 50 Å apart (Yusupov *et al.* 2001) (Figure 3). The simultaneous presence of the tRNA molecules in the A and P-sites is facilitated by their tilted orientation relative to each other that leads to a 45° "kink" in the mRNA between the A- and P-site codons (Yusupov *et al.* 2001).

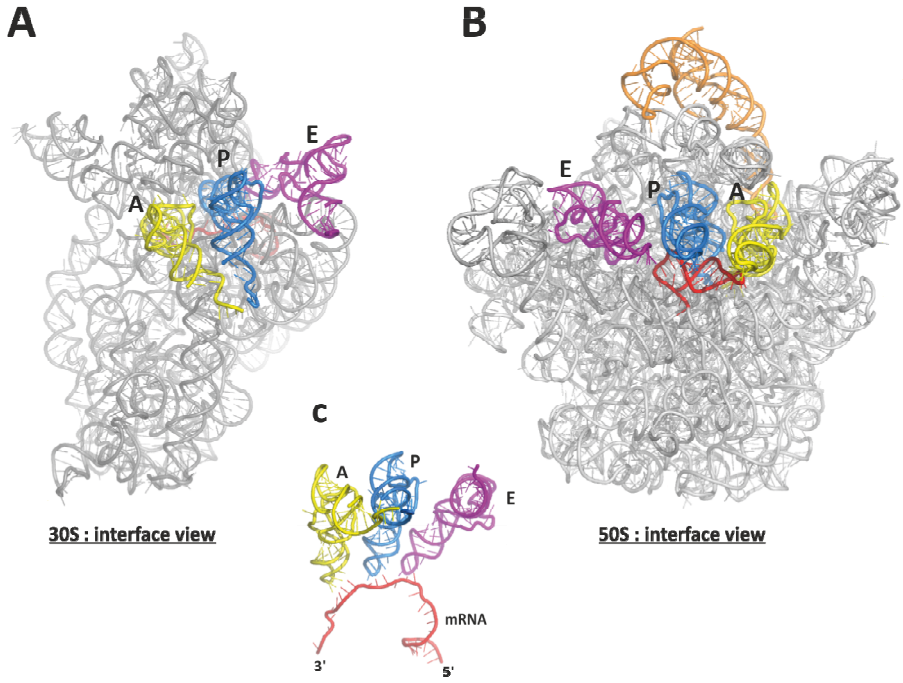

Figure 3. Location of the tRNAs on the ribosome. Panel A, tRNAs on the 30S subunit. Panel B, tRNAs on the 50S subunit. Panel C, location of tRNAs with respect to mRNA. The letters A, P, and E denote the aminoacyl-, peptidyl-, and deacylated tRNA, respectively. The structure was rendered with PyMol using the coordinates from Jenner and coworkers (Jenner *et al.* 2010a; PDB IDs **3I8F** and **3I8G**).

On the 50S subunit the A-site is formed by 23S rRNA helices H38, H69, H71, H89, and H93 and r-proteins L16, L25 and L27 (Yusupov *et al.* 2001). On the 30S subunit it involves the top of helix h44 as well as parts of helices h18, h31 and h34 of 16S rRNA and the r-proteins S12, S13 and S19 (Yusupov *et al.*

2001). H38 (known as the "A-site finger" or ASF) that together with S13 forms the intersubunit bridge B1a, contacts the tRNA at the D and T-stems (Rinke-Appel *et al.* 1995; Yusupov *et al.* 2001) while H89 runs parallel to the entire lenght of the acceptor arm (Yusupov *et al.* 2001). Protein S19 from intersubunit bridge B1b contacts the anticodon stem of the A-site tRNA by its C-terminal extension (Jenner *et al.* 2010a). The C-terminal tail of protein L25 interacts with the T-stem of the tRNA whereas the N-terminal tail of L27 stabilizes the positioning of its acceptor end (Jenner *et al.* 2010a; Voorhees *et al.* 2010). The acceptor end of tRNA is also stabilized by a loop of protein L16 (Jenner *et al.* 2010a). Embedded within the A-site is the decoding centre which controls the correctness of the codon anticodon interaction and is thus crucial for translational accuracy (Ogle *et al.* 2001a; Ogle *et al.* 2001b; Jenner *et al.* 2010a). The decoding centre is formed by adenines A1492/1493 from h44 and guanine G530 from h18 of the 16S rRNA together with residues from the small subunit protein S12 (Ogle *et al.* 2001a; Ogle *et al.* 2001b; Jenner *et al.* 2010a). A1493 and G1494 of 16S rRNA interact with the residues A1912 and A1913 from the loop region of helix 69 of 23S rRNA (Schuwirth *et al.* 2005; Jenner *et al.* 2010a). Binding of the anticodon stem-loop of a cognate tRNA induces a rearrangement in the decoding centre that leads to a stabilization of the 3'-acceptor end of the tRNA in the 50S and a tighter packing of the components of the A-site around the tRNA (Jenner *et al.* 2010a). Many of those stabilizing interactions do not occur with near-cognate tRNA (Jenner *et al.* 2010a).

Forming the P-site on the 50S are 23S rRNA helices H69, H74, H80-85 (with the exception of H82), H90, and H93 together with protein L5 (Rinke-Appel *et al.* 1995; Yusupov *et al.* 2001). The 30S-part of the P-site is formed by 16S rRNA helices h24, h28-31 and r-proteins S9 and S13 (Yusupov *et al.* 2001). The overall pattern of tRNA interaction with the 50S part of the P-site is similar to that of the A-site. Here, the stem part of H69 contacts the D-stem of the P-site tRNA whereas helices H74, H80-81, H90 and H93 form a binding pocket for the acceptor end of the tRNA analogously to helices 71, 89 and 93 in the A-site (Yusupov *et al.* 2001). Differently from the A-site where H38 ("ASF") buttresses the D- and T-stems of the tRNA, the elbow region of the P-site tRNA makes a less extensive contact with protein L5 from intersubunit bridge B1b (Yusupov *et al.* 2001). The C-terminal tails of proteins S9 and S13 interact with the anticodon stem of the tRNA (Yusupov *et al.* 2001; Jenner *et al.* 2010a). Important parts of the P-site are 16S rRNA nucleotides A790 from h24 and G1338 and A1339 from the region between helices 29 and 42 (Abdi and Fredrick 2005; Lancaster and Noller 2005). G1338 and A1339 form type II and type I A-minor interactions with the GC base pairs 30–42 and 29–41 in the P-site tRNA (Selmer *et al.* 2006). Those interactions – stronger with GC than AU base pairs – are likely responsible for the enhanced stabilization of the initiator tRNA over elongator tRNAs in the P-site due to the three consecutive GC base pairs in the anticodon stem of the initiator tRNA (Mandal *et al.* 1996). Consistent with the observation of the interaction of the initiator tRNA with the

G1338 of 16S rRNA, a G to A substitution at this position affected the affinity of fMet-tRNA^{fMet} for the P-site (Qin *et al.* 2007). In addition, the 16S rRNA nucleotides G1338–A1339 as well as A790 are situated in such a way that they form a gate-like structure between the P- and E-sites on the 30S, thus precluding a premature movement of tRNA from the P- to the E-site (Schuwirth *et al.* 2005; Selmer *et al.* 2006). Apparently, this "gate"opens during the swivelling of the 30S "head" during translocation, allowing the movement of the tRNA-mRNA complex to the E-site (Schuwirth *et al.* 2005; Zhang *et al.* 2009; Ratje *et al.* 2010). The binding of initiation factor IF3 to the 30S subunit has been shown to protect A790 from modification by chemical probes (Moazed *et al.* 1995; Dallas and Noller 2001), suggesting a role for A790 in factor-dependent initiation. Consistent with this view, the A790G substitution strongly impaired the binding of IF3 to the 30S and compromized the fidelity of translation initiation in vivo (Qin *et al.* 2007).

The E-site is formed by helices H68, H74–77 and H88 of 23S rRNA plus protein L1 on the 50S side (Rinke-Appel *et al.* 1995; Yusupov *et al.* 2001). On the 30S the anticodon stem of the tRNA interacts with helices h23, h24, 28 and 29 of the 16S rRNA and with the r-proteins S7 and S11 (Rinke-Appel *et al.* 1995; Yusupov *et al.* 2001; Selmer *et al.* 2006). The elbow region (D- and T-stem) of the E-site tRNA interacts with H77 and protein L1 from the mobile "L1 stalk" while the base at the A76 of the tRNA is inserted between G2421 and A2422 of 23S rRNA and hydrogen bonds to the universally conserved C2394 (Bocchetta *et al.* 2001; Selmer *et al.* 2006). Differently from the situation in the A- and P-site where tRNAs make extensive contacts with the 16S rRNA, the 30S-part of the E-site is mostly formed by proteins S7 and S11 (Selmer *et al.* 2006). The pattern of codon-anticodon interaction in the E-site appears to vary during different phases of translation. Thus, in the 70S ribosomal complexes that mimic the initiation phase the mRNA molecule is seen in a strained conformation that precludes the formation of codon-anticodon base pairing interactions in the E-site (Selmer *et al.* 2006; Jenner *et al.* 2007; Jenner *et al.* 2010b). However, in complexes mimicking the ribosome in a post-initiation state, mRNA assumes a relaxed, A-form conformation, and base pairing is observed at the first codon position in the E-site (Jenner *et al.* 2007; Jenner *et al.* 2010b).

1.2. Translation

In all organisms whose physiology has been investigated so far, amino acids are incorporated into polypeptides through the action of ribosome. The sequence of amino acids in the polypeptide is specified by the sequence of nucleotides in a molecule of messenger RNA that is synthesized by an RNA polymerase using the nucleotide sequence of DNA as the ultimate template (in some viruses the role of DNA as information container is fulfilled by RNA). In addition to the ribosome and mRNA, tRNAs and different proteins participate in the formation of a polypeptide. Due to the large number of components involved and the need to assure that the formation of a protein be both rapid and sufficiently accurate, translation does not occur in one or two steps but is composed of numerous part reactions. Those reactions include the association of the two ribosomal subunits with mRNA and tRNA at the beginning of translation, followed by the binding of the aminoacylated tRNA to the ribosome and a concomitant screening for the correctness of the codon-anticodon interaction within the ribosome, formation of the peptide bond between two amino acids, movement of the tRNA-mRNA complex on the ribosome by one codon and repeating the previous steps many times until a stop codon reaches the ribosome and leads to the release of the polypeptide and the dissociation of the ribosomal subunits. Those different phases of translation are commonly described – in temporal order – as initiation, elongation, peptide release and recycling (Figure 4). Through all those phases of translation large parts of the ribosome as well as of the ligands undergo conformational rearrangements and intermolecular movements which must be precisely controlled in order to ensure the correct sequence of the nascent polypeptide. Since the dynamics of the ribosome is influenced by the intersubunit contacts or, more colloquially, intersubunit bridges – the main subject of the present thesis – those contacs have an important role for translation in general.

Initiation is the first phase of translation and involves the factor-catalyzed joining of the ribosomal subunits, mRNA and the initiator tRNA into an elongation competent ribosome (Gualerzi *et al.* 2001; Simonetti *et al.* 2009; Myasnikov *et al.* 2009). Since initiation is the rate-limiting step of translation it is the main target of translational regulation (Sonenberg and Hinnebusch 2009; Simonetti *et al.* 2009; Benelli and Londei 2009). In *E.coli* the rate of initiation (i.e. frequency of ribosome loading on the mRNA) has been estimated as around 1 event per 3.2 s on the lac operon (Kennel and Riezman 1977). Although initiation involves both ribosomal subunits, the early steps of initiation in both pro- and eukaryotes take place on the small subunit (Myasnikov *et al.* 2009; Benelli and Londei 2009). In bacteria the mRNA molecule first docks onto the back of the platform of the 30S subunit where a specific hexanucleotide sequence GGAGG (called “Shine-Dalgarno sequence”) upstream of the start codon of mRNA base-pairs with a complementary

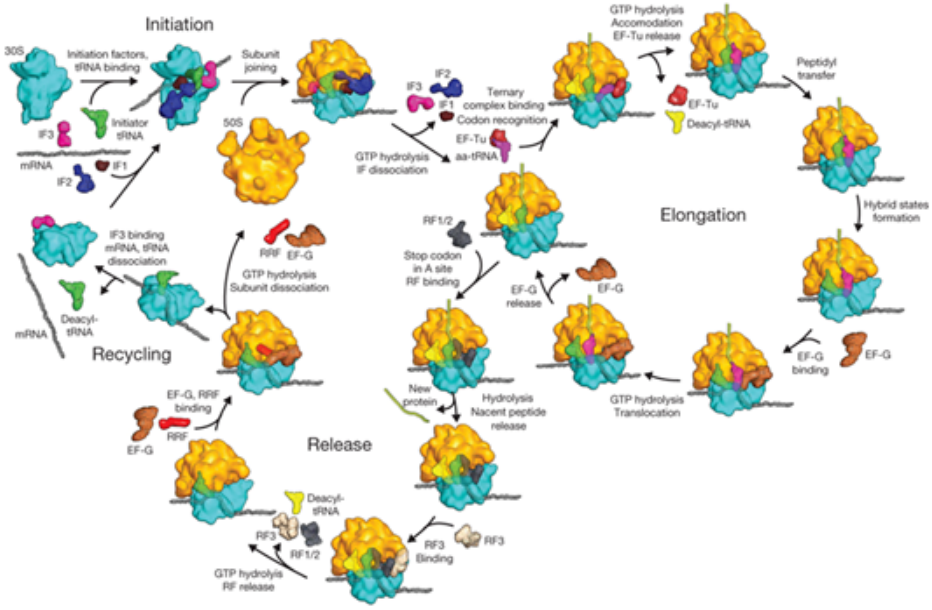

Figure 4. An overview of the ribosomal working cycle in bacteria. The figure shows the four principal steps of translation from initiation to recycling and the most important protein factors participating in the process. Adapted from Schmeing and Ramakrishnan 2009.

sequence ("anti-Shine-Dalgarno sequence") at the 3'-end of the 16S rRNA (Shine and Dalgarno 1974; Steitz and Jakes 1975; Yusupova *et al.* 2001; Kaminishi *et al.* 2007) and a loose 30S preinitiation complex is formed (Yusupova *et al.* 2006). At this stage the mRNA molecule is not yet accommodated into the tunnel around the neck of the 30S and is thus easily displaced from the preinitiation complex (Yusupova *et al.* 2006; Kaminishi *et al.* 2007). Subsequent to the initial binding of mRNA to the 30S, the preinitiation complex undergoes a conformational rearrangement and the region of mRNA downstream of the Shine-Dalgarno sequence is located into the mRNA channel where the AUG start codon is ready to bind the initiator tRNA in the P-site (Yusupova *et al.* 2001; Yusupova *et al.* 2006; Kaminishi *et al.* 2007). As a result of those events a correct reading frame is set on the mRNA. The complex of the small subunit containing mRNA and the initiator tRNA is then bound by the large subunit and an elongation competent ribosome complex is formed (Antoun *et al.* 2006a; Grigoriadou *et al.* 2007; Simonetti *et al.* 2009). In this complex the initiator tRNA is located in the P-site and the A-site is free to accept the elongator aminoacyl-tRNA (Allen *et al.* 2005; Myasnikov *et al.* 2005). Thermodynamic measurements indicate that the rate-limiting step in the formation of the 70S subunit involves a conformational rearrangement in the

30S subunit with an activation barrier of ≈ 80 kJ/mol (Blaha *et al.* 2002). During the transition from the 30S preinitiation to the 70S postinitiation complex the SD-ASD duplex is first extended from 9 to 12 base pairs and undergoes a 70 ^{o} clockwise rotation before being disrupted in the beginning of the elongation phase (Yusupova *et al.* 2006). Various proteins ("initiation factors") are required to make initiation both accurate and sufficiently rapid under cellular conditions (Risuleo *et al.* 1976; Wintermeyer and Gualerzi 1983; Antoun *et al.* 2006a; Antoun *et al.* 2006b; Simonetti *et al.* 2009). While in eukaryotes initiation is a process of astounding intricacy that is catalyzed by more than ten protein factors, including helicases, GTPases and RNA-binding proteins (Kapp and Lorsch 2004; Sonenberg and Hinnebusch 2009; Myasnikov *et al.* 2009), it is considerably simpler in bacteria where only three initiation factors – IF1, IF2 and IF3 are known to be involved (Simonetti *et al.* 2009). The bacterial initiation factors have different but mutually supportive roles in the 70S ribosome formation (Antoun *et al.* 2006a; Antoun *et al.* 2006b; Simonetti *et al.* 2009). IF1 with its molecular weight of around 9 kDa is the smallest among the initiation factors (Carter *et al.* 2001; Simonetti *et al.* 2009). In x-ray and cryo-EM structures of the 30S complexes IF1 is seen to bind in the vicinity of the A-site of the small subunit, thus sterically excluding any other ligand from this region during initiation (Carter *et al.* 2001). One of the functions of IF1 is therefore thought to prevent binding of the initiator tRNA to the 30S A-site (Ramakrishnan 2002; Simonetti *et al.* 2009). In the A-site IF1 interacts with the universally conserved adenines A1492 and A1493 of the decoding centre and causes them to flip-out of helix 44 of 16S rRNA (Carter *et al.* 2001). This is in agreement with an earlier chemical protection study where binding of IF1 to the 30S resulted in the protection of 16S rRNA residues G530 and A1492/A1493 (Moazed *et al.* 1995). A similar flipping-out of A1492 and A1493 during elongator aminoacyl-tRNA binding to the ribosomal A-site is known to induce a large-scale conformational rearrangement in the 30S subunit (Ogle *et al.* 2001). A similar conformational change in the 30S is seen to occur on IF1 binding (Carter *et al.* 2001). The interaction of IF1 with the decoding centre may therefore indirectly regulate the association of the ribosomal subunits by influencing the conformation of the 30S (Ramakrishnan 2002). This is consistent with the results of recent rapid kinetics measurements of the 70S initiation complex formation where the presence of IF1 was seen to induce a conformation of the 30S initiation complex that is unfavorable for the 70S complex formation when a noncognate codon-anticodon interaction occurs in the P-site (Milon *et al.* 2008). Thus IF1 may have a role in ensuring the accuracy of translation initiation. Additionally, IF1 is known to increase the affinity of IF2 and IF3 for the 30S complex and in this way enhance their activities (Wintermeyer and Gualerzi 1983; Antoun *et al.* 2006b; Pavlov *et al.* 2008). Similarly, IF1 promotes the joint activity of IF2 and IF3 during the selection of the initiator tRNA on the 70S initiation complex (Hartz *et al.* 1989). The stimulating influence of IF1 on the functioning of IF2 is supported by cryo-

EM reconstructions of different 70S initiation complexes where IF1 was seen to lie within direct interaction distance with IF2 (Allen *et al.* 2005). However, in a recent cryo-EM map of the 30S initiation complex IF1 and IF2 are located distantly on the 30S, thus excluding any direct interaction between the two factors (Simonetti *et al.* 2008).

IF2 is a 110 kDa GTPase that recruits the initiator tRNA to the 30S initiaton complex (Simonetti *et al.* 2008; Milon *et al.* 2010) and promotes the binding of the 50S to the 30S initiation complex (Godefroy-Colburn *et al.* 1975; Antoun *et al.* 2003a; Grigoriadou *et al.* 2007a). By its C-terminal domain IV IF2 interacts with the formylmethionyl moiety and the acceptor stem of the fMet-tRNA^{fMet} and thereby fixes the latter's location on the 30S (Allen *et al.* 2005; Myasnikov *et al.* 2005; Simonetti *et al.* 2008). The acceptor stem of fMet-tRNA^{fMet} is distorted by 15 ^{o} in the complex with IF2 to increase the interface between the interaction partners (Simonetti *et al.* 2008). A distortion also occurs in the anticodon stem of fMet-tRNA^{fMet} in the 30S initiation complex (Simonetti *et al.* 2008) that is similar to the distorted conformation of the elongator tRNA during initial stages of decoding. The N-terminal part of IF2 contacts regions on the 30S and 50S subunit that form the binding site for GTPase elongation factors EF-Tu, EF-G and RF3 (Allen *et al.* 2005; Myasnikov *et al.* 2005). In the 70S ribosome the GTPase domain of IF2 also interacts with the sarcin-ricin loop and L7/L12 region of the 50S subunit (Allen *et al.* 2005; Myasnikov *et al.* 2005). This interaction may be involved in the activation of the GTPase activity of IF2 immediately after the 50S subunit docks onto the 30S initiation complex (Qin *et al.* 2009), although the GTPase activating role of the L7/L12 region for IF2 has been questioned recently (Huang *et al.* 2010). The overall structure of IF2 in the 30S complex is reminiscent of an arc that is directly anchored to the 30S by its N- and C-termini (Simonetti *et al.* 2008). Although in this conformation IF2 is largely complementary to the interface of the incoming 50S subunit, some clashes can be expected with helices 71 (part of the B3 intersubunit bridge) and 92 (the A-loop) of 23S rRNA (Simonetti *et al.* 2008). In contrast, helix 69 of 23S rRNA that forms an important intersubunit bridge B2a is likely compatible with the IF2-fMet-tRNA^{fMet} complex and is located close to the D-stem of the initiator tRNA (Simonetti *et al*. 2008).

The last of the initiation factors, IF3, serves as a subunit antiassociation factor (Sabol *et al.* 1970; Subramanian and Davis 1970; Godefroy-Colburn *et al.* 1975) and prevents the premature joining of the subunits. The subunit splitting activity of IF3 is stimulated by an mRNA containing a "strong" Shine-Dalgarno sequence and the presence of initiation factor IF1 (Pavlov *et al.* 2008). IF3 is composed of distinct C – and N-terminal domains that are connected by a flexible linker (Moreau *et al.* 1997). Only the C-terminal domain has been shown to be essential for the many functional activities of IF3, the N-terminal domain only increasing the factors affinity for the ribosome (Petrelli *et al.* 2001). The antiassociation activity of IF3 may be explained by the observation that its C-terminal domain binds to the region of the 30S platform that

participate in the formation of intersubunit bridges B2b, B2a and B7a in the 70S ribosome (McCutcheon *et al.* 1999; Dallas and Noller 2001). Accordingly, the presence of the C-terminus of IF3 in those regions of the 30S precludes a stable 70S initiation complex formation (McCutcheon *et al.* 1999; Dallas and Noller 2001). However, the exact location of IF3 on the 30S is a matter of some controversy (Ramakrishnan 2002; Simonetti *et al.* 2009) since in an x-ray structure the C-terminal domain of IF3 was instead seen on the solvent side of the 30S platform (Pioletti *et al.* 2001), thus distant from any intersubunit contacts. The authors of the latter study therefore suggested that IF3 excerts its antiassociation effect by changing the conformational dynamics of the small subunit (Pon and Gualerzi 1974; Gualerzi *et al.* 1977). In addition to its antiassociation activity, IF3 likely assists in the selection and guiding of the initiator tRNA to the P-site of the 30S complex (Risuleo *et al.* 1976; Hartz *et al.* 1989) and acts synergistically with IF2 to promote the 30S initiation complex formation (Wintermeyer and Gualerzi 1983). In light of the thermodynamic study of Blaha and coworkers on the subunit association, the effects of IF3 (and IF1) on the 30S and 70S initiation complex formation may be achieved by influencing the rate-limiting conformational rearrangement of the small subunit in preparation to the 50S binding. The accuracy function of IF3 is explained by a recent rapid kinetics study of the 70S initiation complex formation. Here, when a noncognate codon-anticodon interaction was present in the P-site IF3 was seen to i) slow down the conversion of an initially labile 70S initiation complex into a more stable one and ii) instead enhance the dissociation of the 70S complex (Grigoriadou *et al.* 2007b).

The formation of the 30S initiation complex that contains mRNA, fMet-tRNA^{fMet} and initiation factors IF1, IF2 and IF3 is followed by the binding of the 50S and the eventual formation of an elongation competent 70S post-initiation complex. However, there are indications that the newly formed 70S initiation complex has to undergo a series of conformation changes before "maturing" into the elongation competent ribosome. Those conformational changes include tightening of the intersubunit contacts (Hennelly *et al.* 2005), a ratchet-like movement of the 30S relative to the 50S subunit and relocation of fMet-tRNA^{fMet} into the classical P/P-site from an alternative P/I binding site upon GTP hydrolysis on IF2 and the release of IF3 (Allen *et al.* 2005; Myasnikov *et al.* 2005; Grigoriadou *et al.* 2007a). The importance of the positioning of fMet-tRNA^{fMet} in the 70S initiation complex is highlighted by the presence of a special initiation factor EF-P in *E.coli* (eIF5A in eukarya). In the 70S initiation complex EF-P binds to the region overlapping with the E-site and helps to correctly position fMet-tRNA for the first peptide bond formation (Glick and Ganoza 1975; Blaha *et al.* 2009). After the synthesis of the first peptide bond the 70S ribosome gradually engages in processive elongation. During the transition from initiation to elongation, the SD-anti-SD interaction between mRNA and 16S rRNA is broken after one (Uemura *et al.* 2007) to five (Zavialov *et al.* 2005) codons have been translated and the growing peptide

starts to move into the exit channel in the 50S subunit somewhere at the tetra- to octapeptide stage (Stade *et al.* 1995; Tenson and Ehrenberg 2002).

The ensuing multiple rounds of elongation include aminoacyl-tRNA binding to the ribosomal A-site, peptide bond formation in the peptidyl transferase centre and translocation of the tRNA-mRNA complex by one codon at a time to make the next codon available to a new elongator tRNA (Schmeing and Ramakrishnan 2009). During elongation the polypeptide chain grows in the direction from the N-terminus to the C-terminus (Dintzis 1961) and moves through a channel in the large subunit (Nissen *et al.* 2000; Harms *et al.* 2001). Similarly to other phases of translation, special protein factors participate in elongation (Noble and Song 2008; Schmeing and Ramakrishnan 2009). Principal among them are GTPases EF-Tu that brings the aminoacyl-tRNA to the ribosome and EF-G that catalyzes the translocation of the tRNA-mRNA complex (Noble and Song 2008; Schmeing and Ramakrishnan 2009).

After aminoacylation by the corresponding synthetase in the presence of ATP the aminoacyl-tRNA is bound by the elongation factor EF-Tu (EF-1a in eukaryotes) whose role is to deliver this tRNA to the ribosomal A-site. EF-Tu is a GTPase that in the GTP-bound form ("closed" conformation) binds the aminoacyl-tRNA with a high affinity but has a much lower affinity for aa-tRNA when in complex with GDP ("open" conformation). EF-Tu-GTP binds tRNA at its acceptor arm, forming a ternary complex EF-Tu-GTP-aa-tRNA (Nissen *et al.* 1995). It is this ternary complex that then binds to the ribosome. Within the ternary complex EF-Tu contacts the "L11 stalk" and SRL regions on the 50S while on the 30S it makes contacts to the 16S rRNA helices h5, h8 and h14 on the shoulder as well as protein S12 (Valle *et al.* 2003a; Schmeing *et al.* 2009). At this stage the tRNA within the ternary complex exists in a strained conformation (Valle *et al.* 2003a; Schmeing *et al.* 2009) and is located in a region of the ribosome called "entry" (Hardesty *et al.* 1969), "recognition" (Lake 1977) or "A/T" site (Moazed and Noller 1989; Valle *et al.* 2003a; Schmeing *et al.* 2009). In this state, the anticodon of the tRNA is already in the 30S A-site and interacts with the mRNA codon similarly to a fully accommodated tRNA (Yusupov *et al.* 2001; Schuette *et al.* 2009; Schmeing *et al.* 2009). The acceptor stem of the tRNA that is buried within EF-Tu has a canonical conformation too (Schmeing *et al.* 2009). However, the helix at the anticodon stem and D-stem regions is underwound to allow a bend in the tRNA and the distance between the sugar-phosphate backbones is widened by 1 – 2 Å relative to the canonical/fully relaxed tRNA as seen in a recent crystal structure (Schmeing *et al.* 2009). Also, the D-stem is located farther from the T- and acceptor stem stack than in the relaxed tRNA (Schmeing *et al.* 2009). Similar changes in the D- and T-stems are seen in cryo-EM maps of the EF-Tu-aa-tRNA-ribosome complex (Schuette *et al.* 2009). The strained/bent conformation of the tRNA in the A/T state is stabilized by interactions with 23S rRNA at two regions. The first interaction involves the elbow of tRNA and nucleotides A1067 and U1068 from helix H43 in the "L11 stalk" (Schmeing *et al.* 2009).

The second interaction in the A/T state is between C25 of tRNA and the nucleotide A1914 of helix H69 (Schmeing *et al.* 2009). However, the stabilizing role of H69 for the tRNA in the A/T conformation is questioned by Schuette and coworkers as the interaction between the A/T-state tRNA and H69 in their cryo-EM map is similar to the interaction in the case of fully accommodated tRNA (Yusupov *et al.* 2001; Selmer *et al.* 2006). That the deletion of H69 did not significantly affect ribosomal accuracy or the rate of elongation further speaks against any stabilizing role of H69 (Ali *et al.* 2006).

The formation of a cognate codon-anticodon complex causes a series of rearrangements in the ribosomal decoding centre involving residues G530, A1492 and A1493 of 16S rRNA together with residues from proteins S12 as well as residues A1912 and A1913 from helix H69 of 23S rRNA (Ogle *et al.* 2001; Ogle *et al.* 2002; Jenner *et al.* 2010a). The rearrangement in turn leads to a large-scale conformational change or "domain closure" of the 30S where the shoulder and head of the 30S move inwards with respect to the A-site (Ogle *et al.* 2001). Importantly, near-cognate tRNA was unable to induce a similar conformational change in the 30S (Ogle *et al.* 2001). The inward movement of the shoulder induced by the cognate tRNA is eventually transmitted to the GTPase domain of EF-Tu and leads to the hydrolysis of GTP that is followed by the dissociation of EF-Tu from the aminoacyl-tRNA in the GDP-bound form (the GTP-form of EF-Tu is regenerated by the G-nucleotide exchange factor EF-Ts) (Schmeing *et al.* 2009). This structural view is in good agreement with results from rapid kinetics studies where correct (cognate to the mRNA codon) tRNA was shown to selectively accelerate the GTPase activity of EF-Tu over the near/non-cognate tRNA (Gromadski and Rodnina 2004; Gromadski *et al.* 2006). After the dissociation of EF-Tu the tRNA can either fully accommodate into the 50S in preparation for the peptidyl transferase reaction or alternatively, dissociate from the ribosome. Again, kinetic studies indicate that the accommodation is favored by a correct/cognate codon:anticodon interaction whereas near/noncognate tRNA tends to dissociate from the ribosome (Gromadski *et al.* 2006). However, certain mutations in the D and T-stem regions of the tRNA enable the variant tRNA to accelerate the GTPase activation and accommodation steps more than its WT counterpart on a near-cognate codon (Hirsh 1971; Cochella and Green 2005; Ortiz-Meoz and Green 2010). In contrast, a fragment consisting of the cognate anticodon stem-loop plus D-stem was unable to induce the GTPase activity in EF-Tu (Piepenburg *et al.* 2000). Those results indicate that interactions at tRNA regions distant to the codon:anticodon duplex also contribute to the tRNA selection process.

It must be noted that some aspects of the elegant structural model of tRNA selection described above have been challenged by a recent crystal structure of Thermus thermophilus 70S ribosome (Jenner *et al.* 2010a). Here, the ability to induce both the flipping out of the adenines A1492/1493 from helix 44 and the ensuing domain closure of the 30S was not limited to the cognate tRNA as seen in the earlier structures but was observed with a near-cognate tRNA as well

(Jenner *et al.* 2010a). However, it was only the cognate tRNA that was able to induce a general tightening of ribosomal contacts around the entire tRNA molecule, thus leading to its selective stabilization relative to the near-cognate tRNA (Jenner *et al.* 2010a).

Upon the accommodation of the 3'-end of the aminoacyl-tRNA into the peptidyl transferase center the peptidyl moiety from the peptidyl-tRNA in the P-site is transferred to the α-amino group of aminoacyl-tRNA in an aminolysis reaction involving the ester-bond in the peptidyl-tRNA and the amino group of the aminoacyl-tRNA (for reviews see Polacek and Mankin 2005; Rodnina *et al.* 2006; Simonović and Steitz 2009). The exact mechanism of the peptidyl transferase reaction has been an object of intensive research as well as controversy (Bieling *et al.* 2006; Johansson *et al.* 2008; Wohlgemuth *et al.* 2010; Johansson *et al.* 2010) fuelled by the availability of ribosomal crystal structures with various ligands and the discovery that rRNA itself has an essential part in the process (Noller *et al.* 1992; Khaitovich *et al.* 1999a; Nissen *et al.* 2000; Voorhees *et al.* 2010). The consensus now seems to be that the peptidyl transferase reaction involves an intricate network of precisely positioned ribosomal residues, a water molecule and the 2'- OH group of one of the the substrates – the peptidyl-tRNA – that together shuttle protons between the reaction intermediates (Weinger *et al.* 2004; Schmeing *et al.* 2005a/b; Simonović and Steitz 2009; Wallin and Åqvist 2010). A similar substrate-assisted catalytic mechanism has been observed with the protein enzyme threonyl-tRNA synthetase (Minajigi and Francklyn 2008). A major contribution to the 10^7-fold rate enhancement of peptide bond formation (Sievers *et al.* 2004; Schroeder and Wolfenden 2007) is thought to come from the precise positioning of the tRNA substrates in the peptidyl transferase centre by 23S rRNA and ribosomal proteins (Sievers *et al.* 2004; Schmeing *et al.* 2005a/b; Schroeder and Wolfenden 2007; Jenner *et al.* 2010a; Voorhees *et al.* 2010). The precise positioning of the reactive groups within the ribosome requires a prior re-modeling of the structure of the peptidyl transferase centre that is induced by the binding of ligands like aminoacyl-tRNA or release factors RF1/RF2 into the ribosomal A-site (Schmeing *et al.* 2005a; Jenner *et al.* 2010a; Jin *et al.* 2010). The likely reason for such a substrate-induced fit of the peptidyl transferase center is to avoid a premature release of the nascent peptide from the ribosome that would occur when a water molecule alone reacted with the ester bond in the peptidyl-tRNA (Schmeing *et al.* 2005a). Consistent with the importance of substrate positioning in peptidyl transferase reaction is the finding that the activation energy of peptide bond formation has a large positive entropic component in solution and that ribosome acts by lowering this entropic term in the activation energy (Page and Jencks 1971; Sievers *et al.* 2004; Schroeder and Wolfenden 2007).

After the peptidyl group has been transferred to the aminoacyl-tRNA, the A- and P-sites are transiently occupied by the peptidyl- and deacylated-tRNAs, respectively. This is the "pretranslocation" state that has to be converted into the

"posttranslocation" state where the peptidyl-tRNA is in the P-site and the next mRNA codon in the otherwise empty A-site is ready to accept a new aminoacyl-tRNA. The latter state is achieved through a series of steptwise movements of the tRNAs that are coordinated to various conformational rearrangements in and between the subunits in a process collectively known as translocation. The movements start with the shifting of positions of the acceptor ends of the tRNAs on the large subunit (Hardesty *et al.* 1986; Moazed and Noller 1989; Odom *et al.* 1990). Since the peptidyl- and deacylated tRNAs are known to have higher affinities for the P- and E-sites, respectively, than for the A-and P-sites (Holschuh *et al.* 1980; Lill *et al.* 1989; Semenkov *et al.* 2001) the acceptor ends of the tRNAs spontaneously move to the next sites in the large subunit immediately after the peptidyl transferase reaction. At this stage, the anticodon ends of the tRNAs on the 30S subunit are still bound to the previous, i.e., A and P-sites. The result of this partial dislocation of the tRNAs is an intermediate state of the ribosome where the tRNAs reside in the so-called A/P and P/E hybrid sites (Moazed and Noller 1989; Semenkov *et al.* 1992; Dorner *et al.* 2006). During hybrid site formation the elbows of the tRNAs in the 50S move significant distances of 40 Å from the A-to the P-site and 55 Å from the P- to the E-site (Yusupov *et al.* 2001). In addition to the movement of the tRNAs, the "L1 stalk" moves towards the E-site where it interacts with the D- and T-stems of the tRNA (Valle *et al.* 2003b; Cornish *et al.* 2009; Fei *et al.* 2009; Fischer *et al.* 2010), thus helping to stabilize the location of the tRNA in the P/E hybrid state (Munro *et al.* 2009). In addition to the A/P and P/E sites cryo-EM and FRET analyses of different ribosomal complexes have revealed the occurrence of further intermediate tRNA binding states on the translocation pathway (Munro *et al.* 2007; Pan *et al.* 2007; Fischer *et al.* 2010; Ratje *et al.* 2010). Cryo-EM studies show that the presence of hybrid binding sites of tRNA is not limited to translocation but also occurs in initiation (Allen *et al.* 2005; Myasnikov *et al.* 2005; Grigoriadou *et al.* 2007a) and termination/recycling (Gao *et al.* 2005; Gao *et al.* 2007; Sternberg *et al.* 2009).

Concomitantly with the hybrid site formation, the small subunit rotates counterclockwise (when viewed from its solvent side) by 6–10^{o} relative to the large subunit. This movement is known under the name of "ratchet" (Frank and Agrawal 2000) and is triggered by the formation of deacylated tRNA in the P-site (Zavialov and Ehrenberg 2003). In fact, the energy released during transpeptidation probably serves to power the intersubunit rotation during translocation (Semenkov *et al.* 2001; Marshall *et al.* 2008). Like the hybrid site formation, the "ratchet" occurs through multiple intermediate states (Schuwirth *et al.* 2005; Connell *et al.* 2007; Zhang *et al.* 2009; reviewed in Dunkle and Cate 2010). Although hybrid site formation and the ratchet movement of the subunits occur simultaneously, it is still not resolved whether the two processes are mechanistically coupled (Ermolenko *et al.* 2007; Agirrezabala *et al.* 2008; Julián *et al.* 2008) or can occur independently of each other (Berk *et al.* 2006; Ly *et al.* 2010; Munro *et al.* 2010a). In addition to the ratchet-movement of the

subunits the 30S head moves relative to the body in a process called "head swivelling" (Spahn *et al.* 2004; Taylor *et al.* 2007; Zhang *et al.* 2009). It has been speculated that it is during the "swivelling" of the 30S head that the tRNA-mRNA complex moves to the next sites on the 30S subunit, thus completing translocation (Spahn *et al.* 2004; Schuwirth *et al.* 2005; Zhang *et al.* 2009; Ratje *et al.* 2010). In fact, a recent cryo-EM study of Thermus thermophilus 70S complexes suggests that it is the head movement coupled to the back-ratcheting of the subunits that completes the translocation of the tRNA-mRNA complex and produces the posttranslocation state ribosome (Ratje *et al.* 2010). It is probably during the "head swivelling" that the "gate" formed by the 16S rRNA nucleotides A790, G1338 and A1339 at the head-platform interface widens sufficiently to allow the passage of the tRNA-mRNA complex from the P- to the E-site on the 30S (Schuwirth *et al.* 2005; Munro *et al.* 2009). As suggested by the recent T.thermophilus 70S ribosome cryo-EM analysis, swiveling of the 30S head would also lead to the opening of the "latch" in the mRNA entry channel, further facilitating tRNA-mRNA movement (Ratje *et al.* 2010).

Although recent cryo-EM (Agirrezabala *et al.* 2008) and single molecule FRET studies (Cornish *et al.* 2008) together with earlier biochemical data (Pestka 1968; Gavrilova and Spirin 1971; Bergemann and Nierhaus 1983; Southworth *et al.* 2002; Cukras *et al.* 2003) indicate that the ability to translocate the tRNA-mRNA complex is intrinsic to the ribosome the process needs catalysis to proceed at a physiologically relevant rate. This catalysis is provided by the elongation factor EF-G, although some antibiotics like sparsomycin can also stimulate some of the steps in the translocation pathway (Fredrick and Noller 2003). The EF-G-mediated catalysis has been reported to increase the rate of tRNA-mRNA translocation by 50,000-fold in the presence of GTP (Katunin *et al.* 2002). EF-G is a 5-domain GTPase whose structure resembles that of the aa-tRNA-EF-Tu•GTP ternary complex (Nissen *et al.* 1995) and many of the contacts between EF-G and ribosome are similar to those occurring with other translational GTPases like IF2, EF-Tu and RF2. Thus, like in EF-Tu, the GTP-binding domain I of EF-G interacts with the "L11 stalk" and SRL regions of the 50S that are known to be important for activating the factor's GTPase activity (Spahn *et al.* 2004; Gao *et al.* 2009). Also interacting with the SRL are domains III and V of EF-G (Gao *et al.* 2009). In addition, domain V contacts helix H89 that connects the "L11 stalk" and peptidyl transferase centre regions (Gao *et al.* 2009). On the 30S subunit domain II contacts 16S rRNA helices 5 and 15 on the shoulder region (Spahn *et al.* 2004; Gao *et al.* 2009). Domain IV of EF-G mimics the anticodon end of aa-tRNA and binds to the ribosomal A-site, making extensive contacts with mRNA and the P-site tRNA (Gao *et al.* 2009). The tip of domain IV of EF-G contacts the bridge B2a region (helices h44 and H69) at the decoding centre. Differently from the A-site tRNA, it does not contact the A-site codon in the post-translocation state ribosome (Gao *et al.* 2009). Instead, residues from the apical loops I and II of domain IV of EF-G form hydrogen bonds to the codon-

anticodon duplex in the P-site (Gao *et al.* 2009). Those interactions to loops I and II may be formed when the duplex is still in the A-site (pre-translocation state) and secure the movement of the duplex into the P-site in such a way that the possibility of a slippage of the reading frame is minimized (Gao *et al.* 2009). The presence of domain IV in the A-site probably also helps to prevent a return of the tRNA to the A-site until it is stably bound in the P-site on the 30S (Spahn *et al.* 2004; Gao *et al.* 2009). In addition to the above interactions, domain IV of EF-G interacts closely with helix 34 from the 30S head when the ribosome is in the post-translocation state (Spahn *et al.* 2004; Ratje *et al.* 2010). However, the formation of this contact requires a 12–15 Å inward movement of h34 that is facilitated by the 30S head swiveling and leads to the widening of the mRNA entry channel (Spahn *et al.* 2004; Ratje *et al.* 2010). It is conceivable that the formation of a stabilizing interaction to h34 in the post-translocation state is one of the ways whereby EF-G facilitates tRNA-mRNA movement during translocation.

Despite the wealth of structural and biochemical data on the EF-G ribosome interaction and the ribosomal dynamics, the precise role of EF-G for the translocation of tRNA-mRNA is still open to debate. For instance, does EF-G promote the ratchet movement of the subunits and/or the hybrid site formation? Or is its role to stabilize the already ratcheted state of the ribosome and prevent the backslippage of the tRNA-mRNA complex (acting as a pawl in the thermal ratchet model)? Recent single molecule FRET data indicate that the ratcheted state is achieved by the ribosome itself after which EF-G can productively interact with the ribosome (Munro *et al.* 2010b). According to these data, the catalytic role of EF-G lies in facilitating the steps subsequent to subunit rotation like head swiveling and sliding of the tRNA-mRNA complex on the 30S (Munro *et al.* 2010b). This model is in agreement with cryo-EM analyses where binding of domain IV of EF-G to h34 of 16S rRNA is observed to widen the mRNA entry channel, thus facilitating the mRNA movement (Spahn *et al.* 2004; Ratje *et al.* 2010). Another longstanding question concerns the role of GTP hydrolysis for the functioning of EF-G and translocation at large. According to one model, GTP hydrolysis on EF-G is required for the dissociation of the factor from the ribosome and occurs after the tRNA-mRNA complex has been fully translocated (Inoue-Yokosawa *et al.* 1974). In contrast to this, rapid kinetics measurements indicate that GTP hydrolysis precedes the translocation of the tRNA-mRNA complex, suggesting that the energy in GTP is required for the mechanical movement of the substrates in translocation (Savelsbergh *et al.* 2003).

An interesting turn was given to the translocation research in the last decade when it was shown that in addition to the spontaneous forward translocation the ribosome is able to undergo translocation in the reverse direction (back- or retrotranslocation) (Shoji *et al.* 2006; Konevega *et al.* 2007). At about the same time Qin and co-workers identified a previously known protein LepA (Dibb and Wolfe 1986; Bijlsma *et al.* 2000) as the factor that catalyzes translocation in the

reverse direction (Qin *et al.* 2006). Although structurally similar to EF-G (Evans *et al.* 2008), the LepA-catalyzed backtranslocation proceeds through different intermediate states (Connell *et al.* 2008; Liu *et al.* 2010).

The elongation process is continued until one of the stop codons – UAA, UGA or UAG – is encountered in the ribosomal A-site (for comprehensive reviews on translation termination, see Petry *et al.* 2008 and Loh and Song 2009). Exposure of a stop codon in the ribosomal A-site is a signal for a specific protein factor called class I release factor to bind to the ribosomal A-site and initiate a chain of events that ultimately leads to the hydrolysis of the peptidyl-tRNA in the P-site and the release of the completed polypeptide. In most eukaryotes a single class I release factor – eRF1 – recognizes all three stop codons (Beaudet and Caskey 1971; Konecki *et al.* 1977; Buckingham *et al.* 1997). In bacteria there are two class I release factors – RF1 and RF2 – that differ in the specificity for the stop codon (Scolnick *et al.* 1968; Klein and Capecchi 1971). RF1 is specific for UAG, RF2 for UGA and both factors recognize the UAA codon although there are reports that the RF2-dependent peptide release is weak on the latter codon (Scolnick *et al.* 1968; Klein and Capecchi 1971; Uno *et al.* 1996). Bioinformatic analysis has revealed an additional putative class I release factor in *E.coli* (encoded by the *prfH* pseudogene in *E.coli* K-12) that may recognize atypical mRNA signals under amino acid starvation conditions (Pel *et al.* 1992; Baranov *et al.* 2006). Both RF1 and RF2 are composed of five domains with distinct functional roles in termination (Pel *et al.* 1992). Thus, the N-terminal domain I of RF1/2 is required for their interaction with the class II release factor RF3 and stimulation of the GDP-to-GTP exchange on RF3 (Mora *et al.* 2003a; Gao *et al.* 2007). Domain I of RF1 and RF2 also contacts the "L11 stalk" of the 50S subunit, although the exact location of the contacts differs between RF1 and RF2 (Petry *et al.* 2005). Domains II and IV are involved in stop codon recognition and interaction with the rRNA in the ribosomal A-site while domain III is involved in the stimulation of the hydrolysis of the P-site bound peptidyl-tRNA (Petry *et al.* 2005; Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Jin *et al.* 2010; Korostelev *et al.* 2010). The stop-codon recognition is mediated by a specific tripeptide sequence (a so-called tripeptide anticodon) in domain II of RF1 and RF2 that makes factor-specific interactions with the stop codon (Ito *et al.* 2000; Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010). In RF1 the tripeptide anticodon has the sequence P(A/V)T whereas in RF2 the sequence is SPF (Ito *et al.* 2000; Nakamura and Ito 2000; Nakamura *et al.* 2000; Mora *et al.* 2003). The ribosome-RF cocrystal structures explain the chemical basis of the stop codon recognition. Thus, purines are excluded from the first codon position due to a steric clash with two conserved glycines at the tip of the α5-helix of RF1/RF2; lack of H-bonds to residues in RF1/2 explains the exclusion of C from this position (Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008). Conversely, pyrimidines are excluded from the second codon position due to their inability to form H-bond/hydrophobic interactions to residues in RF1/RF2

(Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008). The inability of a G at the second codon position to donate an H-bond to the threonine in the PVT motif may be the reason for the inability of RF1 to recognize UGA (Laurberg *et al.* 2008). Similarly, lack of H-bonds between a G at the third position and residues R201 and T203 of RF2 may explain the inability of RF2 to recognize UAG (Weixlbaumer *et al.* 2008). The importance of the tripeptide motifs in directing RF1 and RF2 to their cognate stop codons was initially demonstrated in genetic experiments where swapping of the SPF and PVT sequences between RF1 and RF2 led to a change in the stop codon specificity of the factor (Ito *et al.* 2000; Mora *et al.* 2003). However, more recent bioinformatic analyses, genetic experiments and molecular dynamics simulations indicate that the SPF/PVT motifs are not the sole determinants of the stop-codon specificities of RF1 and RF2 and that residues in the entire loop embedding the tripeptide motif as well as residues in rRNA and water molecules are important for the cognate stop codon recognition (Oparina *et al.* 2005; Young *et al.* 2010; Sund *et al.* 2010). Interestingly, certain mutations at positions in domain II of RF2 that are out of interaction distance with the stop codon rendered the factor "ominpotent" – able to decode all three stop codons like its single eukaryotic counterpart eRF1 (Ito *et al.* 1998; Ito *et al.* 2002).

The accuracy of the RF1/2-catalyzed peptide release of 10^{-3}–10^{-6} (meaning one termination event on a sense codon per 10^5 terminations on the stop codon) is on the average a magnitude higher than the accuracy of tRNA selection during elongation (Gromadski and Rodnina 2004; Gromadski *et al.* 2006) and is achieved without any GTP-driven proofreading mechanism comparable to that in tRNA decoding (Jørgensen *et al.* 1993; Freistroffer *et al.* 2000). Thus, differences in the binding energies ($\Delta\Delta G$) of RFs on cognate and noncognate codons, respectively, are higher than the corresponding energetic differences in tRNA-mRNA so as to allow the selection of the correct stop codon by RFs in one step (Freistroffer *et al.* 2000). The high level of accuracy in termination is apparently needed to avoid premature termination events on sense codons that would lead to the accumulation of truncated proteins.

The x-ray and cryo-EM analyses of RF1 and RF2 in complex with the ribosome and the cognate stop codon (Petry *et al.* 2005; Rawat *et al.* 2006; Weixlbaumer *et al.* 2008; Laurberg *et al.* 2008; Korostelev *et al.* 2010) show that both factors exist in an extended conformation that differs from their more compact conformation in solution (Vestergaard *et al.* 2001; Shin *et al.* 2004). It has been suggested that this conformational change may represent an induced fit upon stop codon recognition that contributes to the accuracy of stop codon recognition (Hetrick *et al.* 2009). It must be noted, however, that the compact conformation of the release factors in solution is questioned by a small angle x-ray scattering study that showed RF1 to have a conformation in solution that was compatible with that observed on the ribosome (Vestergaard *et al.* 2005). In the ribosome-bound conformation domain III of RF1/RF2 has moved away from the "core" formed by domains II and IV and is relocated adjacent to the

peptidyl transferase center (Petry *et al.* 2005;Weixlbaumer *et al.* 2008; Laurberg *et al.* 2008; Korostelev *et al.* 2010). The cleft between domains II and III of RF1/2 is occupied by helix 69 of 23S rRNA that may interact with specific residues of the factor (Weixlbaumer *et al.* 2008; Laurberg *et al.* 2008; Korostelev *et al.* 2010). Domain II of RF1/2 contains a conserved GGQ motif that is required for catalyzing the hydrolysis of the peptidyl-tRNA in the P-site (Frolova *et al.* 1999; Seit-Nebi *et al.* 2001; Oparina *et al.* 2005; Jin *et al.* 2010). Together with the residues A2451 and A2602 of 23S rRNA and A76 of the P-site tRNA Q240 of RF1/RF2 forms a pocket that activates a water molecule for a nucleophilic attack at the ester bond between the tRNA and the peptidyl group (Polacek *et al.* 2003; Youngman *et al.* 2004; Brunelle *et al.* 2008; Jin *et al.* 2010).

After the release of the polypeptide chain the ribosomal P-site contains a deacylated tRNA while RF1/RF2 are still bound to the A-site. In the next step RF1/RF2 are released from the ribosome by the action of class II release factor RF3 (eRF3 in eukaryotes) (Buckingham *et al.* 1997; Freistroffer *et al.* 1997; Pavlov *et al.* 1997; Zavialov *et al.* 2002). Initially, RF3 was isolated as a factor that stimulated the RF1/2-dependent fMet release from an fMet-tRNA^{fMet} bound to the initiator AUG triplet (Milman *et al.* 1969; Goldstein *et al.* 1970). RF3 is a GTPase whose overall structure is similar to EF-Tu, although domain III of RF3 contains a unique α/β-fold not seen in other translational GTPases (Gao *et al.* 2007). RF3 is thought to come to the ribosome in the GDP-form and exchange GDP for GTP on the ribosome (Zavialov *et al.* 2001; Gao *et al.* 2007). In the GTP-form RF3 induces a ratchet-like conformational change in the ribosome similar to that occurring during translocation (Gao *et al.* 2007). By disrupting contacts between RF1/2 and the ribosome this ratchet-movement leads to the dissociation of RF1/2 from the ribosome (Gao *et al.* 2007). Despite the stimulatory effect of RF3 on the activity of class I release factors, RF3 is apparently not absolutely required for translation termination. This is indicated by the phylogenetic analysis of Margus and colleagues where RF3 was found to be absent from many groups of bacteria with both minimal-size (e.g. Mycoplasma, Rickettsia) and large genomes (e.g. Bacillus, Mycobacterium) (Margus *et al.* 2007). In line with the dispensability of RF3 for cellular viability, inactivation of the prfC gene coding for RF3 did not completely abolish cell growth in *Escherichia coli* (Grentzmann *et al.* 1994; Mikuni *et al.* 1994).

In the final stage of translation, the subunits are driven apart by the concerted action of ribosomal recycling factor RRF and EF-G (Janosi *et al.* 1994; Karimi *et al.* 1999; Rao and Varshney 2001; Hirokawa *et al.* 2002; Ito *et al.* 2002; Peske *et al.* 2005). RRF is needed for *Cell*ular viability (Janosi *et al.* 1994). In addition to termination, RRF may have a role in ensuring the fidelity of translation initiation by preferentially dissociating noncognate 70S initiation complexes (Seshadri *et al.* 2009). RRF is a small 20 kDa protein that is composed of an α-helical "tail" and α/β globular "head" domain that are connected via a flexible linker (Selmer *et al.* 1999; Kim *et al.* 2000). Genetic

studies have shown that the flexibility of the hinge region is required for the functioning of RRF (Toyoda *et al.* 2000). In cryo-EM reconstructions RRF is seen to occupy different binding sites on the 50S subunits, some of which are incompatible with the presence of the 30S subunit in the ribosome (Barat *et al.* 2007). Movement of RRF into the 30S-incompatible binding sites is induced by the binding of domain IV of EF-G to the ribosomal A-site and results in the disruption of the central RNA-RNA intersubunit bridges B2a and B3 (Gao *et al.* 2005; Pai *et al.* 2008). The dissociating ability of RRF and EF-G is strongly stimulated by the presence of the deacylated tRNA in the ribosome (Pavlov *et al.* 2008). The energy for the separation of the subunits may come from the hydrolysis of GTP on EF-G during its concerted action with RRF (Gao *et al.* 2007). In addition to bridges B2a and B3, there are indications that the binding of RRF to the posttermination ribosome leads to conformational changes in the "head" regions bridges B1a and B1b (Barat *et al.* 2005). Following the dissociation of the subunits, binding of initiation factor IF3 to the 30S leads to the dissociation of mRNA and the deacylated tRNA (Karimi *et al.* 1999; Peske *et al.* 2005; Pavlov *et al.* 2008). The subunits are now ready for another round of translation.

1.3. Ribosomal intersubunit bridges

The two-subunit nature is a general characteristic of ribosomes that may be related to the mechanism of protein synthesis[†]. Although template-directed like DNA/RNA synthesis, protein synthesis differs from the latter processes by the spacial relationship between the template and the growing polypeptide. In DNA/RNA synthesis the monomers to be incorporated into the nascent polymer directly base pair with the template and the polymer therefore grows colinearly to the template (Doublié *et al.* 1998; Kiefer *et al.* 1998; Von Hippel 1998; Yin and Steitz 2004; Steitz 2006). Accordingly, the translocation of the polymerase can be envisaged as being propelled by the growth of the nascent polymer with the help of the energy stored in the phosphoanhydride bond (a "jet-plane" model). During protein synthesis however there is no direct pairing between the template and the nascent polypeptide. The incoming amino acids interact with the mRNA template via tRNA as the adaptor and the polymer grows roughly perpendicularly to the template. Translocation of the ribosome can therefore not

[†] It is interesting to note that the two-subunit structure of the ribosome with its characteristic irregularity came as a surprise to the early molecular biologists who were rather expecting something regular like the tobacco mosaic virus. A quotation from two of the „founding fathers" seems appropriate here : „It was therefore a surprise to find that the 70S particle breaks up into a 50S and a 30S particle. Why should there be two unequal particles, and how are they related" (Crick, F. H. C and Brenner, S. (1959). A Note for the RNA Tie Club).

be accomplished by pushing it forth through the addition of monomers to the nascent polypeptide. Instead, the entire complex of mRNA and tRNAs has to be translocated within the ribosome in a controlled and stepwise manner which requires parts of the ribosome itself to be mobile. It is thus advantageous for a ribosome to be composed of separate subunits that i) bind different parts of tRNA and mRNA and ii) are capable of independent movement since this would facilitate a gradual displacement of the tRNA-mRNA complex between different binding sites. The movements of the subunits relative to each other as well as large conformational changes in subunits themselves during translocation are now well established by many x-ray and cryo-EM studies. In addition, structural studies on different ribosomal complexes indicate that the intersubunit movement is not confined to the translocation of tRNA-mRNA alone but is a widespread phenomenon that occurs during translation initiation and termination. Despite the intrinsically dynamic nature of the two-subunit ribosome the subunits still have to remain connected during the entire process of translation to enable the maintenance of a correct reading frame on mRNA as well as a proper interaction between the anticodon of the incoming aminoacyl tRNA and the mRNA codon. The subunits are held together by contacts between protruding structures from both subunits that form the intersubunit bridges. Depending on the resolution and source of the ribosomes (prokaryotic or eukaryotic) around ten to seventeen of such intersubunit contacts or "bridges" have been observed (Yusupov *et al.* 2001; Spahn *et al.* 2001; Spahn *et al.* 2004; Schuwirth *et al.* 2005; Ben-Shem *et al.* 2010) (Figure 5 and 6). Since the "bridges" must keep the subunits together, the overall strength of interaction contributed by the bridges must be sufficient to withstand the various inter- and intrasubunit movements within the translating ribosome. However, the bridges must still be flexible enough to allow those same movements since the movements are a prerequisite for an efficient translation. Making the intersubunit interaction too strong – by e.g. increasing the Mg^{2+} concentration to 20 mM *in vitro* – has in fact been shown to block the translocation reaction (Belitsina and Spirin 1979) where the intersubunit movement is mandatory. Those considerations indicate that a carefully selected balance must exist in the strength of the intersubunit interaction – to keep the subunits firmly together while simultaneously allowing them to move. How can the ribosome carry out those apparently contradictory tasks? The solution to this problem may lie in the differing composition and dynamics of the bridges and their different localization on the subunit interface. As seen in the cryo-EM and x-ray structures of 70S ribosomes, one class of the intersubunit bridges is composed entirely of RNA ("RNA-RNA bridges") whereas bridges of another class have at least one protein as their component ("protein bridges"). The two classes differ conspicuously in their localization on the interface (Figure 5). While the RNA-RNA bridges tend to be centrally located, the protein bridges are in the periphery of the interface (Cate *et al.* 1999; Yusupov *et al.* 2001), particularly in the "head" region of the small subunit that is known to undergo large

movements during translocation (Zhang *et al.* 2009; Gao *et al.* 2003). On the average, the "RNA-RNA" bridges have a larger contact surface than the "protein bridges". An idea therefore suggests itself that there may be a "division of labor" among the bridges. Thus, the central "RNA-RNA" bridges may be static – i.e. their interactions are not broken or reformed to a significant extent – during elongation and help maintain the subunits in an associated state (Figure 7). The "protein bridges" on the other hand may be sufficiently dynamic – undergoing conformational rearrangements – to allow limited movements within the ribosome (Frank and Agrawal 2000) (Figure 7). In addition, it is very likely that any rearrangements within the bridges during the ribosomal movements occur in a stepwise manner so that only a small number of contacts would be broken at any time (Zhang *et al.* 2009). This stepwise remodeling of the bridges would be in keeping with the stepwise nature of translocation.

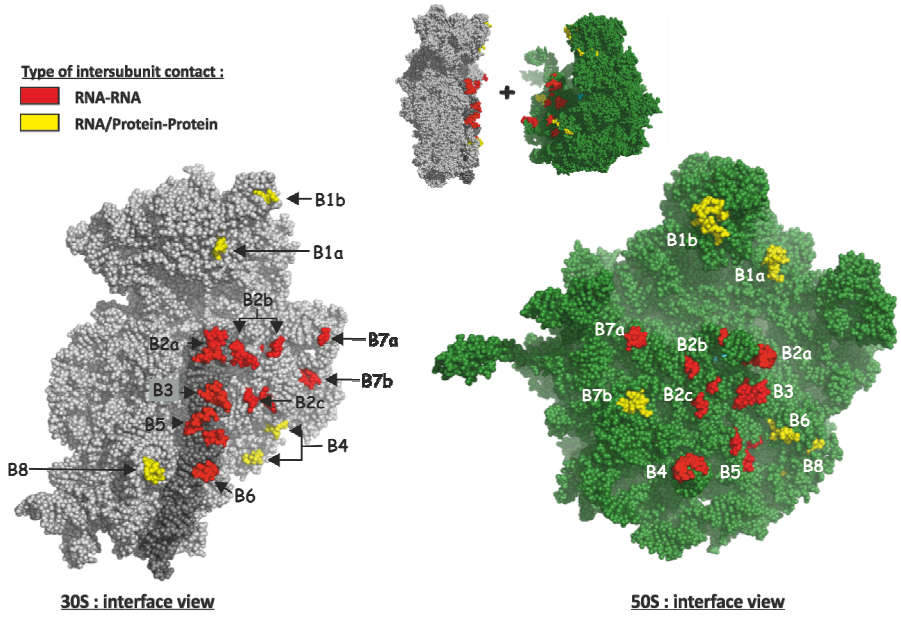

Figure 5. Intersubunit contacts in a prokaryotic 70S ribosome. Intersubunit bridges consisting of RNA-RNA contacts are shown in red, bridges that have a protein component are shown in yellow. The coordinates of the *T.thermophilus* 70S ribosome are from Jenner and coworkers (Jenner *et al.* 2010a; PDB IDs **3I8F** and **3I8G**).

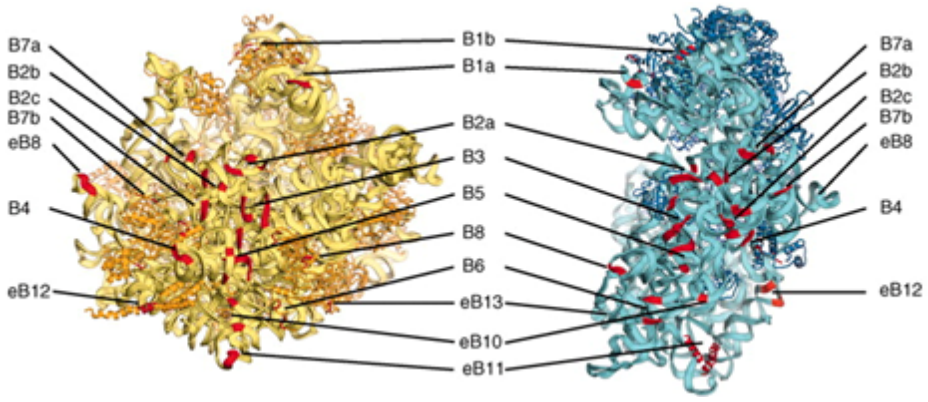

Figure 6. Intersubunit contacts in a eukaryotic 80S ribosome. Intersubunit contacts are shown in red. The prefix "e-" denotes eukaryote specific bridges. Adapted from Ben-Shem *et al.* 2010.

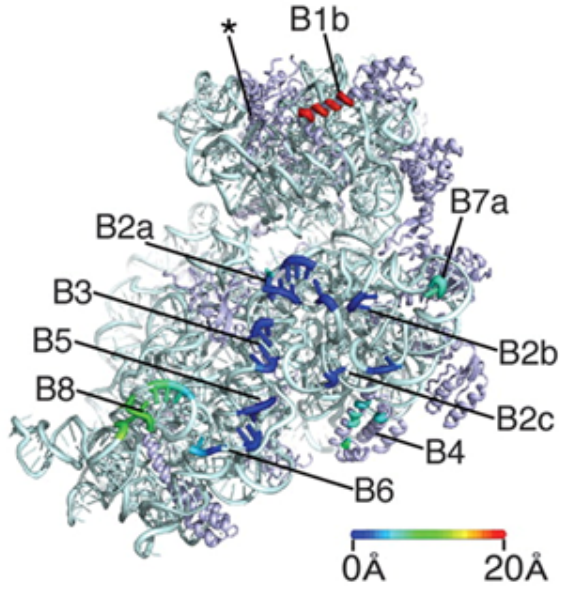

Figure 7. Structural dynamics of the intersubunit bridges in *E.coli.* This figure shows the 30S parts of the bridges. The bridging contacts are color-coded according to their mobility (in Å) as deduced from a comparison of different crystal structures. The asterisk denotes the location of bridge B1a that is invisible in the current structure due to disorder in H38 of 23S rRNA. Adapted from Zhang *et al.* 2009.

Table 1. Molecular interactions in the intersubunit bridges of the 70S ribosome. h- helix; S- small subunit protein; L- large subunit protein, numbers – positions in 16S or 23S rRNA, p-p: protein-protein; R-R: RNA-RNA; R-p: RNA-protein. Data are from Yusupov *et al.* 2001.

bridge	type	30S component	50S component
B1a	p-R	S13	h38/881–883
	p-R	S19	h38/884–885,891–892
B1b	p-p	S13	L5
	p-p	S13	L5
B2a	R-R	h44/1409–1410, 1495–1496	h69/1912–1913
B2b	R-R	h24/783–786,791–792	h67/1836–1838
	R-R	h24/783–786,791–792	h69/1922–1923,1928–1929
	R-R	h45/1514–1516	h67,h71/1833–1834,1932–1933
B2c	R-R	h24/770–774	h66,h67/1793–1794,1830–1833
	R-R	h27/900–901	h67/1832
B3	R-R	h44/1483–1486	h71/1948–1949,1960–1962
B4	R-R	h20/762	h34/715–716
	p-R	S15	h34/714–716
B5	R-R	h44/1418–1419	h64/1718–1719
	R-R	h44/1420–1421	h71/1950–1951
	R-p	h44/1421–1423	L14
	R-R	h44/1473–1476	h62/1689–1690,1702–1703
	R-R	h44/1473–1476	h64/1988–1989
B6	R-R	h44/1429–1431	h62/1704–1705
	R-p	h44/1432–1433,1463–1465	L19
B7a	R-R	h23/698–699,701–703	h68/1847–1848,1896–1897
B7b	R-p	h23/712–714	L2
	R-p	h24/773–776	L2
	R-p	h22/669–671	L2
B8	R-p	h14/339–340,345–346	L14
	R-p	h14/345–346	L19

Is there any experimental evidence to support the idea of the different dynamics of the intersubunit bridges during ribosomal movements? Although it has so far not been possible to directly visualize the process of translation in its entirety, X-ray and cryo-EM studies have provided snapshots of the structures of the ribosome in conformations that are believed to resemble some of the macrostates/intermediates on the actual translation pathway. In those studies, the interaction patterns in the "protein bridges" in the "head" region of the small subunit – B1a and B1b – have indeed been observed to rearrange between the different ribosomal states. Thus, in bridge B1b protein L5 from the central protuberance of the 50S subunit contacts the N-terminal part of protein S13 in the "head" of the 30S prior to the ratchet-movement of the subunits (Gao *et al.* 2003; Zhang *et al.* 2009; Shasmal *et al.* 2010) (Figure 8). During the ratchet and 30S head swiveling L5 shifts its interaction to the central α–helix of S13 (Zhang *et al.* 2009)(Figure 8). This remodeling of the interaction between L5 and S13 helps to retain the B1b bridge during the 30S "head" swiveling towards the E-site that is presumably required for the tRNA-mRNA complex translocation on the 30S (Zhang *et al.* 2009; Gao *et al.* 2003). Analogously, in bridge B1a the 50S component of the bridge – 23S rRNA helix 38 (the "A-site finger") – has been shown to change its interaction from protein S13 in the preratcheting ribosome to protein S19 during the ratchet-movement (Valle *et al.* 2003b). A similar "swinging" of interaction partners occurs in bridge B1a of the yeast 80S ribosome (Spahn *et al.* 2004). However, the remodeling of the interactions during ribosomal movements is not entirely confined to the "protein bridges". This is shown by the example of bridge B7a (Zhang *et al.* 2009). Although an "RNA – RNA" bridge, B7a is located rather peripherally on the "platform" of the 30S subunit (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). It is broken during the rotation of the subunits when the nucleotide A702 of 16S rRNA moves out from its preratcheting location in the minor groove of helix 68 of 23S rRNA (Pulk *et al.* 2006; Zhang *et al.* 2009). Similarly, the high level of solvation between the contacting minor grooves of helix 44 in 16S rRNA and H62 in 23S rRNA at bridge B6 at the base of the 30S body (Schuwirth *et al.* 2005) is suggestive of the capability of this bridge to undergo rearrangement during intersubunit rotation (Schuwirth *et al.* 2005).

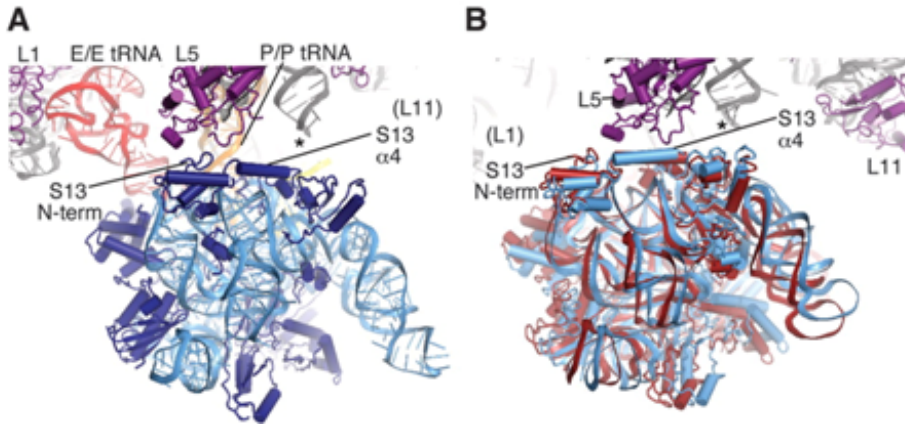

Figure 8. Rearrangements in bridge B1b during intersubunit movement. Panel A – bridge B1a in the pre-ratcheted state R_1. Panel B- bridge B1b in the intermediate states R_2 (light blue) and R_F (red) of ratcheting. Notice the different placement of L5 relative to S13 in panels A and B. Adapted from Zhang *et al.* 2009.

The dynamism of the bridges in the 30S "head" region may be important for the ordered movement of tRNAs during translocation (and thereby maintaining the correctness of the reading frame) since components of the B1a and B1b bridges are seen to interact with the elbow regions of the A- and P-site tRNAs (Yusupov *et al.* 2001; Jenner *et al.* 2010). The contact between the C-terminus of protein S13 from bridge B1b and the P-site tRNA is retained during ratcheting and may serve to stabilize the tRNA in the P/E hybrid site (Zhang *et al.* 2009; Agirrezabala *et al.* 2008). Similarly, in bridge B1a the 23S rRNA helix 38 retains its contact with the elbow of the A-site tRNA during the latter's movement into the A/P hybrid site (Agirrezabala *et al.* 2008). However, the functional importance of the dynamics of the bridges B1a and B1b is not confined to the translocation step but is also used in tRNA selection during the proofreading reaction (Jenner *et al.* 2010a). Here, binding of a cognate tRNA to the A-site induced a complex set of changes in the interactions in bridges B1a and B1b as well as between components of those bridges and the A- and P-site tRNAs (Jenner *et al.* 2010a). Those conformational changes were subsequently transferred to the components of the peptidyl transferase center and led to an overall tightening of the A-site around the cognate tRNA (Jenner *et al.* 2010a). Many of those conformational changes were lacking in the case of near-cognate tRNA, shedding light on the mechanism of tRNA selection in proofreading and highlighting the role of intersubunit contacts in the process (Jenner *et al.* 2010a).

In contrast to the peripheral bridges that display significant dynamism, the centrally located "RNA-RNA" bridges B2a, B2b, B2c, B3 and B5 along helix

44 of 16S rRNA exhibited only minor local rearrangements in a landmark cryo-EM study of the *E.coli* 70S ribosome (Gao *et al.* 2003). The central "RNA-RNA" bridges (B2a, B2b, B2c, B2d, B3 and B5) were also preserved during the ratchet-like movement of the yeast 80S ribosome (Spahn *et al.* 2004). These observations support the idea of the static nature of the central bridges. The importance of the integrity of the central bridges is indicated by the observation that the ribosome even resorts to a temporal bending of helix 44 of 16S rRNA instead of allowing the intersubunit contacts along this helix to break during the ratcheting movement (Van Loock *et al.* 2000; Schuwirth *et al.* 2005; Zhang *et al.* 2009). The importance of the conformation of helix 44 for intersubunit contacts is supported by the results of Tobin *et al.* 2010 where deletion of protein S20 – known to be important for the correct positioning of helix 44 – impaired the association of the ribosomal subunits (Tobin *et al.* 2010). Importantly, when the orientations of the subunits are compared in the ratcheted and nonratcheted states the axis of rotation is found to lie in the region between the same B3 and B5a bridges (Valle *et al.* 2003b). This region is the conformationally least mobile part of the 30S subunit (Zhang *et al.* 2009). Therefore, during the ratcheting movement the relative stability of the central bridges may confer them the role of a spring that keeps the skewed subunits together and forces them to move back to the original orientation after the tRNA-mRNA complex has been fully translocated (Gao *et al.* 2003). It is also noteworthy that the centrally located bridges tend to be evolutionarily more conserved than the bridges in the periphery of the interface. Thus, in a study based on comparative sequence analysis of ribosomal RNA, bridges B2a, B2c and B3 – located close to the decoding centre and making contacts to the A- and P-site tRNAs (Yusupov *et al.* 2001; Belanger *et al.* 2002; Schuwirth *et al.* 2005; Selmer *et al.* 2006) – were found to be present in ribosomes from all three phylogenetic domains as wells as in the organellar (mitochondrial and chloroplast) ribosomes (Mears *et al.* 2002)(Figure 9). In contrast, a lesser degree of conservation was seen in the case of the more peripherally located bridges like B1a, B5, B6 or B7a that were absent from the organellar ribosomes (Mears *et al.* 2002) (Figure 9). In the light of those results, it is tempting to speculate that B2a, B2c and B3 – all of them "RNA-RNA" bridges – may have formed a set of "core" interactions that were present in the ribosome during early stages of evolution.

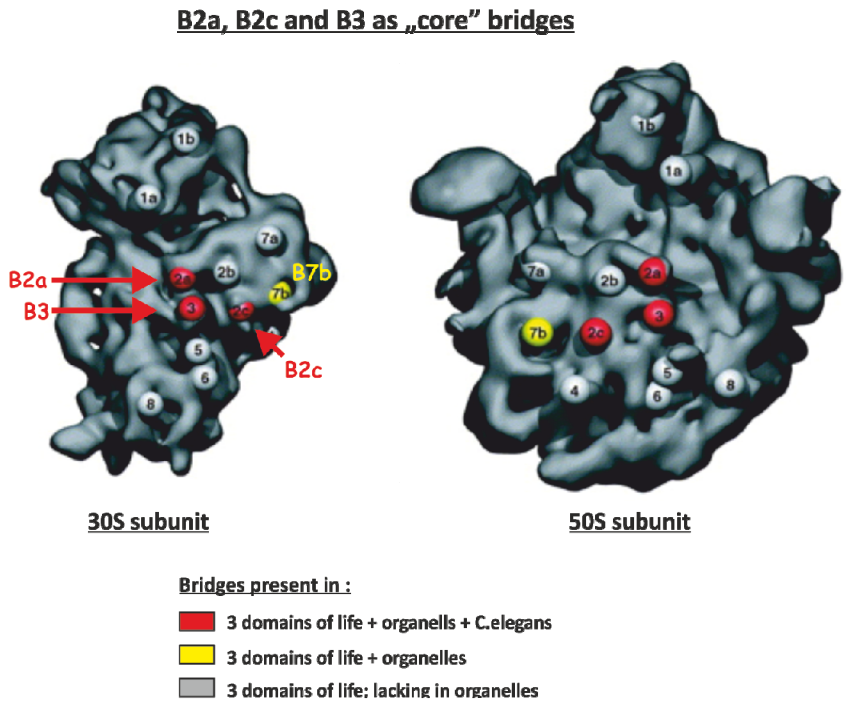

Figure 9. Phylogenetic analysis of the ribosomal intersubunit bridges. The drawing is adapted with modifications from Mears *et al.* 2002.

In addition to their putative role in regulating the intersubunit movement some of the bridges may act as signal transmitters between the subunit interface and the peptidyl transferase centre in the large subunit. This idea is suggested by the ribosomal location of the lateral arm of domain IV of 23S rRNA that contains the components of bridges B2a, B2b, B2c, B3 and B7a (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). This region of 23S rRNA lies beneath the peptidyl transferase centre and forms the floor of the cavity where the acceptor ends of tRNAs are located (Yusupov *et al.* 2001). A direct interaction between the lateral arm of domain IV and parts of the peptidyl transferase center can be seen at helix 71 of 23S rRNA that forms the intersubunit bridge B3 with helix 44 of 16S rRNA. Here, the adenines 1952 and 1953 of the loop of helix 71 are able to form type I and II A-minor interactions with the stem region of the A-loop (helix 92) of the peptidyl transferase centre. It has therefore been speculated that the conformational changes that are induced in 16S rRNA upon tRNA-mRNA interaction in the decoding centre may be transmitted to the peptidyl transferase region via the lateral arm of domain IV of 23S rRNA (Yusupov *et al.* 2001).

The importance of different bridges for the stability of the ribosome has been addressed biochemically and genetically. Thus, using a modification interference strategy a number of nucleotides in the bridge regions of the 16S and

23S rRNA were shown to be important for the 70S formation in vitro (Maiväli and Remme 2004; Pulk *et al.* 2006). Many of the16S rRNA nucleotides whose modification by kethoxal or dimethyl sulfate resulted in the inhibition of subunit association in the study of Pulk *et al.* were located in helix 44 of 16S rRNA – emphasizing the importance of this structure for ribosomal stability (Pulk *et al.* 2006). Similarly, in their mutagenesis study Liiv and O'Connor identified various base replacements in the 23S rRNA components of bridges B2b, B2c, B3, B4, B5 and B7a that impaired subunit association in vivo and in vitro, although the extent of the association defect was different for different bridges. Many of the base replacements in those bridge regions also affected cellular growth rate and increased the level of nonsense suppression (Liiv and O'Connor 2006). Additionally, mutations in the B3 and B7b bridge regions decreased the stringency of the start codon selection (Sun *et al.* 2010). The latter facts – namely, that mutations in bridges (i.e. B2b, B2c, B3, B5 and B7a, B7b) that are out of direct contact range with the tRNAs or other ligands still affect initiator tRNA binding to the P-site and stop codon readthrough underlines the importance of an appropriate positioning of the subunits within the translating ribosome for translational accuracy. However, despite visible defects in subunit association and translational accuracy a majority of the rRNA base changes in the bridge regions had modest effects on cellular viability in the rich medium used in the studies of Liiv and O'Connor (Liiv and O'Connor 2006) and Sun and colleagues (Sun *et al.* 2010). It therefore seems that under favorable growth conditions at least, i) other components of the translational apparatus, especially the tRNAs that span the two subunits also contribute to the maintenance of the subunit association and that ii) disruption of one bridge may in some cases be tolerated provided that other bridges remain intact (Liiv and O'Connor 2006; Sun *et al.* 2010). More intriguingly, there are indications that the negative effect of disrupting one bridge may be suppressed by changes in another bridge(s). Thus, in a study by O'Connor the lethal phenotype due to deleting an A from helix 69 of bridge B2a was partly rescued by a mutation in a distant bridge B6 (O'Connor 2007). The cross-talk between distantly located regions – including intersubunit bridges – on the subunit interface is also exemplified by the results of the mutagenesis studies of Maisinier-Patin and coworkers (Maisnier-Patin *et al.* 2007), McClory and coworkers (McClory *et al.* 2010) and Sun and coworkers (Sun *et al.* 2010). In those three studies, the researchers identified mutations in the B5, B6 and B8 bridge regions that supressed the hyperaccurate and growth-restrictive phenotype conferred by the K42N mutation in protein S12 (Maisinier-Patin *et al.* 2007; Sun *et al.* 2010). As the B5, B6 and B8 bridges are distant to the decoding centre where the K42N mutation is located, their effect on the translational accuracy must be caused by alterations in the conformation of the 30S subunit and/or its interaction with the EF-Tu • GTP • aa-tRNA ternary complex (McClory *et al.* 2010).

The involvement of intersubunit bridges in the part reactions of translation that require the mobility of the subunits – the process of mRNA-tRNA

translocation in particular – is supported by a number of biochemical studies. Of particular importance for understanding the general mechanism of ribosomal translocation and its evolution are the experiments targeting the "head" region bridges B1a and B1b. In one set of experiments carried out by Rachel Green and her coworkers, the interactions in B1a and/or B1b were modified by treating the small subunit with thiol-specific reagents like para-chloromercurybenzoate (known to specifically react with the cysteins of S13) or omitting the S13 protein from the in vitro reconstituted ribosomes. Modification/depletion of S13 regularly resulted in a significantly compromised subunit association (Southworth *et al.* 2002; Cuckras *et al.* 2003) as would be expected in the case of an intersubunit bridge. However, a more interesting observation from those studies was that the same modifications/depletion of S13 strongly promoted the EF-G-independent (i.e. "spontaneous") translocation with the modified ribosomes as compared to the unmodified ones (Southworth *et al.* 2002; Cukras *et al.* 2003). In addition, ribosomes containing variants of S13 exhibited higher rates of –1/+1 frameshift and nonsense suppression (Cukras and Green 2005). Contrary to the effects of the modifications in protein S13 that is involved in both B1a and B1b bridges, deletions in helix 38 of 23S rRNA that forms bridge B1a led to an increased rate of EF-G-dependent translocation while not significantly affecting subunit association (Komoda *et al.* 2006). Insensitivity of subunit association to the disruption of bridge B1a was also observed by Liiv and O'Connor (Liiv and O'Connor 2006) and Sergiev and coworkers (Sergiev *et al.* 2005). The latter results indicate that at least with some bridges the primary role of the bridge is not in assisting the association of the subunits but may lie in subsequent steps of translation. The results of the abovementioned biochemical studies are in line with the X-ray and cryo-EM data about the dynamics of bridges B1a and B1b during subunit ratcheting as well as the importance of their interaction with the P-site tRNA in maintaining the ribosomal reading frame (Yusupov *et al.* 2001; Gao *et al.* 2003; Zhang *et al.* 2009). Perhaps even more importantly, they demonstrate that some of the intersubunit bridges may be involved in the regulation or fine-tuning of the directional movements during ribosomal translocation. As the "regulatory" bridges like B1a and B1b are those that have a protein(s) as their component they may not have been present in the proto-ribosome of the RNA-world but have instead arisen later in evolution to fine-tune the factor-dependent part reactions of translation and/or increase the ribosomal processivity (Cukras *et al.* 2003).

In the central regions of the subunit interface, the effects of base replacements in an RNA – RNA bridge B3 were among the severest observed by Liiv and O'Connor. These effects were not confined to subunit association but also included a significantly increased UGA and UAG readthrough (Liiv and O'Connor 2006). In bridge B3 two sheared G – A base pairs in helix 44 of 16S rRNA form a type I A minor interaction with two G – C base pairs in helix 71 of 23S rRNA (Schuwirth *et al.* 2005). Since bridge B3 contains the largest RNA

– RNA minor groove surface complementarity among the interface contacts, its stability may preclude any large-scale rearrangements in it during translocation. Coupled to its location close to the axis of intersubunit rotation (Valle *et al.* 2003b; Spahn *et al.* 2004), the presumably strong interaction in bridge B3 makes it a good candidate for the spring-like regulator of subunit rotation. It is not surprising then that disrupting interactions in B3 severely affects subunit association and subsequent steps of translation as seen by Liiv and O' Connor and Sun *et al.* 2010. Further supporting the importance of the centrally located RNA-RNA bridges for the stability of subunit association, mutations in the B2b and B2c bridges had a strong effect on subunit association and translational accuray (Belanger *et al.* 2004; Liiv and O'Connor 2006). In contrast to this, mutations in the "protein bridge" B4 had a minor effect on subunit association in in vitro and in vivo and did not significantly affect cellular viability (Liiv and O'Connor 2006).

Along with B3 and B2b-c, one of the intersubunit contacts in the central region of the interface is contributed by bridge B2a (Mitchell *et al.* 1992; Merryman *et al.* 1999; Yusupov *et al.* 2001; Schuwirth *et al.* 2005) (Figure 5). B2a covers the largest fraction of the surface area (about 600 $Å^2$) occupied by any of the intersubunit bridges (Cate *et al.* 1999) and is conserved in all three domains of life (Mears *et al.* 2002). The bridge occurs near the mRNA decoding site on the 30S subunit (Yusupov *et al.* 2001; Schuwirth *et al.* 2005) and some of its positions directly participate in the decoding process (Jenner *et al.* 2010a). Bridge B2a is formed by the interaction of the helix-loop 69 (H69) of 23S rRNA and the top of helix 44 (h44) of 16S rRNA (Figure 10). The bridging interaction involves residues in the minor groove of helix 44 and residues at the stem-loop junction of helix 69 (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). In the 3.5 Å crystal structure of the *E.coli* 70S ribosome 23S rRNA nucleotides A1912 and A1919 in H69 are seen to lie within H-bonding distance to the nucleotides C1407, G1494, U1495 and G1517 in 16S rRNA (Figure 10). Additionally, the 2´-OH group of A1913 was found to be bridged to the nonbonding phosphate oxygens of the 16S A1492 and A1493 via a Mg^{2+} ion in the 2.8 Å crystal structure of *T. termophilus* 70S ribosome (Selmer *et al.* 2006). The crystal structures indicate that the interaction of the 23S rRNA nucleotides A1912 and A1919 with the nucleotides in 16S rRNA is facilitated by a network of intrahelical interactions in H69 (Schuwirth *et al.* 2005). Thus, an interaction between Ψ1911, A1918 and A1919 orients the A1919 in the minor groove of h44 to interact with U1495 and G1517 in 16S rRNA (Schuwirth *et al.* 2005). A similar interaction of A1912 with A1918 and Ψ1917 facilitates the interaction of A1912 with the 16S rRNA nucleotides C1407 and G1494.

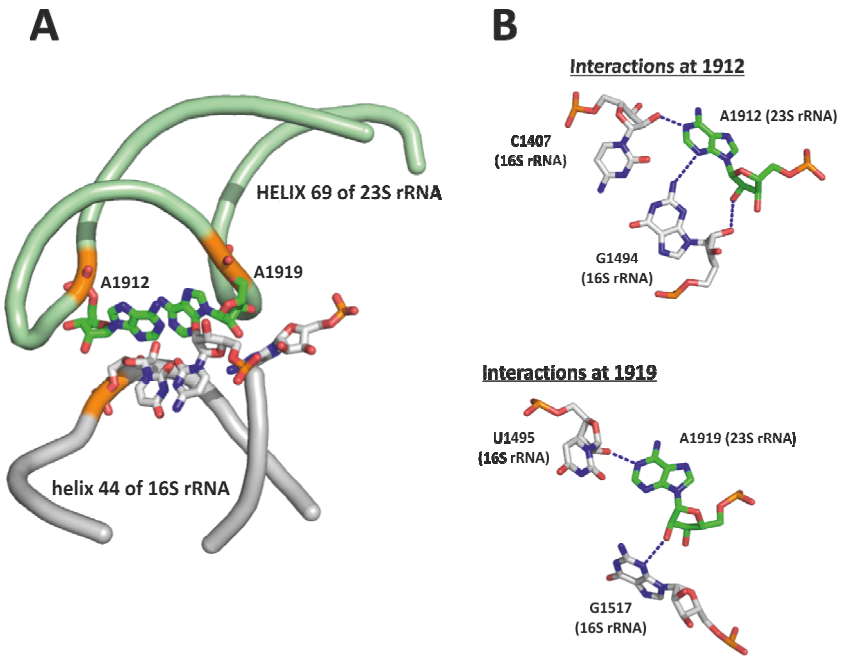

Figure 10. Molecular contacts in bridge B2a. Panel A, placement of helix 69 of 23S rRNA with respect to helix 44 of 16S rRNA. Nucleotides in involved in interhelical contacts are shown as "sticks". Panel B, close-up view of the interactions in bridge B2a. The coordinates are from Schuwirth and colleagues (Schuwirth *et al.* 2005; PDB IDs **2AW4** and **2AVY**) and were rendered with PyMol.

The importance of specific positions in H69 and h44 for the formation of bridge B2a is confirmed by modification interference experiments. Here, methylation of the N1s of A1912 and A1918 in 23S rRNA (Pulk *et al.* 2006) and CMCT modification of the N3 of U1495 in 16S rRNA (Maiväli and Remme 2006) severely impaired the association of the ribosomal subunits *in vitro*. Chemical modification of specific bases in rRNA has also been used to analyze the kinetics of intersubunit bridge formation during subunit association. The changes in the chemical reactivities of the bases were observed to occur with different rates and proceeded more rapidly in regions involved in decoding and ribosome stability (Hennelly *et al.* 2005). Notably, positions in helix 44 involved in bridge B2a were among the first to become protected from chemical modification by the binding of the 50S subunit (Hennelly *et al.* 2005). This points to B2a as one of the earliest intersubunit contacts and is in agreement with the central role of B2a in subunit association. Hydroxyl radical footprinting studies indicate that H69 contacts regions on the 30S subunit which overlap with the binding site of the C-terminal domain of translation initiation factor 3 (IF3) (Dallas and Noller 2001). This suggests that H69 may be

competing with IF3 in binding to the 30S complex during translation initiation (McCutcheon *et al.* 1998; Dallas and Noller 2001).

In the crystal structures of the ribosome-tRNA complexes bridge B2a is seen to form part of both the A- and the P-site (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). In addition, in the 2.8 Å crystal structure of *T. termophilus* 70S ribosome A1913 in the loop-part of H69 interacts directly with the A-site tRNA nucleotides 37 and 38 (Selmer *et al.* 2006). The simultaneous interaction of bridge B2a with the decoding site and the A-site tRNA led to speculations about its role in the accuracy of protein synthesis. According to this view H69 was supposed to be involved in the transmission of the signal of correct codon-anticodon interaction to the 50S subunit.Those speculations are substantiated by a more recent crystal structure of the *T. thermophilus* 70S ribosome where residues of helix 69 are seen to be directly involved in the decoding process (Jenner *et al.* 2010a). For instance, the stacking of A1913 of helix 69 with A1493 of 16S rRNA serves to keep A1493 within helix 44 when the ribosomal A-site is empty (Jenner *et al.* 2010a). This configuration of the A1913-A1493 pair is stabilized by the interaction between A1912 of helix 69 and G1494 of 16S rRNA (Jenner *et al.* 2010a). Those interactions are abolished upon tRNA binding to the A-site when A1493 and A1913 assume new conformations to interact with residues from the anticodon region of tRNA (Jenner *et al.* 2010a). The change in the orientation of A1493 and A1913 occurs irrespective of the nature of the incoming anticodon – i. e. cognate or near-cogante (Jenner *et al.* 2010a). However, the binding of cognate as opposed to near cognate tRNA further leads to the formation of an intricate network that involves residues from tRNA, 16S and 23S rRNAs, the small subunit protein S12 and a number of Mg^{2+} ions (Jenner *et al.* 2010a). This set of specific interactions stabilizes the binding of the cognate tRNA and thereby likely induces the conformational change throughout the A-site that serves to accommodate the acceptor end of the tRNA and thereby ultimately leads to the peptidyl transferase reaction (Jenner *et al.* 2010a). Taken together, these data suggest a central role for helix 69 in the decoding process and ribosomal accuracy in general. The involvement of helix 69 and bridge B2a in ribosomal accuracy has been analyzed in a number of biochemical and genetic studies. Overall, the results of those studies are supprotive of an important role of helix 69 for translational accuracy. Thus, O'Connor and Dahlberg isolated mutations in the loop-part of helix 69 that promoted readthrough of stop codons and caused an increase in the level of frameshifting (O'Connor and Dahlberg 1995). Similar results were obtained by Hirabayashi and coworkers who randomized the sequence of helix 69 and sought for variants capable of supporting cell growth (Hirabayashi *et al.* 2006). Here, all of the viable variants of H69 displayed a more or less increased level of +1/– 1 frameshifting as well as UAG and UGA readthrough (Hirabayashi *et al.* 2006). An increased level of –1 frameshifting and UGA readthrough was also observed by O'Connor in ribosomes containing the Δ1916A mutation and compensatory mutations in 16S rRNA (O'Connor 2007). However, it must be

born in mind that the mechanisms of frameshift, stop-codon readthrough and missense errors may be different. Hence, mutations in helix 69 may not be expected to have a uniform effect on translational accuracy. This is exemplified by the results of Ali and coworkers who deleted the entire helix 69 (nucleotides 1906 – 1931 of 23S rRNA) and analyzed the level of Leu misincorporation at the UUU codon by the ΔH69 variant ribosomes *in vitro* (Ali *et al.* 2006). Under all conditions tested the level of Leu misincorporation by the ΔH69 variants was equal to or even lower than with the WT ribosomes (Ali *et al.* 2006), casting doubt on the role of helix 69 in decoding. The fact these ΔH69 ribosomes were significantly compromised in the RF1-dependent peptide release may even provide an alternative explanation for the increased stop-codon readthrough observed in the earlier studies of helix 69 mutants since defects in release factor functioning are known to promote nonsense supression. In contrast to the results of Ali and coworkers (Ali *et al.* 2006), O'Connor recently identified a number of single and multiple base substitutions in helix 69 that moderately increased the level of missense errors in a mixed ribosomal population *in vivo* (O'Connor 2009). Those results provide evidence favoring a role for helix 69 in ensuring the translational fidelity in the elongation phase.

In the co-crystal structures of ribosomes with various translation factors bridge B2a has been seen interacting with parts of IF2, eEF2 (EF-G), RF2, RF3 and RRF (Klaholz *et al.* 2003; Agrawal *et al.* 2004; Klaholz *et al.* 2004; Allen *et al.* 2005; Myasnikov *et al.* 2005; Wilson *et al.* 2005; Gao *et al.* 2007; Pai *et al.* 2008; Schmeing *et al.* 2009). These interactions suggest that bridge B2a is involved in regulating the activity of the translation factors when they are bound to the 70S ribosome during protein synthesis. This idea is supported by recent biochemical studies where different mutations in helix 69 affected steps of protein synthesis from initiation to termination and recycling (Liiv *et al.* 2005; Ali *et al.* 2006; Hirabayashi *et al.* 2006; Kipper *et al.* 2009). One of the structurally and biochemically best characterized interactions of B2a is with class I polypeptide release factors RF1 and RF2. In the cocrystal structures of *T.termophilus* 70S ribosomes with RF1 and RF2 helix 69 is seen to be located between domains II and III of both factors (Figure 11), presumably helping to stabilize the mutual orientation of those domains in the ribosome-bound release factor (Weixlbaumer *et al.* 2008; Laurberg *et al.* 2008; Korostelev *et al.* 2010). In this state domain III is positioned away from the rest of the factor and lies close to the peptidyl transferase center where its conserved GGQ motif is in position to assist the ribosome in the hydrolysis of the peptidyl-tRNA (Laurberg *et al.* 2008; Korostelev *et al.* 2008; Weixlbaumer *et al.* 2008; Jin *et al.* 2010; Korostelev *et al.* 2010) (Figure 11). Particularly important for the domain rearrangement in RF1 and RF2 is the interaction of A1913 of helix 69 with the decoding centre nucleotide A1493 of 16S rRNA (Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010). During elonagtion A1913 of helix 69 interacts with the A-site tRNA (Selmer *et al.* 2006). Binding of the release factor to the ribosomal A-site forces A1493 of 16S rRNA out of the

decoding center and into a stacking interaction with A1913 of helix 69 (Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010). The formation of this interaction has been suggested to be important for the domain rearrangement in RF1 and RF2 (Laurberg *et al.* 2008; Korostelev *et al.* 2010). Consistent with the structural data on the helix 69–RF interactions, mutations in helix 69 and/or in RF1 strongly impaired the RF1–mediated peptide release (Ali *et al.* 2006; Korostelev *et al.* 2010) and a lack of pseudouridulation in helix 69 positions 1911, 1915 and 1917 caused a defect in RF2-mediated peptide release (Ejby *et al.* 2007).

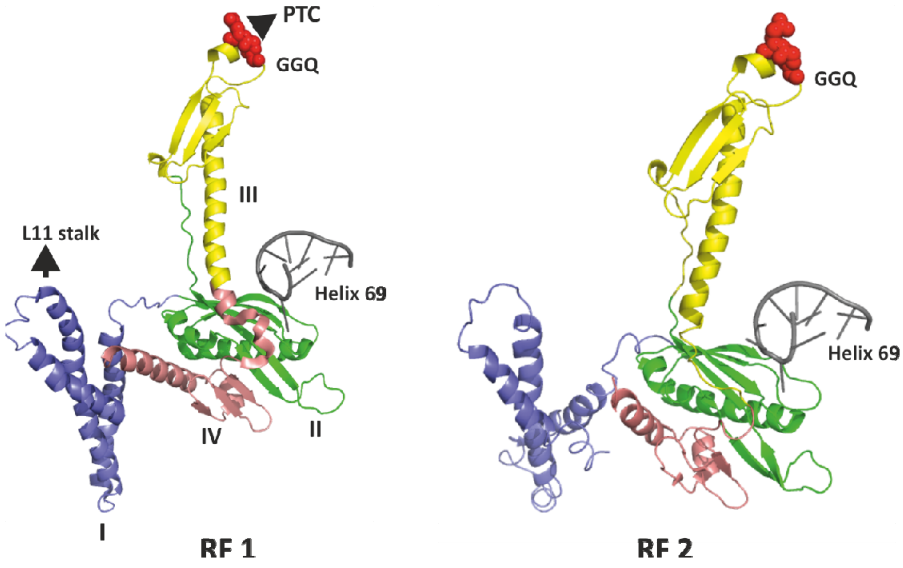

Figure 11. Structure of class I prokaryotic polypeptide release factors RF1 and RF2 on the ribosome. RF1 and RF2 are shown as would be seen from the ribosomal P-site. GGQ denotes the universally conserved tripeptide motif involved in peptidyl-tRNA hydrolysis. The arrow denotes an approximate location of the L11 stalk that interacts with domain I of RF1 and RF2. The structure of RF1 is from Korostelev and colleagues (Korostelev *et al.* 2008; PDB IDs **3D5A** and **3D5B**) and the structure of RF2 is from Weixlbaumer and colleagues (Weixlbaumer *et al.* 2008; PDB IDs **2WHI** and **2WH2**).

1.4. Pseudouridines in 23S rRNA helix 69

In cells rRNA is synthesized as one long transcript by RNA polymerase using adenine, guanine, cytosine and uracil as the building blocks. However, nucleotides in the RNA of the mature ribosome contain different types of chemical modifications that have been added at various stages of rRNA processing and packing into the ribosomal particle. Currently, over 100 different types of chemical modifications varying in number and location are known in rRNA. The modifications are found in the RNAs of both subunits in all three kingdoms of Life (Ofengand and Bakin 1997; Ofengand 2002). Pseudouridylation and base/ribose methylation represent the two major types of nucleotide modification in rRNA (Piekna-Przybylska *et al.* 2007; Piekna-Przybylska *et al.* 2008a). When considered as a single modification (i.e. differently from methylation that may involve both the base and the sugar of a nucleotide) pseudouridine (Ψ) is the most frequent base modification in rRNA (Ofengand and Fournier 1998). Besides rRNA, pseudouridines are also found in tRNA and small nuclear RNAs (Björk 1995; Kiss 2001). The number of pseudouridines and other nucleotide modifications is higher in eukaryotes than in bacteria and archea (Ofengand and Fournier 1998). Thus, while the rRNA of the eubacterium *E.coli* contains a total of 35 modified nucleotides out of which 69% are methylations and 31% pseudouridylations (Piekna-Przybylska *et al.* 2007) there are around 100 rRNA modifications in yeast and over 200 in vertebrates (Maden 1990; Piekna-Przybylska *et al.* 2008a). Modelling of the modified nucleotides into the structures of the ribosomes from *E.coli*, *T.termophilus* and *S.cerevisiae* shows that they are located in the functionally important regions of the ribosome such as the peptidyl transferase center in the large subunit, the decoding center in the small subunit and regions involved in subunit association (Brimacombe *et al.* 1993; Decatur and Fournier 2002; Hansen *et al.* 2002; Ofengand *et al.* 2001; Ofengand 2002; Kirpekar *et al.* 2005; Mengel-Jørgensen *et al.* 2006; Chow *et al.* 2007; Piekna-Przybylska *et al.* 2008b). Interestingly, pseudouridylation and methylation were seen to cluster to the same regions in the large subunit rRNA in *E.coli*, yeast and humans (Ofengand and Bakin 1997).

Pseudouridine was the first modified nucleotide discovered in RNA (Davis and Allen 1957; Cohn 1960). Chemically, pseudouridine (5-β-D-ribofurano-syluracil) is an isomer of uridine where the C-N glycosylic linkage between the ribose and the base moieties is replaced with a C-C glycosylic linkage (Cohn 1960) (Figure 12). Although Ψ adopts a *syn* conformation in the free nucleotide, *anti* conformation of Ψ is predominant in RNA (Neumann *et al.* 1980; Roy *et al.* 1984; Griffey *et al.* 1985; Davis and Poulter 1991; Arnez and Steitz 1994; Davis 1995; Davis 1998). Due to the C-C-glycosylic bond, the N-1 position of Ψ is free to act as an hydrogen bond acceptor, giving Ψ a greater hydrogen bonding capability than is possible for U (Griffey *et al.* 1985; Charette

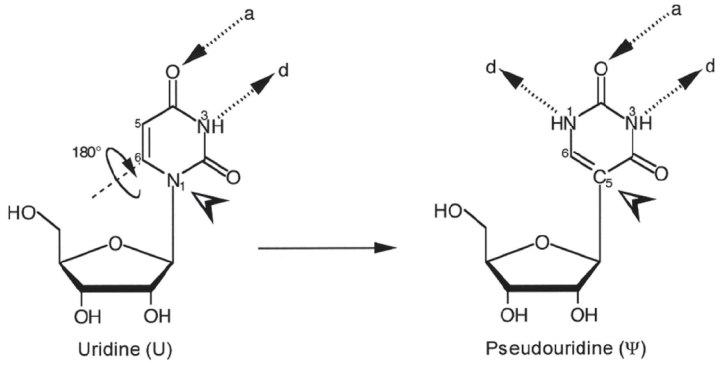

Figure 12. Uridine and pseudouridine. In pseudouridine the uracil and ribose moieties are linked via a C1'- C5 glycosylic bond. As a result of this linkage the N1 of uracil in Ψ can participate in hydrogen bond interactions. "d" and "a" denote hydrogen bond donor and acceptor, respectively. Adapted from Charette and Gray 2000.

and Gray 2000) (Figure 12 and 13). The involvement of the N1-H of Ψ in stable hydrogen bonds has been demonstrated by NMR studies on ^{15}N-labeled tRNAs and synthetic RNA oligonucleotides (Griffey *et al.* 1985; Davies and Poulter 1991; Desaulniers *et al.* 2008). Both NMR, X-ray and molecular dynamics simulation studies indicate that the N1-H of Ψ forms a water-mediated hydrogen bond to the consecutive 5'-oxygens in the sugar-phosphate backbone (Griffey *et al.* 1985; Davies and Poulter 1991; Arnez and Steitz 1994; Auffinger and Westhof 1998) (Figure 13). Such a water-mediated base-to-backbone interaction of Ψ may serve to rigidify the surrounding RNA and therefore contribute to the thermodynamic stability of the structural element embedding the Ψ (Auffinger and Westhof 1998). Incidentally, this bidentate base-to-backbone interaction is probably responsible for locking Ψ in the *anti* conformation in RNA (Davis 1998; Charette and Gray 2000; Desaulniers *et al.* 2005)(Figure 13).

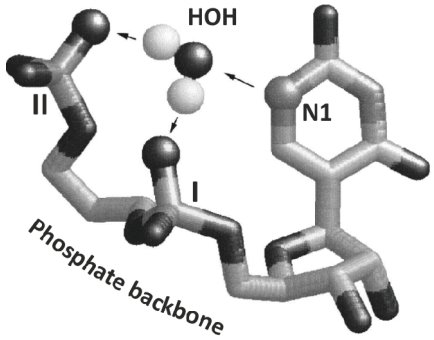

Figure 13. A water-mediated bifurcated hydrogen bond involving the N1 of pseudouridine. Adapted with modifications from Charette and Gray 2000.

Pseudouridines are formed post-transcriptionally by pseudouridine synthases (Gustafsson *et al.* 1996; Koonin 1996; Charette and Gray 2000; Ofengand 2002; Hamma and Ferré-D'Amaré 2006; Ishitani *et al.* 2008). The catalytic mechanism of the synthases is thought to involve breaking of the C-N glycosylic bond, covalent enzyme-substrate complex formation, rotation of the base by 180° and formation of the new C-C bond (Huang *et al.* 1998; Alian *et al.* 2009). Since the C-C glycosylic bond in pseudouridine is by 9 kcal/mol more stable than the C-N linkage in uridine (Preumont *et al.* 2008), pseudouridine synthases do not require the input of external energy for catalysis (Hamma and Ferré-D'Amaré 2006). In *E.coli* pseudouridines are degraded by a two-component system consisting of a pseudouridine kinase and a glycosidase (Preumont *et al.* 2008). The pseudouridine synthases of bacteria and eukaryotes differ by their mechanism of substrate recognition (Ofengand 2002). The eukaryotic synthases are ribonucleoprotein complexes where the catalytic activity resides in the protein component and the RNA component (called "guide") confers substrate specificity to the enzyme (Ofengand and Fournier 1998; Kiss 2001; Ofengand 2002). The mechanism of pseudouridine formation in archea is similar to eukaryotes, involving snoRNP-like ribonucleoprotein enzymes (Watanabe and Gray 2000; Tang *et al.* 2002; Ofengand 2002). In eubacteria the synthases are protein enzymes that contain both the catalytic activity for Ψ formation and specificity for a given rRNA region (Ofengand 2002). In *E.coli* there are 7 different Ψ-synthases that make a total of 12 Ψ-s in rRNA (Ofengand *et al.* 2001; Ofengand 2002). Those synthases belong to the RsuA and RluA families and contain a highly conserved aspartate residue which is thought to be important for catalysis (Koonin 1996; Gustafsson *et al.* 1996; Conrad *et al.* 1998; Raychaudhuri *et al.* 1999; Ofengand *et al.* 2001; Ofengand 2002). The RsuA-familiy contains synthases RluB, RluE, and RluF that each make a single pseudouridine at the positions 2605, 2457, and 2605 of 23S rRNA, respectively (Ofengand 2002). Synthases RluA, RluC and RluD belong to the RluA family (Ofengand 2002). *In vivo* every synthase pseudouridylates a specific region of rRNA so that no Ψ is made by more than one synthase, although one synthase can make more than one Ψ as shown by the examples of RluC (pseudouridylation at the positions 955, 2505, and 2580 of 23S rRNA) and RluD (pseudouridylation at 1911, 1915, and 1917) (Ofengand 2002).

The location of pseudouridines in the large subunit and their conservation in different organisms leads one to expect that they are involved in important ribosomal functions (by analogy to e.g. the universally conserved A1492 and A1493 of 16S rRNA that have a structurally and biochemically precisely defined role in the decoding process). Accordingly, pseudouridines have been implicated in various rRNA and translation-related processes like processing of the nascent rRNA transcript, rRNA folding and ribosome assembly as well as different part reactions of translation (Ofengand and Fournier 1998; Ofengand 2002). In the early 1990-s it was even hypothesized that the pseudouridines clustered in the peptidyl transferase region are directly involved in catalyzing

peptide bond formation (Lane *et al.* 1992). This view has now been discarded (Ofengand 2002).

By now, the roles of pseudouridines in different rRNA regions have been examined in various genetic and biochemical studies and some conclusions about the function (s) of pseudouridines can therefore be drawn. A general observation from studies where pseudouridine formation has been blocked in *E.coli* and yeast is that single or multiple depletions of pseudouridines – although reducing cellular growth rates in many cases – do not result in lethal phenotypes. This indicates that despite their conservation and location in the functionally important regions of the ribosome, pseudouridines are not absolutely required for any of the "core" functions of the ribosome such as peptide bond formation, decoding or translocation. The dispensability of the pseudouridines (and other rRNA modifications) is in line with the observation that reconstituted 30S or 50S subunits containing in vitro transcribed (and thus unmodified) rRNA retain a certain level of functional activity in vitro (Cunningham *et al.* 1993; Khaitovich *et al.* 1999b). However, the fact that the ribosome can perform some of its functions in the absence of pseudouridines does not exclude a stimulatory role of the pseudouridines for ribosomal activity. Indeed, depletion of pseudouridines has regularly been observed to adversely affect various ribosomal functions. Notable among these are ribosomal accuracy (Baudin-Baillieu *et al.* 2009) and A-site binding of tRNA (Baxter-Roshek *et al.* 2007) as well as the process of large subunit assembly and rRNA processing (Liang *et al.* 2007; Liang *et al.* 2009). Usually, the *in vivo* rate of protein synthesis (as measured by ^{35}S incorporation) was decreased by 10–60% upon pseudouridine deletion, the extent of reduction depending on the exact location of the pseudouridine and the number of deleted modifications (King *et al.* 2003; Liang *et al.* 2007; Piekna-Przybylska *et al.* 2008b). Although in the majority of cases deletion of pseudouridines compromised the ribosomal accuracy, deletion of certain pseudouridines from the helix 69 region of the yeast 25S rRNA rendered the ribosomes hyperaccurate (Baudin-Baillieu *et al.* 2009). In a similar vein, blocking the formation of a specific pseudouridine (Ψ2920) in the A-loop of the yeast 25S rRNA was observed to increase both the affinity of the aminoacyl-tRNA for the ribosomal A-site and the rate of peptide bond formation (Baxter-Roshek *et al.* 2007). The latter result that was interpreted as a widening of the A-site upon Ψ2920 depletion is so far the most explicit demonstration of a role for pseudouridines in a process related to peptide bond formation (Lane *et al.* 1992; Baxter-Roshek *et al.* 2007). The effects seen on multiple pseudouridine depletion were generally more pronounced than with single pseudouridine depletions. However, in some cases, depletion of one specific pseudouridine had a greater impact on ribosomal functioning than multiple deletions, indicating a combinatorial nature of the effects of at least some modifications (Baudin-Baillieu *et al.* 2008; Piekna-Przybylska *et al.* 2008b). Although the involvement of pseudouridines in ribosomal functioning seems now to be biochemically established, then differently from e.g. the case

of A1492/1493 of 16S rRNA a situation has not been observed where a single pseudouridine forms a specific molecular interaction whose presence/absence could be linked to the occurrence/nonoccurrence of a specific ribosomal process. Overall, it appears that the role of pseudouridines lies in the fine-tuning of various ribosomal reactions and not in being an irreplaceable participant of an essential process.

In all analyses of pseudouridine localization so far, the region with the highest pseudouridine conservation has turned out to be helix 69 of the large subunit RNA (Ofengand and Bakin 1997). Helix 69 of the *E.coli* 23S rRNA contains pseudouridines at the positions 1911, 1915 and 1917 of 23S rRNA (Ofengand and Bakin 1997; Raychaudhuri *et al.* 1998; Huang *et al.* 1998). All those pseudouridines are made by the helix 69-specific synthase RluD during late stages of ribosomal large subunit assembly (Leppik *et al.* 2007; Vaidyanathan *et al.* 2007; Siibak and Remme 2010). Although located in the same secondary structure element, the three pseudouridines of helix 69 differ subtly in their structural environment. Thus, whereas the pseudouridine at the 1911 position is located at the stem-loop junction (class A according to Ofengand and Fournier 1998), pseudouridines at the 1915 and 1917 position occur in the loop region of helix 69 (class D location) (Ofengand and Bakin 1997; Ofengand and Fournier 1998). While pseudouridines at 1915 and 1917 are conserved in all three domains of life, archea lack pseudouridine at the 1911 position (Ofengand and Bakin 1997; Sumita *et al.* 2005). In many eubacterial species including *E.coli*, Ψ1915 is further modified by methylation at N3 (Kowalak *et al.* 1996; Ero *et al.* 2008; Purta *et al.* 2008). A universally conserved location of the H69 pseudouridines in an important intersubunit bridge near the decoding and tRNA binding sites led to suggestions about their involvement in intersubunit association and tRNA binding (Ofengand and Bakin 1997). The participation of the helix 69 pseudouridines in interactions forming bridge B2a and in tRNA binding is supported by the analyses of the crystal structures of the 70S ribosomes of T.thermophilus and *E.coli* (Yusupov *et al.* 2001; Schuwirth *et al.* 2005). Thus, in the *E.coli* 70S ribosome the reversed Hoogsteen base pair between A1919 and Ψ1911 allows A1919 to interact with nucleotides U1496 and G1517 of 16S rRNA (Schuwirth *et al.* 2005). Similarly, in the reversed Hoogsteen base pair between Ψ1917 and A1912, A1912 is positioned to contact nucleotides C1407 and G1494 of 16S rRNA (Schuwirth *et al.* 2005). Both of those interactions are important for the formation of bridge B2a (Schuwirth *et al.* 2005). In turn, Ψ1915 is located close to the D-stem of the P-site tRNA in the T.thermophilus 70S structure (Yusupov *et al.* 2001). Important for the understanding of the role of pseudouridines for the structural dynamics of helix 69 are the results of the physicochemical studies on the oligonucleotide analogues of helix 69. The properties of the chemically synthesized oligonucleotides containing one or multiple pseudouridines in locations corresponding to those in rRNA were monitored by thermal melting profiles as well as circular dichroism and 1H-NMR spectra. A novel finding of those

studies was that pseudouridines can in some cases have a destabilizing – though not extensive – effect on RNA structure (Meroueh *et al.* 2000). Thus, incorporating a pseudouridine in the loop region of a synthetic RNA hairpin (corresponding to Ψ1915 and Ψ1917 of helix 69) led to a 0.3 – 0.7 kcal/mol decrease in RNA stability relative to the fully unmodified RNA (Meroueh *et al.* 2000). On the other hand, the presence of a pseudouridine at the stem-loop junction stabilized the same helix by 1.0 kcal/mole relative to the unmodified RNA (Meroueh *et al.* 2000). Together, these findings indicate that the stabilizing/destabilizing influence of pseudouridines is context dependent, complementing the previously held view about the uniquely stabilizing role of pseudouridines on RNA structure (Davis 1995; Auffinger and Westhof 1998). Another notable effect of the pseudouridines was that their presence led to an increased base stacking and decreased solvent accessibility of the loop region of helix 69, as if making the structure more compact (Desaulniers *et al.* 2008). Concerning the role(s) of the pseudouridines for helix 69 functioning it is interesting that the destabilizing effect on the helix structure was exerted by the evolutionarily more conserved Ψ1915 and Ψ1917 (Ofengand and Bakin 1997). It is therefore conceivable that the destabilizing influence of Ψ1915 and Ψ1917 is required for ensuring the conformational flexibility of helix 69. The conformational flexibility of helix 69 may in turn be needed for its interaction with translation factors and/or participating in the structural rearrangements during translocation (Van Loock *et al.* 2000; Zhang *et al.* 2009).

The role of the helix 69 pseudouridines has also been addressed bio-chemically and genetically. In the genetic studies the role of helix 69 pseudouridines and their synthase RluD has regularly been investigated by first disrupting the chromosomal gene encoding RluD and then following the effect of functional RluD deletion on cell growth, ribosome biogenesis and helix 69 pseudouridylation. These studies have regularly been conducted in derivatives of *E. coli* strain K-12 (Mora *et al.* 2007). Collectively, the studies have shown that the deletion of functional RluD protein leads to a severe growth impairment of the cells. As expected from the substrate-specificity of RluD, ribosomes from the *rluD*$^{-}$ cells were found to lack pseudouridines at all three positions in helix 69 (Raychaudhuri *et al.* 1998). In rescue experiments where the functional RluD enzyme was expressed from a plasmid, the growth rate was restored to the WT-level, demonstrating that the growth impairment was related to the absence of functional RluD (Gutgsell *et al.* 2001; Ofengand *et al.* 2001; Gutgsell *et al.* 2005). The strong growth impairment caused by RluD deletion stands in contrast to the generally minor effects on cell growth observed upon deleting other large subunit pseudouridine synthases and indicates an important role for RluD in cellular metabolism. However, the strong impact of RluD deletion on cellular viability does not by itself conclusively prove that the effect is due to the absence of pseudouridines in helix 69 since other roles for RluD in rRNA metabolism cannot be excluded. It is for instance conceivable that RluD has an additional substrate either in rRNA or somewhere else. A “dual specificity” has

been demonstrated for pseudouridine synthase RluA that makes a pseudouridine at the position 746 of 23S rRNA but also synthesizes a pseudouridine at position 32 in $tRNA^{Phe}$, $tRNA^{Cys}$, and $tRNA^{Leu}$ (Wrzesinski *et al.* 1995; Raychaudhuri *et al.* 1999) as well as for Pus1p in S.cerevisiae that pseudouridylates both tRNA and U2 snRNA (Massenet *et al.* 1999). It has also been suggested that in addition to its pseudouridine synthase activity RluD may be involved in the 50S assembly process (Gutgsell *et al.* 2001; Ofengand *et al.* 2001). This suggestion has mainly been based on the following observations. First, intermediates of the large subunit assembly have been seen to accumulate in the absence of functional RluD (Ofengand *et al.* 2001). This was taken as an indication that RluD has a direct role in the 50S subunit assembly which is independent of its role in pseudouridine formation (Ofengand *et al.* 2001). However, this conclusion can be questioned by arguing that the assembly defect observed in the absence of RluD is indirect and is caused by the suboptimally functioning protein synthesis in the absence of helix 69 pseudouridines that leads *e.g.* to an imbalance in the ratio of ribosomal proteins to rRNA. More convincing appeared the demonstration that the growth impairment of the $rluD^{-}$ strain could be alleviated by the plasmid-borne expression of a variant of RluD that was unable to catalyze the formation pseudouridines in helix 69 (Gutgsell *et al.* 2001). Unfortunately, the latter finding was challenged by a later study of the same group (Gutgsell *et al.* 2005). Namely, the plasmid-borne expression of the catalytically inactive variant of RluD did not restore the WT growth of the $rluD^{-}$ cells (Gutgsell *et al.* 2005). The reasons for the discrepancy between the two studies are not known. Taken together, a direct role of RluD in the large subunit assembly is still debatable.

An interesting phenomenon regularly observed in the RluD-deletion studies is the occurrence of pseudoreversion. During pseudoreversion a subpopulation of the rluD-minus cells acquires the growth characteristics of WT cells despite still lacking the functional RluD enzyme and pseudouridines in helix 69 (Raychaudhuri *et al.* 1998; Gutgsell *et al.* 2005). In a detailed analysis by Ejby *et al.* 2007 this phenomenon was traced back to a single amino acid replacement in the prfB gene coding for class I polypeptide release factor RF2 (Ejby *et al.* 2007). The likely structural reasons for the mechanism of this compensatory mutation have been discussed in the previous paragraph. The importance of the finding of Ejby and colleagues – i.e. that a mutation in a polypeptide release factor could compensate for the lack of RluD/helix 69 pseudouridines – lies in the fact that it indicates a role for helix 69 pseudouridines in a specific ribosomal process beyond that of a mere structural modulator.

During the writing of this thesis, the results of the genetic studies by two independent research groups have provided unexpected insights into the roles of pseudouridines in helix 69 in translation termination and their combination with the genetic background of the parent strain. It was namely shown that the previously reported deleterious effects of the RluD deletion on cellular viability is restricted to the strain K-12 of *E.coli* and does not occur with other bacterial

species or with other strains of *E.coli* (Schaub and Hayes 2011; O'Connor and Gregory 2011). Differently from other *E.coli* strains the strain K-12 contains a variant of RF2 that has a threonine instead of alanine at the position 246 of RF2 (Uno *et al.* 1996; Dinçbas-Renqvist *et al.* 2000). This Ala246Thr substitution renders RF2 in K-12 less active in peptide release than its Ala246 counterpart in other strains (Dinçbas-Renqvist *et al.* 2000; Mora *et al.* 2007). In addition to a variant RF2, ribosomal protein S7 is also different in K-12 (Schaub and Hayes 2011). In K-12 protein S7 has a 23-amino acid extension at its C-terminus that i) compromises the RF2-dependent release of S7 from the peptidyl-tRNA during termination of its own synthesis and ii) leads to its enhanced tmRNA-dependent SsrA-tagging compared to that in other strains (Schaub and Hayes 2011). The less efficient RF2-dependent translation termination in K-12 due to variant RF2 and S7 proteins is apparently tolerated as long as the cell still possesses a functional RluD enzyme and pseudouridines in helix 69. However, upon deletion of RluD from K-12 the accumulation of otherwise moderate termination defects apparently passes a threshold and leads to defects in downstream processes that result in significantly reduced cellular viability. Those downstream events may include i) an increased readthrough of the stop codon in the open reading frame of RNase III (involved in the maturation of the 23S rRNA) and the ensuing defect in the ribosomal assembly (Ejby *et al.* 2007), ii) disruption of the expression of the str-operon coding for ribosomal protein S12 and elongation factor G (Schaub and Hayes 2011), as well as iii) sequestration of release factor RF1 on the UAA codons with the consequent read-through of the UAG codons (O'Connor and Gregory 2011).

It thus appears that similarly to the other pseudouridine synthases RluD and/or its reaction products are dispensable for cell growth in strains other than *E.coli* K-12, at least in the commonly used laboratory media. However, the strong conservation of the helix 69 pseudouridines in three domains of life indicates that their presence may be required under more stringent growth conditions.

2. RESULTS AND DISCUSSION

2.1. Reference 1. The role of helix 69 of 23S rRNA in factor-dependent initiation and elongation

By the year 2005 when this study was begun a sufficient number of cryo-EM and X-ray structures of prokaryotic ribosomes and ribosomal complexes had accumulated to allow the interpretation of various stages of ribosomal working cycle in structural terms. One salient feature that was observed in different ribosomal structures (Cate *et al.* 1999; Yusupov *et al.* 2001; Bashan *et al.* 2003; Schuwirth *et al.* 2005) was a conserved stem-loop in domain IV of 23S rRNA that bears the number 69. Already at the low-to-medium resolution level helix 69 was seen to contact helix 44 of 16S rRNA at the decoding centre and form one of the largest RNA-RNA intersubunit contacts – bridge B2a (Cate *et al.* 1999; Yusupov *et al.* 2001). The participation of helix 69 in an intersubunit contact had previously been established with the help of RNA-RNA cross-linking and chemical protection experiments (Mitchell *et al.* 1992; Merryman *et al.* 1999; Maiväli and Remme 2004). Collectively, the structural and bio-chemical data had firmly established the role of helix 69 in subunit association and this view was only reinforced by later biochemical and genetic studies (Hennelly *et al.* 2005; Liiv *et al.* 2005; Ali *et al.* 2006; Hirabayashi *et al.* 2006; Pulk *et al.* 2006; O'Connor 2007). However, by showing the interaction of helix 69 with various ribosomal ligands, structural analyses pointed to other possible roles of helix 69 in translation in addition to its involvement in an intersubunit interaction. For instance, this helix was seen to contact the D-stem regions of both the A- and P-site tRNAs (Yusupov *et al.* 2001), implicating that it may have a role in the binding and movement of tRNAs within and between the ribosomal sites during elongation (Bashan *et al.* 2003). By 2005 helix 69 had also been observed to lie within interaction distance with major translation factors like the initiation factor IF2 (Allen *et al.* 2005; Myasnikov *et al.* 2005), elongation factor EF-G (Spahn *et al.* 2004) and termination factors RF1/2 and RF3 (Selmer *et al.* 2005) Later structural studies expanded the list of helix 69 interaction partners to elongation factor EF-Tu (Schmeing *et al.* 2009) and ribosome recycling factor RRF (Gao *et al.* 2007; Pai *et al.* 2008).

Given this impressive list of helix 69 interactions with the factors that function in the central stages of the ribosomal cycle and its known importance for the stability of the functional 70S ribosome, it was reasonable to hypothesize that helix 69 itself has a regulatory role in one or more steps of the ribosomal cycle. For this reason, we decided to biochemically test the structure-based hypotheses about the role of helix 69 in translation. An earlier pilot study by our group had already established the importance of the intactness of helix 69/bridge B2a for cellular viability and ribosomal functioning in vivo (Liiv *et al.* 2005). To probe the role of helix 69 in translation in more detail we devised

a mutagenesis study where we made base replacements at selected positions in helix 69. In adopting the mutagenesis strategy we reasoned that by disrupting interactions in bridge B2a we may in fortunate cases also interfere with its function in the ribosomal working cycle. The positions selected for site-directed mutagenesis included nucleotides 1912 and 1919 of 23S rRNA in helix 69. Those nucleotides were selected on the basis of their central location in intersubunit contacts as deduced from the crystal structure of the *E.coli* 70S ribosome (Schuwirth *et al.* 2005). As a negative control, we constructed a 50S variant (ΔH69) lacking the entire helix 69 (nucleotides 1906 – 1931) similarly to Ali and coworkers (Ali *et al.* 2006). The H69 variant 23S rRNAs/50S subunits were expressed from the ptBB plasmid (Lewicki *et al.* 1993) in *E.coli* in the background of the chromosomally encoded WT 50S. The 50S subunits (WT and H69 variant) were extracted from the 70S fraction of a 10–25% sucrose gradient. The H69 variant 50S subunits were purified to (near) homogeneity by affinity chromatography using a streptavidine-binding aptamer in helix 25 of the H69 variant 23S rRNA (Leonov *et al.* 2003). In agreement with earlier data (Liiv *et al.* 2005) the expression of the H69 variant 50S subunits - even in the presence of the WT ribosomes – conferred a slow growth phenotype on the cells (Ref.1 Figure S1), indicating a trans-dominant effect of the H69 mutations on the cellular protein synthesis. The purified H69 variant ribosomes were subsequently tested in various *in vitro* assays that monitored separate steps of the ribosomal working cycle.

As a first screen for *in vitro* activity we analyzed the ability of the H69 variant ribosomes to catalyze the formation of trichloroacetic acid precipitable polyphenylalanine on the poly (U) template in the presence of elongation factors EF-Tu and EF-G (and GTP as the energy source). The poly(U) assay reflects the ability of the ribosomes to elongate a polypeptide chain but bypasses the factor-dependent initiation step. In this assay a notable reduction in the level of poly(Phe) formation was observed with all of the single-point H69 variant ribosomes, the effect being severest with the 1919 variant ribosomes (Ref. 1 Figure 2). Interestingly, the smallest impairment in poly(Phe) synthesis was observed on the ΔH69 ribosomal variant that lacks the entire helix 69. The strongly reduced elongation activity of the H69 variant ribosomes in the poly(U) assay supports the notion that positions 1912 and 1919 of 23S rRNA in helix 69 are important for the ribosomal functioning as deduced from their central location in the interaction network in bridge B2a. However, the results of the poly(Phe) assay alone do not permit to specify which step(s) of the ribosomal cycle is/are affected in the H69 variant ribosomes since the reduced activity may be due to a defect in a number of steps, such as:

- transpeptidylation,
- aminoacyl/peptidyl-tRNA binding
- tRNA-mRNA complex translocation
- association of the subunits into functional 70S complexes.

In view of the many interactions between bridge B2a and ribosomal ligands, it is reasonable to expect that many steps of the ribosomal cycle are affected simultaneously by base changes in helix 69. Additional experiments allow us to shed more light on which steps are or are not affected by mutations in helix 69. Thus, the observation that the H69 variant ribosomes were sufficiently robust in the formation of the NAc-Phe-puromycin dipeptide on the nonenzymatically formed 70S complexes (Ref.1 Figure 3) indicates that the strong reduction in their elongation activity in the poly(Phe) assay cannot be ascribed to a defective peptidyl transferase. Alternatively, it can be suggested that an impairment in the 70S formation with the H69 variant ribosomes is the root cause of their reduced poly(Phe)-forming activity. We acknowledge that the H69 variant 50S subunits did display a notable defect in the 70S complex formation during the *in vitro* reassociation assay (Ref.1 Figure 4) and this can therefore be taken as an explanation of their reduced elongation activity in the poly(Phe)-assay. However, we have reasons to believe that – relative to the other assays – the reduced reassociation ability of the H69 variant 50S exaggerates the effects of the base replacements in helix 69 on the stability of the 70S ribosome. First, it has been shown that the excess hydrostatic pressure generated during ultracentrifugation drives the equilibrium of the 70S formation towards the dissociation of the subunits (Infante and Baierlein 1971: Van Diggelen *et al.* 1971). This phenomenon may therefore amplify the otherwise moderate differences in the subunit association of the WT and H69 variant 50S subunits. Secondly, naked subunits were used in the reassociation assay whereas tRNA and mRNA were present in the poly(Phe) assay (and in all the other *in vitro* assays). As has been observed by us and by others (Liiv and O'Connor 2006; Southworth *et al.* 2002), ligands like tRNA and mRNA are able to compensate for defects in subunit association, at least under near-physiological conditions. In line with this reasoning (i.e. that ribosomal ligands support the association of the subunits), the fraction of the 70S ribosomes active in the NAcPhe-puromycin formation was close to the WT value (Ref.1 Figure 3). In conclusion, the robust behavior of the H69 variant ribosomes in the NAcPhe-puromycin synthesis appears to rule out defective subunit association and defective transpeptidylation as the major causes of the elongation defect in the poly(Phe) assay (compare Figures 2 and 3 in Ref.1).

One of the shortcomings of the poly(Phe) assay is its lack of requirement for initiation factors for the 70S complex formation. Yet both cryo-EM and biochemical analyses indicate that helix 69 interacts with initiation factors. For example, helix 69 is known to be located close to the C-terminal domain IV of IF2 in the 70S initiation complex (Allen *et al.* 2005; Myasnikov *et al.* 2005) and may compete with IF3 for the binding site on the 30S subunit (Dallas and Noller 2001). To probe the involvement of helix 69 in the factor-mediated translation initiation the H69 variant 50S subunits were tested for their ability to form a functional 70S initiation complex in the presence of all three initiation factors (Figure 14).

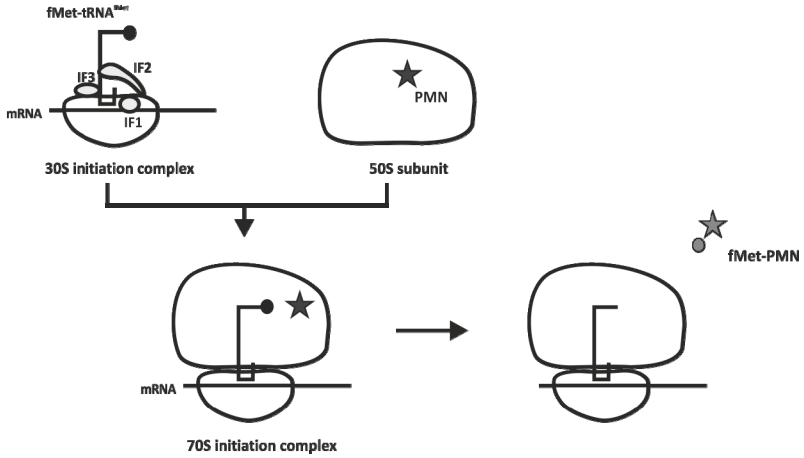

Figure 14. Principle of the 70S initiation assay. The preformed 30S initiation complex containing 30S subunits (5 pmol), mRNA (40 pmol), [^{35}S]fMet-tRNA^{fMet} (10 pmol) and initiation factors IF1 (20 pmol), IF2 (10 pmol), and IF3 (20 pmol) in the presence of 1 mM GTP in 15 µL polymix buffer (see Appendix 1) was mixed with a solution of 5 µL containing the H69 variant 50S subunits (5 pmol) and puromycin (5 mM) in polymix buffer at 37°C. The formation of the functional 70S initiation complex in time was analyzed by monitoring its ability to catalyze the formation of the [^{35}S]fMet-puromycin (fMet-PMN) adduct. The puromycin reaction was stopped by the addition of KOH to a final concentration of 1.0 M and the fMet-PMN product extracted into ethyl acetate. ½ of the ethyl acetate fraction was counted for the ^{35}S radioactivity.

The "functionality" of the 70S complex was defined as its ability to catalyze the formation of the fMet-puromycin dipeptide between the fMet-tRNA^{fMet} in the P-site and the antibiotic puromycin. Puromycin is a mimic of the acceptor end of aminoacyl-tRNA to which a peptidyl group (or its analogue) can be transferred in the transpeptidylation reaction (Monro 1967; Monro and Marcker 1967; Green and Noller 1996; Nissen *et al.* 2000). The puromycin-based assay was chosen i) due to its relative simplicity and ii) because it had been previously used to monitor the 70S initiation complex formation under similar conditions (La Teana *et al.* 1996; Cuckras and Green 2005). In this reaction, i) preformed 30S initiation complexes (30SIC) are mixed with a solution containing 50S subunits and puromycin, ii) the incubation is continued for varying times and iii) the reaction is then stopped by the addition of alkali. A prerequisite for an efficient transpeptidylation reaction between fMet-tRNA^{fMet} and puromycin is the proper positioning of the fMet-tRNA^{fMet} in the P-site of the newly formed 70S initiation complex (La Teana *et al.* 1996; Blaha *et al.* 2009). Therefore, the puromycin reactivity of the fMet-tRNA^{fMet} can be used to monitor the process of 70SIC formation. We emphasize here that our puromycin-based assay most likely monitores the unimolecular rearrangements

within the 70S initiation complex and not the bimolecular association of the 30S and 50S subunits. The latter reaction has been shown to occur on a more rapid time scale than the 10 to 60 s timeframe of the present assay (Antoun *et al.* 2003b; Antoun *et al.* 2006a; Grigoriadou *et al.* 2007a).

To facilitate the kinetic analysis we first tried to establish conditions where the 70S initiation complex formation would follow single-turnover kinetics. Under single-turnover conditions a 70S initiation complex is formed only once during the reaction and there is no dissociation and subsequent reformation of it (in the presence of excess starting components) which would render the kinetic analysis more complicated. We reasoned that this situation would occur when the concentration of the 50S were close to the concentration of the 30SIC or even exceeded it. Under those conditions a stoichiometric combination of the 30SIC with the 50S subunits occurs and there will be little recycling of the 50S between the 30SIC – a situation that likely occurs when the 30SIC are in excess (Figure 15). We therefore monitored the formation of fMet-puromycin in a preliminary experiment where the 50S concentration was varied at a fixed 30SIC concentration. The results were expressed as the fraction of fMet-puromycin formed per 50S subunits. Under single-turnover conditions this fraction should never exceed unity since on the average each molecule of 50S (within the 70S ribosome) could maximally form one molecule of fMet-puromycin. A value above unity, on the other hand would indicate that single turnover conditions do not hold and there is some recycling (multiple turnover) of the 70S initiation complex.

As seen from our results, the fraction of fMet-puromycin per 50S depends on the 50S-to-30SIC ratio (Figure 16). The fraction is significantly above unity at low 50S: 30SIC ratios (< 0.5) where the 30SIC is in excess over 50S but drops below unity at higher 50S: 30SIC ratios with a tendency to leveling off (Figure 16). Thus, the reaction has more single-turnover character at higher 50S:30SIC ratios and it would therefore be reasonable to use 50S subunits in excess over the 30S initiation complex as has been done in Grigoriadou *et al.* 2007a. However, we opted for a 1 : 1 ratio of 50S to the 30SIC in the subsequent initiation experiments with the H69 variant ribosomes. This ratio reflects a compromise between efforts to achieve single turnover conditions for 70SIC formation on one hand and the parsimonious expenditure of the variant 50S subunits that were regularly obtained in low yields during purification. Incidentally, this 1:1 ratio has been used in many of the previous *in vitro* initiation studies (La Teana *et al.* 1996; Cuckras and Green 2005; Antoun *et al.* 2006a/b) (see Appendix I).

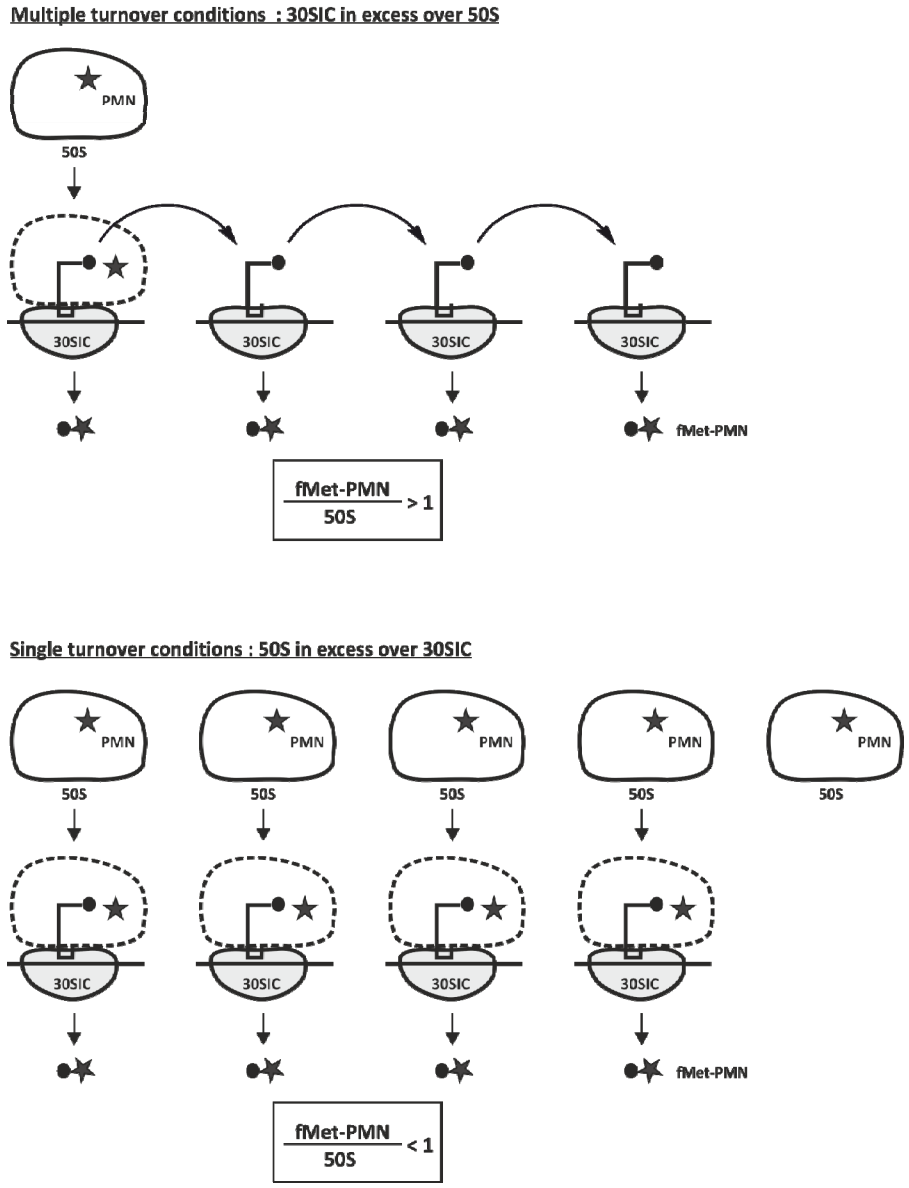

Figure 15. Differences between a single turnover and a multiple turnover mechanism in the 70SIC formation.

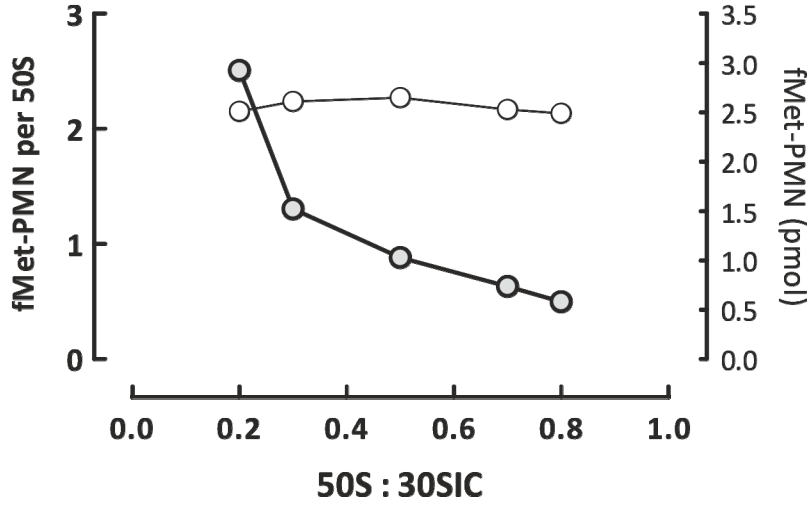

Figure 16. f[^{35}S]Met-puromycin formation at different 50S : 30SIC ratios. 30S initiation complexes (max. 6 pmol) were mixed with a solution containing 1 mM puromycin and varying amounts (1 – 5 pmol) of WT 50S subunits and incubated in a polymix buffer at 37°C for 60 s. The reaction was stopped with 1.0 M KOH and the samples treated identically to Figure 13. The amount of f[^{35}S]Met-puromycin formed (left axis, open circles) was divided by the amount of 50S to express reaction yield as a fraction of fMet-puromycin formation per molecule of 50S (right axis; grey circles).

The formation of 70SIC was monitored in the presence and in the absence of the initiation factors. The time-course of fMet-puromycin formation $F_{fMet–PURO}$ (t) could be fit to a single exponential in the form of

$$F_{fMet–PURO}(t) = F_0*(1 – \exp[-k_{obs}*t])$$

where k_{obs} is the apparent[†] first-order rate constant for the 70S complex formation and F_0 (the final extent of the reaction) is the amount of fMet-puromycin formed at longer incubation times ($t >> 1/k_{obs}$). Those two parameters were used to characterize the effects of the base replacements in helix 69 on the 70S initiation complex formation. The following results were obtained.

[†] „apparent“ because in a more detailed analysis this rate constant turns out to be a combination of other rate constants and depend on the concentration of the reacting species

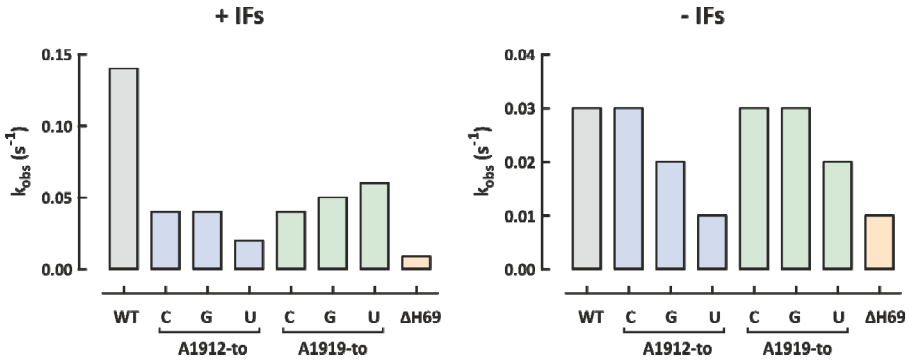

Figure 17. Rate-constants for the 70S initiation complex in the presence ("+IFs") and absence of the ("-IFs") initiatation factors. Data have been taken with modifications from Ref.1 Figures 5 and 6.

In the presence of 3-fold excess of initiation factors over the 30S subunit (conditions similar to Cuckras and Green 2005), both the rate and the final extent of the puromycin reactive 70S complex formation were decreased on the H69 variant ribosomes relative to the WT (Ref.1 Figure 5). The greatest decrease in the kinetic parameters (rate constant k_{obs} and the final extent of the reaction) was seen with the ΔH69 variant ribosomes (Figure 17; Ref.1 Figure 5). In line with the stimulating role of the initiation factors, the rate constant of the 70SIC formation was slowed down 5-fold with the WT (with a concomitant 3-fold decrease in the final extent of fMet-puromycin formation) when the factors were omitted from the reaction mixture (Figure 17; Ref.1 Figure 6). However, in the absence of the initiation factors the base substitutions in bridge B2a caused a much smaller impairment in the 70S initiation complex formation as compared to the situation in the presence of the factors (Figure 17; Ref.1 Figure 6). The decreased 70S-forming capability of the H69 variant 50S subunits in the presence of the initiation factors supports the structure-based suggestions about the functional importance of the interactions between helix 69 and initiation factors. As for the way how the base replacements in helix 69 exert their effect on the factor-dependent initiation, three scenarios can be envisaged.

First, it is possible that due to the altered conformation of helix 69 in the H69 variant 50S this helix is unable to efficiently compete with IF3 in binding to the 30S. As a consequence, the formation of the 70S initiation complex occurs more slowly and with a lower yield with the H69 variant 50S. The smaller effect of the base replacements in helix 69 on the initiation process in the absence of the factors can then be explained as due to the lack of competition with IF3. This "direct competition" model fits in with the data of McCutcheon and coworkers (McCutcheon *et al.* 1998) and Dallas and Noller (Dallas and Noller 2001) which indicate that IF3 and helix 69 share an

overlapping binding site on the 30S. The dissociation of IF3 would therefore be a prerequisite for the docking of the 50S subunit on the 30S initiation complex. The orthogonality of IF3 and 50S in the 70S ribosome is supported by the kinetic studies of Grunberg-Manago and coworkers (Godefroy-Colburn *et al.* 1975) and more recently by Ehrenberg and coworkers (Antoun *et al.* 2006a). Nevertheless, the competition model with IF3 could hold even if IF3 bound to a region on the opposite side of the 30S (as suggested by the results of Piolietti *et al.* 2001) as long as the binding of IF3 to the 30S induced a conformational change in the 30S that is incompatible with the docking of helix 69 onto the small subunit. To further test the "IF3-competition" model, we monitored the amount of the puromycin-reactive 70SIC at increasing IF3 : 30SIC ratios. Unexpectedly, addition of IF3 in up to 5-fold excess over the 30S did not notably impair the 70SIC formation with any of the H69 50S subunits tested (Figure 18). This result appears to disfavor an IF3-related "competition" model because at increasing concentrations IF3 would be more efficient in out-competing the 50S in docking to the 30SIC.

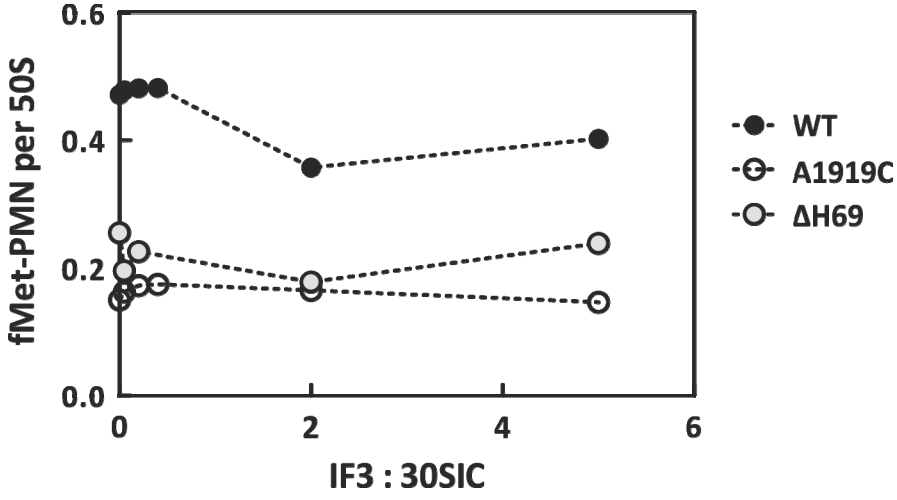

Figure 18. 70S initiation complex formation at different IF3 : 30SIC ratios. The experimental conditions were identical to Figure 14 except for IF3 the concentration of which was varied from 0.05 pmol IF3/pmol 30SIC to 5 pmol IF3/pmol 30SIC. Reaction time was 60s at 37°C. The subsequent treatment of the samples was identical to Figure 14.

According to a second scenario, changes to the conformation of helix 69 would interfere with the IF2-catalyzed docking of the 50S and/or the accommodation of the fMet-tRNA^{fMet} into a puromycin-reactive binding site within the 70S complex. This model is in agreement with the results of the cryo-EM analyses where helix 69 was seen to lie within interaction distance with the initiator tRNA and the C-terminal domain IV of IF2 (Allen *et al.* 2005; Myasnikov *et al.*

2005). This second interpretation is also in qualitative agreement with the results of Grigoriadou and coworkers on the 70S initiation complex formation (Grigoriadou *et al.* 2007a). In the latter study, IF2 was shown to be involved in the conversion of the newly formed and labile 70S initiation complex into a more stable complex with the concomitant placement of fMet-tRNA^{fMet} into the "classical" P-site (Grigoriadou *et al.* 2007a). Importantly for the interpretation of our results, factors interfering with the proper functioning of IF2 – like replacement of GTP with the nonhydrolyzable analogue GDPCP – were shown to perturb the accommodation of fMet-tRNA^{fMet} into the P-site in the study of Grigoriadou and coworkers. It is conceivable that the effect of the base replacements in helix 69 in our study parallels the effect of GDPCP on the IF2-mediated events in the initial 70S complex. Considering the synergistic interaction of the initiation factors the two mechanisms – the IF2 and IF3-related one – need not be mutually exclusive and may both be operative on the H69 variant ribosomes.

The third scenario to explain the adverse effect of the base replacements in helix 69 on the factor-dependent initiation is related to different mRNA conformations. It has namely been shown – by biochemical (Canonaco *et al.* 1989; La Teana *et al.* 1995) as well as structural studies (Kaminishi *et al.* 2007) – that the mRNA molecule assumes two different binding states on the 30S subunit. In the first or "stand-by" state (Canonaco *et al.* 1989; La Teana *et al.* 1995) the mRNA is anchored to the 3'-end of 16S rRNA by the formation of the Shine-Dalgarno-anti-Shine-Dalgarno (SD-aSD) duplex that sits snugly in a "chamber" formed by proteins S2, S11, and S18 and 16S rRNA residues U723 and G1530 (Kaminishi *et al.* 2007). However, in this state the initiation codon is not yet properly placed in the P-site and cannot therefore interact with the initiator tRNA (Kaminishi *et al.* 2007). In the second binding state, the SD-aSD duplex is extruded from the "chamber" and the initiation codon is accommodated into the P-site (Kaminishi *et al.* 2007). Importantly, initiation factors and fMet-tRNA^{fMet} have been shown to favor the second binding state of mRNA (Canonaco *et al.* 1989; La Teana *et al.* 1995). Thus, according to the third scenario, in the absence of the initiation factors the mRNA is in the "stand-by" position and cannot therefore stabilize the fMet-tRNA^{fMet} in a puromycin-reactive orientation in the 70S complex regardless of the conformation of helix 69. On the other hand, when the initiation factors are included in the reaction mixture, the mRNA assumes the second binding state with the AUG codon in the P-site. The initiator tRNA is now placed in such a fashion in the 70S complex that it interacts with helix 69 and is therefore sensitive to the conformation of the helix. Now the impact of the changes in the conformation of helix 69 on its interaction with the fMet-tRNA^{fMet} is revealed in the different kinetic behaviour of the H69 variant ribosomes during the 70S initiation complex formation.

However, a closer inspection of our results on the 70S initiation complex formation in the light of the results of recent rapid kinetics studies on translation

initiation (Antoun *et al.* 2003b; Antoun *et al.* 2005; Antoun *et al.* 2006a/b; Grigoriadou *et al.* 2007a) seems to raise a serious objection to the validity of our interpretation of the effects of helix 69 mutations on the initiation process regardless of the scenario chosen. Namely, in the rapid kinetics studies the formation of the 70S particle (as monitored by changes in the light scattering) and the ensuing conformational rearrangements within the 70S initiation complex – including the accommodation of fMet-tRNA^{fMet} into the P-site – are seen to be completed within a couple of seconds. For instance, the slowest step – the rate-limiting accommodation of fMet-tRNA^{fMet} in the 70S – proceeds with a rate of 2.3 s^{-1} in the study of Grigoriadou and coworkers and is essentially complete by the time of 1–2 seconds (Grigoriadou *et al.* 2007a). Now, in our experiments the formation of fMet-puromycin (which we believe reflects the formation of the elongation competent 70S) on the WT 70S ribosomes proceeds with a 40–50-fold lower rate constant even in the presence of all three initiation factors (k_{obs} = 0.14 s^{-1}). The rate of 0.14 s^{-1} also falls short of the *in vivo* initiation rate of 0.3 s^{-1} (approximately 1 inititiation event every 3 s) as measured on the lac operon (Kennel and Riezman 1977). Examination of the reaction conditions (temperature, buffer, reactant concentrations) used in our study and that of others (see Appendix 1) does not reveal any differences that could account for the 50-fold variation in the rate of the 70S complex formation. The only significant difference in the experimental conditions was in the study of Grigoriadou and coworkers where time courses were taken at 20°C (Grigoriadou *et al.* 2007a) and not at 37°C as in our study[†]. However, this fact leads one to expect even greater differences in the rates at 37°C and thus makes the slowness of our ribosomes only more conspicuous. If differences in the reaction conditions are excluded, what then could be the reason for the slow rate of 70S formation in our experiments? The heterogeneity of the ribosomal preparations comes to mind as the most straightforward explanation. According to this interpretation, we are monitoring the 70S complex formation with a less active ribosomal fraction both in the case of the WT as well as the H69 variant 50S subunits. With the more active ribosomal fraction – presumably studied by the aforementioned authors in their more sophisticated equipment – the reaction has reached completion within the first time-point and we are unable to follow it there. The author acknowledges that it is difficult to rule out the heterogeneity of the ribosomal preparations considering the many purification steps they have undergone. Nevertheless, our recent rapid kinetics measurements of fMet-puromycin formation (under conditions similar to those described above) show

[†] Another difference between our study and that of Grigoriadou and colleagues was their use of a 10- to 20-fold higher concentration of 50S subunits (Grigoriadou *et al.* 2007a; see Appendix I). This difference could partly explain the lower rate of the 70SIC formation in our experiments. Unfortunately, the published data about the dependence of the rate constants of the 70SIC formation on the concentration of 50S (Figure 4b and 5c in Grigoriadou *et al.* 2007a) do not permit to precisely extrapolate the rate constants measured by Grigoriadou and colleagues to lower 50S concentrations.

that on preformed 70S initiation complexes fMet-puromycin is synthesized with a rate of 0.6 s^{-1} (Figure 19). This value is close to the rate of 0.5 s^{-1} for fMet-puromycin formation on preformed 70S initiation complexes as reported by Wohlgemuth and colleagues (Wohlgemuth *et al.* 2006). Although this congruence of the rate constants does not – strictly speaking – disprove the heterogeneity of our ribosomal preparations it at least indicates that our preparations can be as active as those of other research groups. In addition, the (nearly) monophasic behavior of the WT ribosomes in the synthesis of NAcPhe-puromycin (Ref.1 Figure 3) speaks against the presence of two or more ribosomal fractions with widely differing activities.

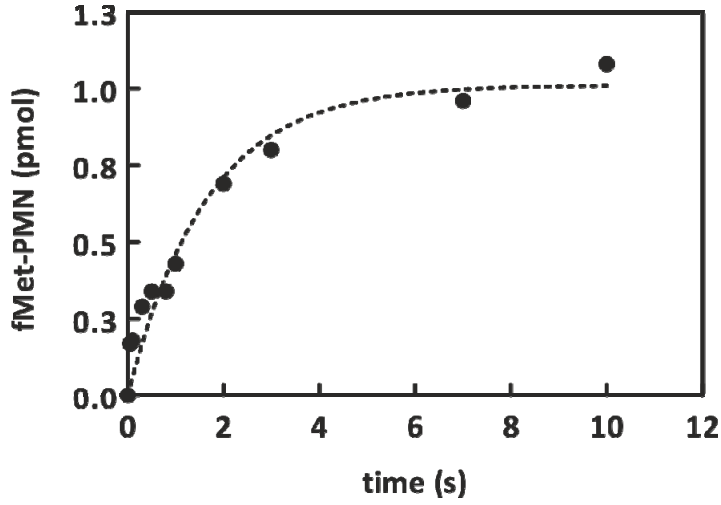

Figure 19. Time-course of fMet-puromycin formation on the WT 70S ribosome. The 70S initiation complexes containing [^{35}S]fMet-tRNA^{fMet} in the P-site were formed under conditions similar to Figure 14 and were subsequently mixed with puromycin (f.c. 0.5 mM) in KinTek RQF4 "quench-flow" apparatus. The reaction was stopped by the addition of KOH to a final concentration of 0.5 M and the fMet-puromycin adduct extracted into ethyl acetate. Subsequent treatment of the time-points was identical to Figure 9. The time-course was fitted to a single exponential of the form $F_{fMet–PURO} = F_0*(1 – \exp [–k_{obs}*t])$ where k_{obs} denotes the apparent first-order rate constant of fMet-PMN formation and F_0 is the final extent ("plateau") of the reaction (in pmol). A value of 0.6 s^{-1} for k_{obs} was found from the above analysis for fMet-PMN formation on the WT 70S initiation complex.

As an alternative way to explain the low rate of the 70S complex formation it may be suggested that what we are measuring under the name of a single turnover 70S complex formation is actually a multiple round process that involves a rate-limiting decomposition of the 70S initiation complex and its reformation in the presence of excess starting components (Figure 20).

According to this explanation we are not measuring the rate of the 70S initiation complex formation but its slow decomposition instead. Although plausible in view of the association defect of the H69 variant 50S subunits, the observation that the rate of fMet-puromycin formation was slower with the H69 variant ribosomes than with the WT (Ref.1 Figure 5) does not support this interpretation. This is because disrupting interactions in an important intersubunit bridge is likely to facilitate the decomposition of the 70S complex and consequently lead to a more rapid turnover of the ribosomes. The increased turnover would in turn lead to higher rates of fMet-puromycin formation with the H69 variant ribosomes – a prediction that is refuted by our observations (Ref. 1 Figure 5). We note that the "multiple turnover" hypothesis could be tested in an experiment where the formation of fMet-puromycin on the preformed 70S complexes is followed in the presence of excess unlabelled fMet-tRNA^{fMet} ("chase") added to the reaction mixture prior to puromycin. If the "multiple turnover" mechanism is operative, the amount of [^{35}S]fMet-puromycin should decrease with time due to the replacement of the [^{35}S]fMet-tRNA by the unlabelled initiator tRNA in excess.

As a third explanation for the slow rate of the 70S initiation complex formation in our assay one could suggest that the reaction of fMet-tRNA with puromycin in the peptidyltransferase center is slow relative to the initiation complex formation. According to this suggestion we are not monitoring the effect of helix 69 mutations on translation initiation but rather on the peptidyl transferase reaction. However, our preliminary quench-flow experiments on the formation of fMet-puromycin on preformed 70S initiation complexes indicate that the apparent rate constant of this reaction under conditions similar to those described in Ref. 1 is around 0.6 s^{-1} (Figure 19). This value is about 4-fold higher than the rate constant of 0.14 s^{-1} for the fMet-puromycin formation in the experiments described in Ref. 1 and indicates that in the initiation assay we are not monitoring the peptidyl transferase reaction itself but some other process(es).

Taken together, the reasons for the significantly slower rate of initiation in our experiments remain unclear. The discrepancy in the rates is made even more puzzling by the fact that the same puromycin-based assay has been used by other groups and in their experiments similar (Cuckras and Green 2005) or even lower rates (La Teana *et al.* 1996) for the 70SIC formation have been reported. Nevertheless, the impact of the base replacements in helix 69 on the kinetics of the first peptide bond formation and especially, its dependence on the presence of the initiation factors, supports – regardless of the exact mechanism – the structure-based suggestions about the involvement of helix 69 in factor-dependent translation initiation (Dallas and Noller 2001; Allen *et al.* 2005; Myasnikov *et al.* 2005).

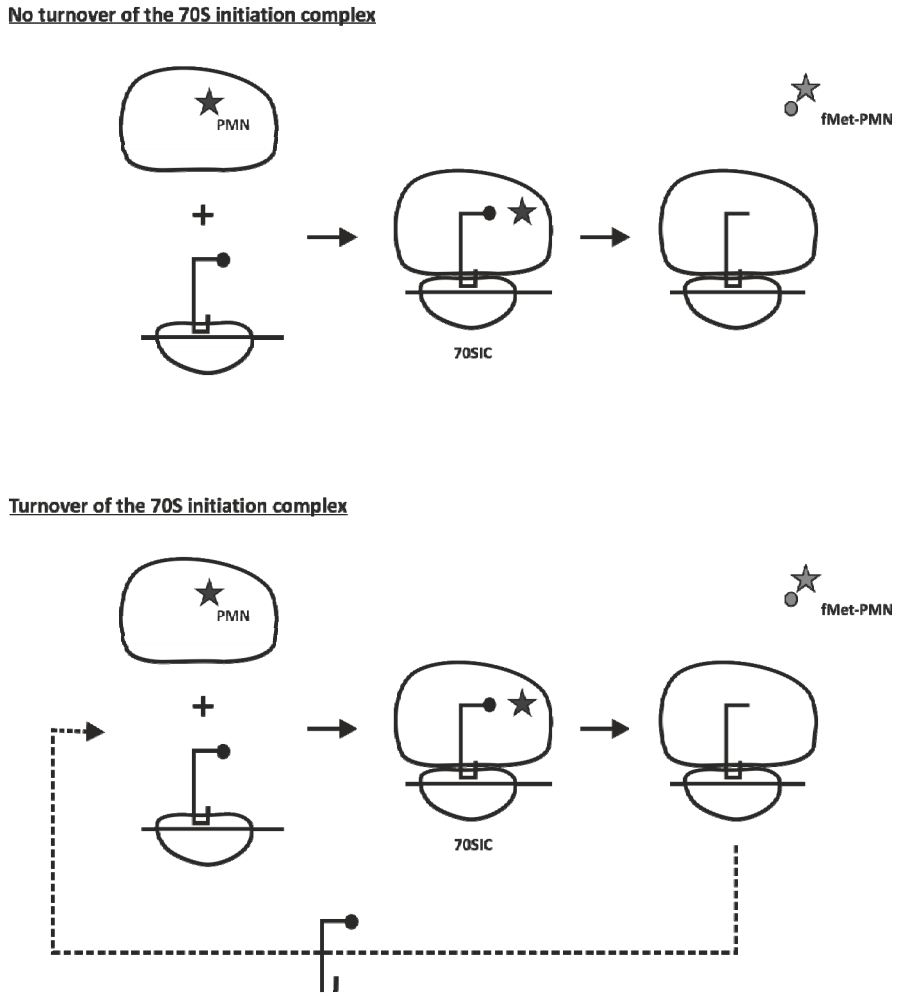

Figure 20. A "dissociative" mechanism of the 70SIC formation.

The possible involvement of helix 69 in steps beyond the formation of the 70S initiation complex is suggested by its contacts to the tRNAs in the A- and P-site (Yusupov *et al.* 2001; Selmer *et al.* 2006) as well as its proximity to domain IV of the ribosome-bound elongation factor EF-G (Spahn *et al.* 2004). Thus, helix 69 may modulate the dynamics of the tRNAs and/or EF-G on the ribosome. Alternatively or in addition to regulating the dynamics of the tRNAs and/or EF-G, helix 69 as part of one of the central intersubunit bridges may be involved in regulating the "ratchet" movement of the subunits during translocation (Gao *et al.* 2003). We reasoned that mutations in helix 69 may help to reveal this (putative) involvement by interfering with the processive elongation. The interference may be due to e.g. increased dissociation of tRNAs from the ribosome (tRNA "drop-off") or defective translocation of the tRNA-

mRNA complex. We therefore examined the effect of helix 69 mutations on elongation in vitro by following the stepwise formation of a peptide containing two to five amino acids on the H69 variant ribosomes. In this experiment (Figure 21) the ribosomes were loaded with a synthetic mRNA that contained a strong Shine-Dalgarno sequence (5'-AGGAGGU-3') followed by a 5-nucleotide spacer and a sequence coding maximally for the fMet-Phe-Val-Ala-Ser pentapeptide. The peptides (di- to penta-) were then formed by providing the mRNA-loaded ribosomal complexes with the corresponing aminoacyl-tRNAs and incubating the reaction mixture for 1.5 min at 37°C. The yield of oligopeptide formation was used as an estimate of the capability of the H69 variant ribosomes to carry out processive elongation. At the fMet-Phe dipeptide level this yield was moderately (by 20 40%) decreased on the H69 variant ribosomes as compared to the WT (Ref.1 Figure 8). However, the decrease in the level of oligopeptide formation became more pronounced with the increasing length of the peptide with most of the H69 variant ribosomes (Ref.1 Figure 8). In the case of the A1912U variant a sharp decrease in the oligopeptide formation occurred after the dipeptide with no further changes in the synthesis of longer peptides (Ref.1 Figure 8). Notably, out of all of the H69 variant ribosomes oligopeptide formation was least impaired with the ΔH69 variant (Ref.1 Figure 8).

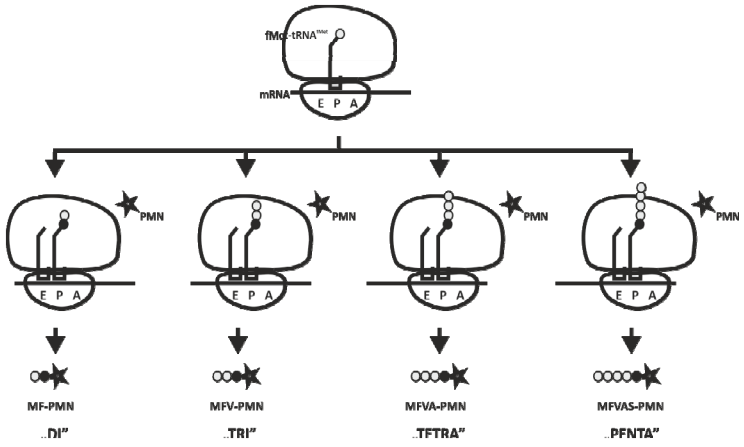

Figure 21. Principle of the oligopeptide formation ("elongational processivity") assay. The preformed 70S initiation complexes (see legend to Figure 14) were mixed with a solution containing the corresponding aminoacyl-tRNAs, elongation factors EF-Tu and EF-G and GTP at 37°C to start oligopeptide formation. The mRNA contained a strong Shine-Dalgarno sequence, a five-nucleotide spacer and a sequence coding for fMet-Phe-Val-Ala-Ser-STOP. The C-terminal amino acid was ^{3}H-labeled except for the dipeptide where [^{14}C]Phe was used. The complexes were subsequently reacted with 1 mM puromycin for 10 min and the reaction stopped by the addition of KOH to a final concentration of 1.0 M. The subsequent treatment of the samples was identical to Figure 14.

As mentioned above, this gradual decrease in the efficiency of oligopeptide formation with the H69 variant ribosomes can be caused by defects in a number of ribosomal processes, either acting alone or in combination. For us, the most plausible explanations for the decreased processivity of the H69 variant ribosomes were the following: i) an increased tendency of the H69 variant 70S to dissociate into subunits, ii) an increased dissociation of tRNA from the ribosome ("drop-off") (Menninger 1976; Dong and Kurland 1995) and/or iii) a blockage in the translocation of the tRNA-mRNA complex. As for i, an increased tendency to dissociate into subunits does not explain the remarkable robustness of the ΔH69 variant ribosomes in the oligopeptide formation assay since in other experiments this ribosomal variant displayed the severest defects in the stability of the 70S (Ref. 1 Figure 4 and Figure 5). In a similar vein, the A1919C variant ribosomes that were the ones most severely compromised in the subunit reassociation assay (Ref.1 Figure 4) again displayed the smallest impairment of oligopeptide synthesis as compared to the other 1919 variants (Ref.1 Figure 8). Further arguing against defective subunit association as the primary cause of the reduced processivity of the H69 variant ribosomes is their relatively high efficiency in the fMet-Phe dipeptide formation (Ref.1 Figure 8). Thus, looking at the gradual loss of the peptide synthesizing ability of the H69 variant ribosomes, one rather gets the impression that those variant ribosomes are able to form an initial elongation competent 70S complex but for some reason get stuck thereafter. One reason for this getting stuck may be the increased tendency of tRNA to dissociate from the H69 variant ribosomes where some of the stabilizing interactions to the tRNAs (Yusupov *et al.* 2001; Selmer *et al.* 2006) are likely to be no longer formed due to changes in the conformation of helix 69. That base changes in helix 69 have the potential to affect the stability of the peptidyl-tRNA in the ribosome is shown by our observation that the dissociation of fMet-Phe-tRNA^{Phe} from the A-site was increased up to 6-fold with the 1919 variant ribosomes (Ref. 1 Figure 7). However, this observation cannot explain the uniform decrease in processivity with the 1912 and the ΔH69 variants where the A-site binding of fMet-Phe-tRNA^{Phe} was even somewhat more stable than on the WT ribosomes (Ref.1 Figure 7). Therefore, it seems that at least with some of the H69 variant ribosomes the major cause of the gradual loss in processivity must be related to the movement of the mRNA-tRNA complex within the ribosome. In this respect it is significant that with the A1912U variant ribosomes the extent of oligopeptide formation decreases sharply after the second codon has been translated (2–3 fold lower yield of tripeptide relative to dipeptide) (Ref. 1 Figure 8). It is the third codon whose translation in the A-site first requires the translocation of the tRNA-mRNA complex. It is also possible that a remodeling of the interactions between mRNA and the 30S subunit is defective on the H69 variant ribosomes and this affects the oligopeptide formation. According to one tempting scenario, the defect in the tRNA-mRNA complex movement may be related to the transition of the ribosome from the initation to the elongation

phase (Tenson and Hauryliuk 2009). During this transition the mRNA is known to assume a more "relaxed" conformation with a simultaneous "closure" of the 30S (shoulder moving towards the A-site) (Yusupova *et al.* 2006; Jenner *et al.* 2007; Jenner *et al.* 2010b). Indeed, Jenner and coworkers even speculate that the 30S "closure" may be caused by tRNA binding to the A-site. An impairment in the latter process – caused by e.g. an improper conformation of helix 69 in the H69 variant ribosomes – may therefore interfere with the events leading to the transition of the ribosome from initiation to elongation.

We note that the attribution of the processivity impairment in the oligopeptide assay to a defect in the translocation of the tRNA-mRNA complex is in agreement with the results of our in vivo assay where the distribution of the H69 variant 50S in polysomal fractions was analyzed. Here, four out of seven of the H69 variant 50S were incorporated into the 70S fraction to an extent similar to the WT variant, indicating that the *in vivo* initiation process is functional in those variants (Ref. 1 Figure 9). However, the same H69 variants were progressively underrepresented in the polysomal fractions (di- to tetrasome) as compared to the WT control (Ref.1 Figure 9). Since recent estimates (Brandt *et al.* 2009) indicate that the spacing between the A and E-sites of the successive 70S ribosomes in bacterial polysomes is around 46 nucleotides on an mRNA molecule, a number of translocation rounds are required to accommodate a new 70S ribosome on the initiation codon in a polysome. The progressively reduced level of the H69 variant 50S subunits in the polysomal fractions is therefore compatible with defective translocation of the H69 variant ribosomes, although an impairment in the binding of the EF-Tu-GTP-aa-tRNA ternary complex to the ribosomal A-site (Hirabayashi *et al.* 2006) can also contribute to the elongation defect.

As a way of conclusion, it is interesting to compare the effects exerted on the steps of the ribosomal cycle by i) the single base replacements in helix 69 and by ii) the deletion of the entire helix 69. Thus, while the absence of helix 69 had a relatively more severe impact on subunit association and factor-dependent initiation as compared to single-base replacements in helix 69, single base replacements were in turn less tolerated in the elongation assays where the ΔH69 variant ribosomes fared much better (Table 2). This trend was retained under in vivo conditions, where the ΔH69 variant 50S subunits were poorly incorporated into the 70S fraction – indicating a disturbed initiation process – but displayed a roughly 70S-level representation in the successive polysomal fractions. It therefore appears that the mere presence of helix 69 – in whatever conformation – is important for the processes involving the formation of the 70S particle whereas this structure is more dispensable in the elongation phase where the 70S particle has already been formed and part of the role of bridge B2a in keeping the subunits associated has likely been taken over by other bridges and tRNAs. It is conceivable that during initiation helix 69 is required for the positioning of IF2 and/or fMet-tRNA^{fMet} in the newly formed 70S initiation complex and can fulfill its role even in a suboptimal conformation. In

elongation the situation may be reversed and it is better for the ribosome not to have bridge B2a at all than have it in an improper conformation that interferes with the conformational dynamics of the particle. We note that this interpretation is in agreement with the study of Ali and coworkers (Ali *et al.* 2006) where lack of helix 69 had no major effect on the elongation phase reactions but led to a defective subunit recycling in the termination phase. The cellular growth rate was similarly impaired upon expression of the 23S rRNA harboring either single-base replacements in helix 69 or lacking the helix altogether (Ref. 1 Figure S1). This fact indicates that although different mutations in helix 69 seem to affect different part reactions of translation, translation must remain intact in its entirety to support a WT-level cell growth.

Table 2. A qualitative estimation of the effects of H69 mutations on various steps of the ribosomal working cycle.

Assay	Effect of:	
	Base replacement in H69	Deletion of H69
Factor-dependent initiation	* *	* * *
Subunit reassociation	* *	* * *
NAcPhe-puromycin synthesis	*	*
Poly(Phe) synthesis	* * *	*
Oligopeptide synthesis	* * *	*
fMet-Phe-tRNA "drop-off"	* * *	*

* – modest effect
* * – medium-level effect
* * * – severe effect

2.2. Reference 2. Elucidation of the role of pseudouridines in helix 69 of 23S rRNA for factor-dependent peptide release

This study was a continuation of the biochemical investigations into the role of helix 69 pseudouridines and the synthase RluD that had been previously conducted in our laboratory. When we started the study the following facts about the pseudouridines in helix 69 were known. First, their universal conservation in all three domains of life (Ofengand and Bakin 1997). This conservation indicates that the presence of the pseudouridines in helix 69 makes some part of the translation apparatus of the pseudouridine-containing ribosome more efficient compared to the unmodified ribosome. Without a benefit from having the pseudouridines, it would be difficult to explain the expenditure of metabolic energy for the synthesis of the corresponing modification enzyme

RluD[†]. The importance of the pseudouridines in helix 69 for cellular functioning was demonstrated by the finding that inactivation of the corresponding synthase RluD confers a notable growth defect on the cells (Huang *et al.* 1998; Ofengand *et al.* 2001; Gutgsell *et al.* 2005). A plausible explanation for the *rluD*$^{-}$ growth phenotype seemed to be that by eliminating the synthetase activity from the cell and consequently the pseudouridines from helix 69 a crucial step in translation was affected – the very step for the sake of which the pseudouridines had been kept in helix 69 over the course of evolution. However, the identity of this step still remained elusive. A significant step forward in assigning a specific role to the pseudouridines in helix 69 was made by Ejby and coworkers in 2007. In their genetic study Ejby *et al.*2007 sought for mutations in the genome of *E.coli* that would allow a WT-level growth of the cells in the absence of RluD (and pseudouridines in helix 69) (Ejby *et al.* 2007). In other words, they tried to find second-site mutations that would compensate for the lack of a functional RluD enzyme. The rationale behind this strategy is that if the lack of RluD and helix 69 pseudouridines is compensated by a mutation in a factor whose function in a cellular process is known, it indicates that the pseudouridines of helix 69 participate in the same process. The finding by Ejby and colleagues that the lack of pseudouridines in helix 69 can be compensated by the E172K mutation in polypeptide release factor 2 (RF2) therefore points to a role of those pseudouridines in translation termination. Since the publication of the results of Ejby and colleagues further compensatory mutations have been found in RF2 and also in the class II release factor RF3 (O'Connor and Gregory 2011). The conclusion of Ejby *et al.* 2007 about the involvement of the helix 69 pseudouridines in factor-depependent peptide release is strenghtened by recent x-ray crystallography data showing a close interaction between helix 69 and release factors RF1 and RF2 that is required for peptide release (Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010).

After the publication of the results of Ejby *et al.* 2007 the question about the function of the pseudouridines of helix 69 in translation seemed to be settled. However, there were two considerations that motivated us to embark on the investigation of the role of the helix 69 pseudouridines in termination.

First, the strategy of Ejby and colleagues (Ejby *et al.* 2007) was based on the implicit assumption that peptide release is the only cellular function of RF2. Consequently, if a mutation in RF2 compensates for the lack of pseudouridines in helix 69, this indicates that the pseudouridines function in peptide release,

[†] The physiological importance of pseudouridine was suspected by early molecular biologists soon after its discovery in the mid-1950s. Quoting again the „founding fathers" : „It is impossible to believe that this (i.e. Ψ) is an accident or is done for trivial reasons. A little thymine may slip into RNA, or a methyl group may be tacked on here and there, and nobody get excited. But to forge a carbon-carbon bond, and to provide an appreciable amount in one special type of RNA ..... one does not have to be a theoreticiant to hear Nature when she shouts at one " (Crick, F. H. C and Brenner, S. (1959). A Note for the RNA Tie Club)

too. If, on the other hand, RF2 has a second ribosome-related function in translation in addition to peptide release, the E172K mutation in RF2 may instead exert its compensatory effect via this second process. The results of Ejby *et al.* 2007 do not therefore completely rule out the possibility that the true role of the helix 69 pseudouridines is not in peptide release but, for instance, in ribosome assembly. A possibile connection between the release factors and ribosome assembly is suggested by the studies of Rydén-Aulin and Kaczanowska where mutations in ribosomal assemby factors were found to compensate for mutations in release factor RF1 (Kaczanowska and Rydén-Aulin 2004 and 2005). The finding by O'Connor and Gregory that some mutations in RF2 are able to restore the otherwise defective subunit association in the *rluD*$^{-}$ cells to the WT-level (O'Connor and Gregory 2011) is also compatible with a role of RF2 in ribosomal assembly. Although somewhat tentative, these suggestions bring out the limitations of a purely genetic approach in elucidating the role of helix 69 pseudouridines and justify a complementary biochemical approach that has the advantage of monitoring – under precisely defined in vitro conditions – the RF2-dependent peptide release without interference from other cellular processes.

The second and more intriguing observation motivating our study was that neither Ejby and coworkers nor our laboratory had managed to find any compensatory mutations in the other class I release factor – RF1. A similar lack of compensatory mutations in RF1 has later been reported by O'Connor and Gregory. Now, if pseudouridines in helix 69 are indeed required for peptide release, one would expect to find compensatory mutations in RF1 as well since RF1 and RF2 share a similar tertiary structure and interaction pattern with the ribosome (Rawat *et al.* 2003; Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010). Hence, RF1 and RF2 would presumably respond similarly to changes in the structure of helix 69 incurred by the lack of pseudouridines. The inability to recover compensatory mutations in RF1 therefore indicates that either i) the mechanisms of peptide release by RF1 and RF2 are different or again that ii) the pseudouridines in helix 69 are not required for peptide release but some other process in which RF2, but not RF1, participates. In perusing the relevant literature it did not escape our notice that compared to RF1 release factor RF2 behaves differently from RF1 (Tate *et al.* 1983) and is generally more susceptible to mutations in different parts of the ribosome (Arkov *et al.* 1998; Arkov *et al.* 2000; Bouakaz *et al.* 2006). This observation is in agreement with proposal i, namely that the mechanism of peptide release is different with RF1 and RF2.

To analyze the interaction of the helix 69 pseudouridines with release factors RF1 and RF2 we monitored the release of the fMet-Phe dipeptide in an in vitro system consisting of purified components of the *E.coli* translation apparatus. Throughout the experiments, the more widespread and more active Ala246 variant of RF2 was used (Mora *et al.* 2000; O'Connor and Gregory 2011). As RF2 was produced by overexpresion from a plasmid, it was undermethylated at

Gln252 (M. Leppik, unpublished data) as reported previously (Mora *et al.* 2007). Three types of 50S subunits were used – those lacking all three pseudouridines in helix 69 ("-3Ψ" variant), those where pseudouridines had been reintroduced by *in vitro* treatment with purified RluD ("+RluD" variant) and the WT 50S subunits as controls. As an additional control 50S subunits lacking the entire helix 69 ("ΔH69" variant) were used in the study. The 70S prerelease complexes were formed by mixing the EF-Tu•GTP•[^{3}H]Phe-tRNA^{Phe} ternary complex with the 70S initiation complex containing fMet-tRNA^{fMet} in the P-site and the UUU codon in the A-site followed by the UAA stop codon. The UAA stop codon was chosen beacause it i) is efficiently decoded by RF2 and ii) is also recognized by RF1, thus enabling the use of the same mRNA in experiments with both release factors. The fMet-[^{3}H]Phe-tRNA^{Phe} was translocated to the P-site by EF-G present in the mixture of the ternary complex – yielding the prerelease complex with the UAA codon in the A-site. In most of the cases the prerelease complex was not purified from the starting components and was used directly in the peptide release experiments. The amount of the dipeptidyl-tRNA present in the prerelease complex prior to the termination reaction was estimated by its puromycin reactivity. The peptide release reaction was started by the addition of release factor RF1 or RF2. The reaction was quenched at different times by the addition of 5 volumes of ice-cold 5% trichloroacetic acid (TCA), the ribosomal complexes were spun down and the released fMet-[^{3}H]Phe was extracted into ethyl acetate from the supernatant. The resulting time courses of fMet-Phe release were generally biphasic and were therfore analysed as a sum of an exponential and a linear term in the form of

$$F_{fMet-Phe} = \Pi*[1-\exp(-k_{RAPID}*t)] + [RF]*k_{SLOW}*t.$$

Here, k_{RAPID} and k_{SLOW} are the apparent first-order rate constants for the exponential phase and the ensuing linear phase, respectively, and Π is the amount of fMet-Phe formed at a time t = 0 when extrapolated back from the linear phase. Although biphasic kinetics is frequently encountered in ribosome studies and may reflect the heterogeneity of the ribosomal preparation, we have additional information to support our interpretation of this kinetics as being due to the rapid RF-induced peptidyl-tRNA hydrolysis reaction that is followed by a rate-limiting dissociation of the factor from the postrelease complex. First, the slow phase disappeared (and the rapid phase was accelerated to a virtual completion within the first 10 seconds) when the release factors were used in excess over the 70S complex (Ref. 2 Figure S2). Under the conditions of excess RF, the release of fMet-Phe is a single-turnover reaction and is consequently not limited by the dissociation of the factor. Hence, using RF2 in excess over the 70S complex would lead to the disappearance of the slow phase if our inter-pretation were valid. Instead, if the biphasic behavior were caused by the presence of two kinds of ribosomes with different activities, one would expect

to see this biphasic behavior independently of the RF2:70S complex ratio. The observation that the slow phase disappeared when RF2 was used in excess therefore supports our kinetics scheme for the RF2-dependent fMet-Phe release. As a second argument in support of our interpretation, the rate of the slow phase was increased when class II release factor RF3 was included in the reaction mixture in the presence of GTP (Ref.2 Figure S1). This is exactly what one would expect if the slow phase were due to the slow dissociation of RF1/2 from the postrelease ribosome since the function of RF3 is generally held to be in facilitating this dissociation (Freistroffer *et al.* 1997; Zavialov *et al.* 2002).

Another possible objection to our interpretation of the biphasic kinetics of fMet-Phe release – that it is a process consisting of rapid dipeptidyl-tRNA hydrolysis and the following slow dissociation of the factor from the ribosome – would be that the slow linear phase in fMet-Phe release is not due to the rate-limiting dissociation of the factor but is instead caused by a spontaneous dissociation and reformation of the ribosomal complex in the presence of excess starting components (translation factors, tRNAs and mRNA). According to this scenario ("multiple turnover of the substrate") the postrelease complex slowly falls apart and a new prerelease complex is formed in the reaction mixture. The "multiple turnover of the substrate" mechanism is likely independent of the RF2:70S ratio. Hence, the disappearance of the slow phase of fMet-Phe release in the presence of excess RF2 (Ref. 2 Figure S2) seems to disfavor the "multiple turnover" mechanism. However, to be more confident in rejecting the "multiple turnover of the substrate" scenario we purified the 70S prerelease complexes by gel-filtration on a Sephacryl S-300 column as described by Zavialov *et al.* 2002. The purified complexes were used as substrates for the RF1/2-catalyzed peptide release under conditions where the 70S complexes were in excess over RF2. If the slow linear phase in fMet-Phe were caused by a turnover of the substrate (i.e. the ribosomal complex), it would not be observed on the purified complexes since the complex cannot reform in the absence of the starting components. The fact that the linear phase was present on the purified prerelease complexes (Ref. 2 Figure S4) argues against the "multiple turnover of the substrate" scenario but is compatible with the notion of the rate-limiting dissociation of the release factor from the ribosome. Collectively, the control experiments described above allowed us to be fairly confident that in our assays we monitor the effect of pseudouridine depletion on the hydrolysis of fMet-Phe-tRNA and the dissociation of the factor from the ribosome.

The analysis of the RF1- and RF2-dependent peptide release on the H69 variant ribosomes revealed that in the absence of pseudouridines i) peptide release is indeed impaired but ii) only with RF2 (Ref.2 Figure 1). This defect in RF2-dependent peptide release was alleviated when the "-3Ψ" 50S were treated with RluD prior to their inclusion into the termination complex (Ref.2 Figure 1). The latter fact serves to further strengthen the link between the efficiency of peptide release by RF2 and the pseudouridylation state of helix 69. With RF1 an essentially WT-level fMet-Phe release was observed on the ribosomes lacking

the pseudouridines in helix 69 (Ref. 2 Figure 1). All of the H69 variant ribosomes, including the "-3Ψ" variant, displayed an essentially WT-level activity in the fMet-Phe-puromycin assay (Ref. 2 Figure S5). Our results thus provide further support for the suggestion by Ejby *et al.* that the pseudouridines in helix 69 are involved in peptide release and argues against their involvement in other RF2-related processes (if any there be). On the other hand, the resilience of the RF1-catalyzed peptide release to the depletion of pseudouridines from helix 69 indicates that the mechanisms of peptide release by RF1 and RF2 are different, at least in the case of short peptide sequences. Since the different kinetic behavior of RF1 and RF2 is most likely related to (subtle) differences in their structure and interaction with the ribosome, we analyzed the interactions between the nucleotides of helix 69 and amino acid residues from RF1 and RF2 using the published ribosome-RF cocrystal structures of Laurberg *et al.* 2008 and Weixlbaumer *et al.* 2008 The interactions between helix 69 and the release factors probably help to shift the equilibrium towards the more extended conformation of RF1/2 that is required for docking the GGQ motif in domain III into the ribosomal peptidyl transferase centre (Laurberg *et al.* 2008; Weixlbaumer *et al.* 2008; Korostelev *et al.* 2010). In line with the different behavior of RF1 and RF2 in peptide release, our analysis revealed differences in the interactions between helix 69 and RF1 or RF2. Overall, RF1 was found to form more contacts with helix 69 than did RF2 (Ref. 2 Figure 4; Table S1 and S2). With RF1 amino acid residues from the "switch region" between domains III and IV were found to line the loop part of helix 69 from the A-site side (Ref.2 Table S2) whereas similar interactions were lacking in the case of RF2 (Ref. 2 Figure 4; Table S1). It is therefore conceivable that due to the higher number of interactions between RF1 and helix 69 there is a redundancy in those contacts. Consequently, disrupting some of those interactions by mutations in helix 69 will not significantly affect the activity of RF1 since enough binding energy is provided by the interactions that are retained. With RF2 on the other hand, there may not be any "back-up" interactions that would take over the function of the contacts that are broken by mutations in helix 69. Loss of stabilizing interactions with helix 69 would in turn lead to a less efficient stimulation of peptide release by RF2 and an enhanced dissociation of the factor from the ribosome. This is in agreement with the experimental results where the rate of the rapid phase of the RF2-dependent fMet-Phe release (interpreted as the hydrolysis of fMet-Phe-tRNA^{Phe}) was decreased and the rate of the slow phase (interpreted as the dissociation of the factor) increased upon depletion of pseudouridines from helix 69 (Ref. 2 Table 1). In the absence of the entire helix 69 the peptide release is expected to be severely impaired with both factors since none of the abovementionled stabilizing interactions can be formed. This expectation is largely confirmed by the very low level of fMet-Phe release observed on the "ΔH69" variant ribosomal complexes with both RF1 and RF2 (Ref 2. Figure S6). However, even here it was RF1 that exhibited a slightly higher residual activity than RF2 (Ref. 2 Figure S6). This may be due to

additional differences in the interactions of the ribosome with RF1 and RF2, e.g. differences in the interactions at the L11 "stalk" region (Tate *et al.* 1984; Petry *et al.* 2005).

Since our analysis did not reveal the involvement of any of the pseudouridines themselves in the contacts between helix 69 and RF1 or RF2 (Ref.2 Table S1 and S2), we reason that their effect on the factor-dependent peptide release must be mediated via the conformation of helix 69. This suggestion is compatible with the results of the studies by Chow and coworkers on the synthetic analogues of helix 69 which indicate that the presence/absence of pseudouridines in different locations in helix 69 has indeed an influence on the conformation of the helix (Meroueh *et al.* 2000; Desaulniers *et al.* 2008). Though the conformational changes in helix 69 caused by the depletion of the pseudouridines are apparently not extensive, they may be sufficient to disrupt the interactions that RF2 makes with helix 69 and lead to a partially defective peptide release. Admittedly, the 3-fold decrease in the rate of the RF2-dependent fMet-Phe hydrolysis appears modest in comparison with the three to four orders of magnitude decreases in peptide release that are observed when rRNA residues directly participating in peptidyl-tRNA hydrolysis are mutated (Polacek *et al.* 2003; Youngman *et al.* 2004). However, we note that the moderate extent of the impairment of the RF2 function upon pseudouridine depletion as observed by us parallels the magnitudes of the effects observed with pseudouridine depletions from other parts of the ribosome (King *et al.* 2003; Baxter-Roshek *et al.* 2007; Liang *et al.* 2007; Piekna-Przybylska *et al.* 2008b). Collectively, the moderate effects of pseudouridine depletions indicate that the ribosomal pseudouridines do not directly participate in the "core" reactions of translation (like e.g. the 23S rRNA residues A2451 or A2602 that have a well-defined role in the proton shuttle during peptide bond formation) but rather exert their influence through subtly modifying the conformation of rRNA in those regions that do participate in those "core" processes. In other words, the pseudouridines contribute to the ribosomal functioning by fine-tuning the structural environment of the catalytic reactions. The small improvement to the translational machinery that is provided by the presence of pseudouridines may assume a greater importance under more adverse growth conditions. Release factor RF2 itself is a good example here. Similarly to the ribosome, RF2 is specifically modified – the modification being a methylation of the Gln residue at the position 252 by the methylase PrmC. This methylation is important for catalyzing the hydrolysis of the ester bond between tRNA and the polypeptide C-terminus (Nakahigashi *et al.* 2002; Graille *et al.* 2005; Mora *et al.* 2007; Jin *et al.* 2010). Though undermethylation of RF2 has been shown to impair its in vitro activity (Mora *et al.* 2007), inactivation of the PrmC methylase did not cause a significant growth defect of the cells when they were cultivated in the rich growth medium (Mora *et al.* 2007). However, the absence of the methylase was shown to confer a clear growth disadvantage to the cells in a minimal medium and even more so when solid media were used (Mora *et al.*

2007). Similarly, deletion of RluA, an *E.coli* pseudouridine synthase that forms a pseudouridine at the position 746 in 23S rRNA adversely affected cellular viability in stationary phase when the *rluA*$^-$ strain of *E.coli* was cultivated together with WT cells (Raychaudhuri *et al.* 1999). In the latter study, a smaller but still observable growth defect was also seen with the *rluC*$^-$ and *rsuA*$^-$ strains of *E.coli* when grown in competition with the WT. In light of this knowledge, it can be imagined that the small deficiencies in the functioning of the termination apparatus which are caused by the lack of pseudouridines from helix 69 and are tolerated under favorable growth conditions become growth-limiting when a cell of *E.coli* experiences environmental stress or has to compete with its better-equipped cousins. Inefficient translation termination may for instance interfere with the expression of an essential stress-response protein via mechanisms like ribosome queuing (Jin *et al.* 2002) or tmRNA-mediated peptide tagging and mRNA cleavage (Collier *et al.* 2002; Liu *et al.* 2007). This in turn may lead to an insufficient expression of a gene or set of genes required for the adaptation to the stationary phase. In addition, when discussing the role pseudouridines in helix 69 it is important to bear in mind that although the negative effect of their depletion on the translation termination was modest *in vitro*, it may be enhanced in vivo through the interplay with a particular genetic background and therefore still significantly affect cellular viability even under favorable growth conditions. In other words, the modest effects regularly seen upon pseudouridine deletions at least in the exponential phase may be due to the otherwise optimally functioning translational apparatus. The interplay of the pseudouridylation state of helix 69 and the genetic background of the bacteria is exemplified by the the mutational studies of O'Connor and Gregory (O'Connor and Gregory 2011) and Schaub and Hayes (Schaub and Hayes 2011). In those studies the lack of pseudouridines in helix 69 affected cellular viability on the condition that the process of termination was already functioning suboptimally due to the Ala-to-Thr mutation at position 246 of RF2 and/or an extension in the C-terminus of ribosomal protein S7 (both in *E.coli* strain K-12). Thus, when defects in different components of the translational apparatus – by itself not sufficient to impair the ribosomal functioning – accumulate over a certain threshold, they start to have pronounced physiological effects. Conversely, a cell can be made more resistant to the effect of mutations (like the Ala-to-Thr conversion at position 246 of RF2) by making its translational apparatus more resilient to the disruption of molecular interactions that those mutations cause. This resilience may be achieved, among other means, by modulating the conformational dynamics of a region of rRNA by the presence of pseudouridines.

In the end, two general conclusions can be drawn from the previous discussion. First, that one has to retain a certain degree of caution when interpreting and generalizing results obtained in one particular model organism. Apparently, the dictum of Jaques Monod that what is good for *E.coli* is also good for an elephant need not always be true. As a model organism, *E.coli* strain K-12 has

been living in an environment that – as far as the authors experience goes – is far more favorable than the harsh environment of *omne bellum contra omnes* inhabited by the majority of microorganisms. Thus, the example of *E.coli* K-12 and its RF2 shows that when evolutionary pressure is releaxed, *Cell*s may acquire phenotypes that do not adequately reflect the general metabolism under field conditions. Secondly, the termination troubles in *E.coli* K-12 highlight the fact that results obtained *in vivo* are not always unambiguous and therefore justify a complementary in vitro approach as a *primus inter pares*.

2.3. Reference 3. Factors determining the substrate specificity of the pseudouridine synthase RluD

When the study on the substrate-specificity of the pseudouridine synthase RluD was started in our laboratory, the following facts about this enzyme were known. First, RluD is solely responsible for making the three pseuouridines at the positions 1911, 1915 and 1917 in helix 69 of 23S rRNA. With regard to this "promiscuous" specificity RluD is similar to two other pseudouridine synthases RluC and TruA that are also able to form pseudouridines at multiple sites in rRNA/tRNA (Ofengand 2002). Second, the pseudouridines in helix 69 appeared to have an important role in translation as suggested by i) their presence in the ribosomes of representative organisms from all three domains of life (with a slight exeption in the case of Ψ1911 that is absent in Archea) (Ofengand and Bakin 1997; Ofengand 2002) and ii) the observation that the inactivation of the synthase RluD (and the consequent lack of pseudouridines in helix 69) adversely affects cellular viability (Gutgsell *et al.* 2005). However, at that time little was known about the factors influencing the substrate specificity of RluD and the timing of its action in vivo. Therefore, our group devised a study to answer the following four questions:

A. Is there any interdependence in the formation of the pseudouridines in helix 69; in other words, does the formation of a pseudouridine at one position influence pseudouridylation at the other two positions in helix 69? In the case of TruA that synthesizes pseudouridines at the positions 38, 39, and 40 in the anticodon stem loop of tRNA, there are indications that pseudouridylation at one position influences the process at the other two positions (Hur and Stroud 2007).
B. Is the in vivo pseudouridylating activity of RluD restricted to the positions 1911, 1915 and 1917? In other words, if uridines were present at other positions in helix 69, would RluD be able to convert them into pseudouridines?
C. In case the answer to the previous question is negative and RluD is specific for the positions 1911, 1915 and 1917, are there any residues in

helix 69 that determine this specifity of RluD? The importance of neighboring residues in specifying the site of pseudouridylation has been demonstrated for the synthases TruB and RluA. With TruA the formation of a pseudouridine at the position 55 in the elbow of tRNA is determined by the neighboring U54: A58 reverse Hoogsteen base-pair (Hoang and Ferré-D'Amaré 2001) and a similar base pair between U33 and A36 is crucial for making Ψ32 in the anticodon stem loop of tRNA by RluA (Hoang *et al.* 2006).

D. When approximately are the pseudouridines formed in helix 69 in vivo; i.e. are they formed on the newly transcribed 23S rRNA, on the large subunit assembly intermediates, on the (nearly) mature 50S particle or as late as in the 70S ribosome

To answer those questions, base replacements (single or double) were generated at selected positions in helix 69 and their impact on the RluD-catalyzed pseudouridylation of helix 69 in vivo was then analyzed using the method of Ofengand and colleagues (Ofengand *et al.* 2001). The base replacements were made at two kinds of positions in helix 69 – i) those not containing U/Ψ in the WT 23S rRNA (positions 1912, 1913, 1914, 1916, 1919, and 1919) and ii) at pseudouridine positions 1911, 1915, and 1917. The H69 variant 50S subunits were expressed in *E.coli* in the presence of functional RluD and purified from the 50S and 70S fractions of the 10–25% sucrose gradients. 23S rRNA was extracted from the 50S using standard protocols and subjected to the treatment with CMCT [1-cyclohexyl-3-(2-morpholinoethyl)carbo-diimidemetho-p-toluenesulfonate] followed by an alkali treatment of the samples. Under those conditions CMCT specifically modifies pseudouridines (Naylor *et al.* 1965; Ho and Gilham 1971; Ofengand *et al.* 2001). The presence of the covalently attached CMCT (and thus pseudouridines) in the 23S rRNA was analyzed by reverse transcription since CMCT, when incorporated into the 23 rRNA, sterically blocks the cDNA synthesis by reverse transcriptase (Ofengand *et al.* 2001). This blockage is revealed by the smaller lenght of the cDNA products in a polyacrylamide gel. In addition to the H69 variant 23S rRNA, WT 23S rRNA and the 23S rRNA extracted from the *rluD*$^{-}$ cells (lacking pseudouridines in helix 69) were used as a positive and a negative control, respectively. In the experiment where the timing of pseudouridine formation with respect to the 50S assembly was analyzed, the 50S subunits were extracted from an *E.coli* strain (*deaD*$^{-}$), negative for the RNA helicase DeaD that has been demonstrated to be deficient in the assembly of the ribosomal large subunit (Charollais *et al.* 2004; Peil *et al.* 2008).

Although the analysis of pseudouridylation was relatively straightforward at the 1911 and 1917 positions, it was not possible to unambiguously analyze pseudouridylation at the position 1915 at the time of our study. This difficulty was caused by two facts. First, at the time of our study we were not sure whether the N3-methylation of the nucleobase at the position 1915 was limited

to pseudouridine (Kowalak *et al.* 1996) or could occur with uridine as well. Since a methyl group at a U/Ψ residue would block primer extension regardless of the identity of the nucleobase (i.e. U or Ψ) the occurrence of a reverse transcriptase stop at 1915 could not be taken as indicative of a pseudouridine at that position. This ambiguity was later resolved by Ero and colleagues (Ero *et al.* 2008) and Purta and colleagues (Purta *et al.* 2008) who showed that the N3-methylation at 1915 occurs only with pseudouridine and not with uridine. We therefore now know that the presence of a reverse transcriptase stop corresponding to the 1915 position – caused by the $–CH_3$ or CMCT group – does unambiguously testify to the presence of a pseudouridine at the 1915 position. However, the absence of a stop at the 1915 position cannot be unequivocally interpreted as due to the absence of Ψ at the 1915 position. This is because in the *syn* conformation $m^3Ψ1915$ can form a Watson-Crick base pair by the N1 of pseudouridine and allow a low level readthrough by the reverse transcriptase (Siibak and Remme 2010). Due to the difficulties with the 1915 position the main focus of our analysis is therefore on the pseudouridylation of the remaining two positions – 1911 and 1917 of 23S rRNA – in helix 69. This analysis allowed us to give following answers to the questions A – D stated above:

A. **The pseudouridines at the positions 1911, 1915 and 1917 in helix 69 are made independently of each other**. This is shown by the observation that despite a U-to-C transversion at one (or two, as in the case of the U1911C/U1915C variant) of the three wild-type pseudouridine positions (i.e. 1911, 1915, 1917), the pseudouridines were present at the two remaining positions (Ref. 3 Figure 3)
B. **RluD is unable to form pseudouridines at positions in helix 69 other than the wild-type positions 1911, 1915 and 1917 *in vivo***. This is shown by the fact that additional reverse transcriptase stops were not observed with the H69 variant 23S rRNAs that had U inserted at one of the positions 1912, 1914, 1916, or 1919 (Ref.3 Figure 2).
C. **Most of the base replacements at the "non-pseudouridine" positions (A1912C, A1913G, C1914A, A1918G, A1919G) did not affect pseudouridylation at 1911, 1915 or 1917** (Ref. 3 Figure 4). However, the A-to-G and A-to-U substitutions at the 1916 position practically abolished pseudouridylation at the 1911 and 1917 positions on the 50S subunits and reduced it strongly in the 70S (Ref.3 Figure 4). In contrast, the A1916C mutation had a minor effect on the pseudouridine formation at the 1911 and 1917 positions (Ref.3 Figure 4). **Taken together, those results point to A1916 as a specificity determinant of RluD in vivo**
D. Analysis of the 23S rRNA from the 50S subunit assembly intermediates that accumulate in the deaD-deficient strain showed that pseudouridines were absent from helix 69 in those 50S precursors (Ref. 3 Figure 5). This finding, together with the fact that pseudouridines were present in the mature 50S subunit, indicates that RluD makes pseudouridines at a time

when the assembly of the large subunit has reached a phase where differences in the particle shape in a density gradient no longer allow to distinguish it from a mature 50S subunit. In a looser sense, it can therefore be concluded that **RluD makes pseudouridines in helix 69 at a late stage of the 50S assembly**. The same conclusion has been reached by Vaidyanathan and colleagues, based on their observation that RluD is much more efficient in pseudouridylating the structured 50S particle than naked 23S rRNA (Vaidyanathan *et al.* 2007).

Because there is currently no RluD-helix 69 cocrystal structure available, the structural basis of our results can be discussed only tentatively. As for the importance of the residue at the 1916 position in directing RluD to its site of action, we note that pseudouridylation of helix 69 was abolished with the A-to-U and A-to-G mutations but not with the A-to-C mutation at the aforementioned position (Ref. 3 Figure 4). Since the pattern of hydrogen bond donors and acceptors along the Watson-Crick edge is similar with A and C (except for the O2 of C) but is reversed with U and G, our mutagenesis results point to a possibility that a base-pairing interaction between A1916 and an other residue in helix 69 is somehow involved in determining the specificity of RluD. Indeed, the formation of a reversed Hoogsteen base pair between U33 and A36 in the anticodon stem loop of $tRNA^{Phe}$ in the course of the pseudouridylation reaction has been shown to be important for ensuring the specificity of the synthase RluA that makes Ψ32 in the ASL (Hoang *et al.* 2006). We note in this respect that the anticodon stem loop of tRNA and helix 69 share a similar tertiary structure, though not a similar sequence. Morover, the location of U32 in the ASL of $tRNA^{Phe}$ is in many respects similar to that of U1917 (located next to A1916) in helix 69. Inspection of the structure of helix 69 in the context of the isolated 50S subunit from *D. radiodurans* (Bashan *et al.* 2003) revealed A1913 as the most plausible interaction partner of A1916. In helix 69 A1913 occupies a position qualitatively similar to that of A36 in the ASL of $tRNA^{Phe}$ in the absence of the modification enzyme (Hoang *et al.* 2006). Thus, it is at least conceivable that a noncanonical base pair involving the Watson-Crick edge of A1916 (or C for that matter) is formed between A1913 and A1916 and that this base pair contributes to the flipping-out of U1917 into the active site of RluD analogously to the situation in RluA (Hoang *et al.* 2006). Although speculative, this idea is valuable as a prediction that will be proved or disproved once a crystal structure of RluD in complex with helix 69 becomes available. Alternatively, A1916 may interact with an amino acid residue from RluD and this way either stabilize helix 69 in the transition state or provide an initial docking point for the enzyme.

Interestingly, base replacements at the positions 1912 and 1919 did not affect the RluD-mediated pseudouridylation at the 1911, 1915, and 1917 position (Ref. 3 Figure 4). In ribosomal crystal structures A1912 and A1919 are seen to interact with other helix 69 residues as well as residues from 16S rRNA

(Schuwirth *et al.* 2005; Selmer *et al.* 2006; Jenner *et al.* 2010a) and are therefore expected to be important for the conformational dynamics of helix 69 and hence for subunit association. This expectation is corroborated by the results of our mutagenesis study (Ref.1) where base replacements at the 1912 and 1919 positions impaired ribosomal functioning in a number of part reactions of translation (Ref. 1). This impairment in ribosomal activity was most likely due to changes in the structure of helix 69. If true, it means that the structural perturbations caused by mutations at the 1912 and 1919 positions – which did not affect RluD activity – are distinct from those induced by base replacements at the 1916 position. The example provided by our study – namely that changes in different parts of a relatively small rRNA element such as helix 69 can result in disctinct conformational states of the element underlines the potential for structural flexibility of RNA in general.

A comparison of the pseudouridine content of helix 69 in the 23S rRNAs extracted from i) the deaD-deficient large subunit assembly precursor particles and ii) the mature 50S subunits revealed that the helix 69 pseudouridines were present in the latter but absent in the former (Ref. 3 Figure 5). Our group has recently provided additional evidence that the *deaD*$^{-}$ particles are authentic intermediates in the 50S assembly pathway and not degradation products of the misassembled 50S subunits (Peil *et al.* 2008). The lack of pseudouridines in helix 69 in the large subunit assembly precursors therefore indicates that RluD acts during late stages of the 50S subunit assembly. This conclusion is in agreement with the observation of Vaidyanathan and coworkers that RluD acts much more efficiently on the 50S and 70S particles than on naked 23S rRNA (Vaidyanathan *et al.* 2007). Another recent study by our group has demonstrated that RluD is the only *E.coli* pseudouridine synthase that acts during late stages of the large subunit assembly, similarly in this respect to the methyltransferases RlmE and RlmH (Siibak and Remme 2010). The different timing of the action of RluD during the assembly of the 50S is likely due to sterical reasons. Thus, whereas the other pseudouridine modifications in 23S rRNA are located in regions that would become more or less inaccessible for the respective synthases as the large subunit assembly proceeds, 23S rRNA in helix 69 is easily accessible even within the mature 50S subunit. Although the region corresponding to helix 69 is also accessible during early steps of the large subunit assembly it is possible that the docking of RluD – likely through its S4 domain – requires a particular fold in the 23S rRNA around helix 69 – e.g. the three-way junction of helices 68, 69 and 70. Supporting the role of domain S4 in guiding RluD to the helix 69 region is the observation that upon the deletion of S4 RluD acquired the ability to pseudouridylate U2457 of 23S rRNA – the usual substrate of RluE (Vaidyanathan *et al.* 2007).

2.4. APPENDIX I

Reaction conditions in the 70S initiation assays

- **Present study (Kipper *et al.* 2009):**
  - Buffer: 20 mM Hepes-KOH (pH 7.6), 95 mM KCl, 5 mM NH_4Cl, 5 mM $Mg(OAc)_2$,0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 1 mM DTE
  - Temperature: 37°C
  - 30S : 0.3 µM (*E.coli* MRE600)
  - 50S : 0.3 µM (*E.coli* MRE600)
  - f[^{35}S]Met-tRNA^{fMet} : 0.5 µM
  - mRNA : 2.0 µM
  - IF1 : 1.0 µM
  - IF2 : 0.5 µM
  - IF3 : 1.0 µM
  - GTP : 1 mM
  - Puromycin: 0.2 mM
- **La Teana *et al.* 1996:**
  - Buffer: 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 7 mM $Mg(OAc)_2$, 1 mM DTT
  - Temperature : 37°C
  - 30S : 0.3 µM (*B.stearotermophilus*)
  - 50S : 0.3 µM (*B.stearotermophilus*)
  - fMet-tRNA : 0.15 µM
  - mRNA : 0.5 µM
  - IF1 : 0.5 µM
  - IF2 : 0.5 µM
  - IF3 : 0.5 µM
  - GTP : 1 mM
  - Puromycin : 1 mM
- **Cuckras and Green 2005:**
  - Buffer: 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 8 mM putrescine, 0.5 mM spermidine, 2 mM DTT
  - Temperature: 37°C
  - 30S : 1 µM (*E.coli* MG1655)
  - 50S : 1 µM (*E.coli* MG1655)
  - fMet-tRNA : 0.8 µM
  - mRNA : 5 µM
  - IF1 : 3 µM
  - IF2 : 3 µM
  - IF3 : 3 µM
  - GTP : 1 mM
  - Puromycin: 4 mM
- **Antoun *et al.* 2006a:**
  - Buffer: 5 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 95 mM KCl, 5 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 1 mM DTE

- Temperature: 37°C
- 30S : 0.9 µM (*E.coli* MRE600)
- 50S : 0.9 µM (*E.coli* MRE600)
- fMet-tRNA^{fMet} : 2.0 µM
- mRNA : 1.6 µM
- IF1 : 3.0 µM
- IF2 : 1.6 µM
- IF3 : 1.4 µM
- GTP : 1 mM

- **Antoun *et al.* 2006b:**
  - Buffer: Buffer: 5 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 95 mM KCl, 5 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 1 mM DTE
  - Temperature: 37°C
  - 30S : 0.3 µM (*E.coli* MRE600)
  - 50S : 0.3 µM (*E.coli* MRE600)
  - fMet-tRNA^{fMet} : 0.6 or 3.0 µM
  - mRNA : 0.6 µM
  - IF1 : 2.0 µM
  - IF2 : 0.45 µM
  - IF3 : 0.45 µM
  - GTP : 1 mM
- **Grigoriadou *et al.* 2007a:**
  - Buffer: 25 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM DTT
  - Temperature: 20°C
  - 30S : 0.3 µM (*E.coli* MRE600)
  - 50S : 0.5 – 6.0 µM (*E.coli* MRE600)
  - fMet-tRNA^{fMet} : 0.45 µM
  - mRNA : 0.9 µM
  - IF1 : 0.45 µM
  - IF2 : 0.15 µM
  - IF3 : 0.45 µM
  - GTP : 0.1 mM

CONCLUSIONS

The present dissertation has had two main objectives. The first one was to elucidate the role of helix 69 of 23S rRNA in the factor-dependent reactions of the ribosomal working cycle (Ref. 1). Secondly, we analyzed the functional importance of the pseudouridines in helix 69 for the factor-dependent peptide release (Ref. 2). The author also participated in the study where the factors determining the substrate specificity of the large subunit pseudouridine synthase RluD were investigated (Ref. 3).

Our experimental approach followed a similar pattern throughout the three studies. First, positions in helix 69 that seemed to be important for the structural integrity of the helix were selected using information from the published crystal structures. Base replacements were then made at the selected positions in helix 69 and the variant 23S rRNAs were expressed from a plasmid in the presence of chromosomally encoded WT 23S rRNA. The 50S subunits containing the variant 23S rRNA were affinity-purified using a streptavidine-binding apatmer inserted in a region of the variant 23S rRNA that did not interfere with the ribosomal functioning. The purified variant 50S subunits were used in in vitro assays that had been designed to reflect various part reactions of the ribosomal working cycle. In Ref. 2 the 50S subunits lacking pseudouridines in helix 69 were expressed from the chromosomal rrnB gene in an *rluD*$^{-}$ strain of *Escherichia coli*. This strain (a derivative of *E.coli* K-12) lacks the helix 69-specific pseudouridine synthase RluD but contains a compensatory mutations that allows a near-normal growth of the cells despite the absence of RluD.

The most important findings of the publications are summarized below:

Reference 1:
Disrupting the native intra- and interhelical interaction pattern in helix 69 affects the factor-dependent translation initiation to a greater extent than initiation in the absence of the factors. According to our interpretation this indicates that helix 69 is involved in the regulation of the interactions between the initiation factors and the ribosome.

Base replacements in helix 69 cause a progressive decrease in the yield of oligopeptide formation. We interpret this as an impairment in the ability of the ribosome to pass from the initiation phase to processive elongation. We suggest that this transition may include the disruption of the Shine-Dalgarno-anti-Shine-Dalgarno interaction. Mutations in helix 69 may block this disruption.

In vivo we observe a progressive underrepresentation of the H69 variant 50S in polysomal fractions. This finding supports our notion that disrupting the native structure of helix 69 adversely affects ribosomal processivity.

Reference 2:
The presence of pseuoduridines in helix 69 stimulates peptide release by class I release factor RF2. This is shown by a 3-fold decrease in the rate of the RF2-

dependent fMet-Phe release on ribosomes lacking all three pseudouridines in helix 69. However, the absence of pseudouridines in helix 69 does not affect peptide release by release factor RF1. Thus, RF1 and RF2 respond differently to alterations in the structure of helix 69. Our analysis of the structures of the release factors indicates that despite sharing a similar tertiary structure, RF1 and RF2 differ in their interaction pattern with helix 69. According to our structural analysis, RF1 forms a greater number of contacts with helix 69 than does RF2. Therefore, RF1 – differently from RF2 – may be better "buffered" against changes in the structure of helix 69 that are caused by the lack of pseudouridines. Taken together, the results of our study provide additional evidence that the structural effects exerted by the helix 69 pseudouridines are important for at least one particular ribosomal process, namely peptide release.

Reference 3:
The large subunit pseudouridine synthase RluD is specific for helix 69 under in vivo conditions. The RluD-dependent pseudouridylation at any one of the positions 1911, 1915, and 1917 is independent of the pseudouridylation at the two remaining positions. The position 1916 of 23S rRNA is important for the substrate specificity of RluD. RluD-dependent pseudouridylation in helix 69 occurs in the late stages of the 50S subunit assembly.

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SUMMARY IN ESTONIAN

23S rRNA heeliksi 69 roll translatsiooni initsiatsiooni, elongatsiooni ja terminatsiooni faktorsõltuvates etappides

Käesoleva doktoritöö teemaks on struktuuri ja aktiivsuse vaheliste seoste selgitamine bakteriaalse ribosoomi ühe struktuuriüksuse – 23S rRNA heeliksi 69 – näitel. Kuigi tõsiasi, et molekuli struktuur määrab ära tema funktsioneerimise, tundub keemikute ligi kahe sajandi pikkuste pingutuste järel peaaegu iseenesestmõistetavana, pole struktuur-aktiivsus sõltuvuse selgitamine ribosoomisuuruse molekuli korral kaugeltki triviaalne ülesanne. Juba ribosoomi enda ruumilise struktuuri kindlaks määramiseks kulus aega ligi pool sajandit – alates James Watsoni ja Alexander Richi esimestest katsetustest 1950. aastate keskpaigas kuni esimeste keskmise lahutusvõimega kristallstruktuurideni selle aastatuhande esimesel kümnendil. Tänu edusammudele röntgenkristallograafias ja krüoelektronmikroskoopias, samuti täiustatud kristalliseerimismeetoditele, on viimase kümne aasta jooksul kogunenud märkimisväärne teave ribosoomi ruumilise ülesehituse kohta. See teave on võimaldanud siduda ühtsesse tervikusse varasemal ajal ribosoomide kohta biokeemias ja geneetikas kogutud andmed ning andnud neil struktuurse põhjenduse. Veel enam, ribosoomi struktuuri tundmine on võimaldanud välja pakkuda uusi hüpoteese selle suure ribonukleoproteiinse ensüümi ja tema osade funktsioneerimise kohta. Uued – ribosoomi struktuuri analüüsil põhinevad – hüpoteesid vajavad aga kontrollimist biokeemia ja geneetika meetoditega. Säärane biokeemiline analüüs ongi olnud selle doktoritöö eesmärgiks.

Tulenevalt ribosoomi suurusest ja komplekssusest keskendusid käesoleva töö autor ja tema kolleegid ühele väiksemale ribosoomi struktuuriüksusele – suure alaüksuse 23S rRNA heeliksile 69. Meie huvi selle näivalt väikse ribosoomiosa vastu äratasid mitmed asjaolud. Esmalt osutas 70S ribosoomi struktuuri analüüs, et heeliks 69 kuulub ühe kõige suurema ribosoomi alaüksuste vahelise kontakti – silla B2a – koosseisu. Sildavad interaktsioonid alaüksuste vahel on vajalikud funktsionaalse 70S ribosoomi moodustumiseks ja koospüsimiseks valgusünteesi ajal. Sestap võis eeldada, et heeliksil 69 on B2a silla komponendina oluline roll 70S ribosoomi stabiilsuse tagamisel. Samuti on oluline tähelepanek, et sild B2a (ja seega heeliks 69) paikneb 70S ribosoomi nö. funktsionaalses "südames". Näiteks osalevad heeliks 69 nukleotiidid koos väiksema alaüksuse 16S rRNA-ga nn. "dekodeeriva" tsentri moodustumisel. Dekodeerivas tsentris aga toimub kogu geneetilise informatsiooni vigadevaba edasiandmise seisukohalt ülioluline ribosoomi siseneva ja aminohapet kandva tRNA antikoodoni mRNA koodonile vastavuse kontroll. 70S ribosoomis kontakteerub heeliks 69 tRNA-de ja mitmete valgusünteesil osalevate valkude, nn. translatsioonifaktoritega. See asjaolu osutab võimalusele, et heeliks 69

osaleb lisaks subühikute füüsilisele kooshoidmisele valgusünteesi nende etappide regulatsioonil, kus osalevad translatsioonifaktorid. Lisaks juba mainitud omadustele muudavad heeliksi 69 huvitavaks ka temas leiduvad kolm modifitseeritud nukleotiidi – kaks pseudouridiini ja üks metüülpseudouridiin. Peale heeliks 69 leidub pseudouridiine ainult vähestest 23S rRNA osades, mis huvitaval kombel paiknevad teiste ribosoomi fuktsionaalsete tsentrite läheduses. Kuigi pseudouridiinid heeliksis 69 on väga konserveerunud, s.t. esinevad organismides kõigist kolmest elusloouse riigist, oli nende (ja teiste 23S rRNA) pseudouridiinide roll valgusünteesil käesoleva töö algusajal veel ebaselge.

Kogu eelmainitud – valdavalt struktuuril põhinevat – informatsiooni arvesse võttes formuleerisime me hüpoteesi, mille kohaselt heeliks 69 osaleb regulaatorelemendina valgusünteesi neis etappides, kus osalevad ka translatsioonifaktorid. Heeliks 69 regulaatorelemendina toimimisel on oluline tema konformatsiooniline dünaamika. Meie teise hüpoteesi kohaselt on selle dünaamika regulatsioonis oluline roll pseudouridiinidel. Järelikult mõjutavad pseudouridiinid ka heeliks 69 interkatsiooni translatsioonifaktoritega. Nende hüpoteeside eksperimentaalse kontrollimise strateegia oli põhiosas järgnev. Kui heeliks 69 on vajalik translatsioonifaktoritega interakteerumiseks ja nende aktiivsuse regulatsiooniks, siis muutused selle heeliksi struktuuris suure tõenäosusega mõjutavad nende faktorite osalusega kulgevate valgusünteesietappide kineetilisi parameetreid. Järelikult – muutes heeliksi 69 struktuuri suunatud mutageneesi abil ning mõõtes valgusünteesi eri etappide kineetikat muteeritud ribosoomidel, on võimalik eelmainitud hüpoteesi kontrollida.

Eksperimetaalse töö käigus vahetati heeliksi 69 teatud positsioonides suunatud mutageneesi abil metsiktüübis esinev lämmastikalus ära kolme ülejäänud lämmastikalusega. Suunatud mutageneesiks valiti välja sellised positsioonid heeliksis 69, mis 70S ribosoomi kristallstruktuuri põhjal otsustades olid olulised heeliks 69 natiivse struktuuri jaoks ning silla B2a moodustumiseks. Variantset heeliksit 69 sisaldav 23S rRNA geen kloneeriti ekspressioonivektorisse ptBB tac promootori kontrolli alla. Variantse 23S rRNA ekspressioon toimus bakteris *Escherichia coli* koos metsiktüüpi 23S rRNA ekspressiooniga kromosomaalselt rrnB operonilt. Variantsed 50S subühikud puhastati metsiktüüpi 50S subühikutest affiinsuskromatograafia teel. Nimelt sisaldas variantne 23S rRNA streptavidiiniga seonduvat järjestust heeliksis 25, mis võimaldas variantsete 50S subühikute affiinsuspuhastamist strepavidiinsefaroosil. Ribosoomide aktiivsust streptavidiini siduv järjestus ei mõjutanud. Affiinsuspuhastatud 50S subühikuid kasutati järgnevalt erinevaid valgusünteesi etappe kajastavates in vitro katsestes.

Töö käigus saadud tulemused ja nende tõlgendused on esitatud viidatud publikatsioonide kaupa:

Publikatsioon 1 (Ref. 1):
Heeliksi 69 natiivse struktuuri ja B2a silla jaoks oluliste interaktsioonide katkestamine pärsib translatsiooni initsiatsiooni tunduvalt tugevamine initsiat-

sioonifaktorite juuresolekul kui nende faktorite puudumisel. Meie tõlgenduse kohaselt osutab see tõsiasi sellele, et heeliks 69 osaleb initsiatsioonifaktorite aktiivsuse regulatsioonil 70S initsiatsioonikompleksi moodustumisel.

Lämmastikaluste asendused heeliksis 69 vähendasid oligopeptiidide moodustumise saagist. Seejuures oli saagis seda madalam, mida pikema oligopeptiidiga tegemist oli. Meie tõlgenduse kohaselt häirivad mutatsioonid heeliksis 69 ribosoomi üleminekut initsiatsioonifaasist protsessiivsesse elongatsioonifaasi. On võimalik, et heeliksi 69 mutatsioonide blokeeriv toime sellel üleminekule on seletatav häiretega Shine-Dalgarno-anti-Shine-Dalgarno interaktsiooni katkemises, mis on vajalik elongatsioonifaasi minekuks.

Heeliks 69 mutatsioonide pärssiv toime ribosoomide protsessiivsusele oli jälgitav ka in vivo tingimustes. Siin oli silmatorkav H69 variantsete 50S subühikute kasvav alaesindatus polüsoomides. 70S fraktsioonis seevastu oli valdav osa variantsetest 50S subühikutest esindatud metsiktüübiga võrreldaval tasemel.

Publikatsioon 2 (Ref. 2):

Pseudouridiinide olemasolu heeliksis 69 stimuleerib RF2-sõltuvat fMet-Phe dipeptiidi vabanemist peptidüül-tRNA küljest. Seda näitab asjaolu, et pseudouridiinide eemaldamisel heeliksist 69 langeb RF2-sõltuva fMet-Phe vabanemise erikiirus ligi 3 korda. Seevastu RF1-sõltuvat fMet-Phe vabanemist pseudouridiinide puudumine praktiliselt ei mõjutanud. Seega reageerivad RF1 ja RF2 struktuursetele muutustele heeliksis 69 erinevalt. Meie analüüs osutab, et kuigi RF1 ja RF2 ruumiline struktuur on väga sarnane ning "suures skaalas" on sarnane ka nende interaktsioon ribosoomiga, erinevad nende "interaktsioonimustrid" heeliksiga 69. Meie analüüsi kohaselt moodustab RF1 võrreldes RF2-ga heeliksiga 69 rohkem kontakte ja on seetõttu heeliksi 69 struktuuris aset leidavate muutuste vastu paremini "puhverdatud" kui RF2. Kokkuvõtvalt näitavad meie in vitro tulemused koos varasemate geneetiliste katsete tulemustega, et heeliksis 69 esinevad pseudouridiini mängivad olulist rolli ühes kindlapiirlises valgusünteesi etapis, nimelt terminatsioonil.

Publikatsioon 3 (Ref. 3):

In vivo tingimustes on pseudouridiini süntaas RluD spetsiifiline heeliksi 69 suhtes – s.t. ta katalüüsib pseudouridiinide moodustumist ainult selles 23S rRNA osas. Seejuures on pseudouridiini moodustumine ühes positsioonidest 1911, 1915 ja/või 1917 sõltumatu pseudouridiinide olemasolust või puudumisest kahes ülejäänud positsioonis. RluD substraadispetsiifilisuse jaoks in vivo on oluline adeniin heeliksi 69 1916. positsioonis, sest lämmastikaluste asendused seal takistavad pseudouridiinide sünteesi RluD poolt. Heeliksi 69 pseudouridüleerimine toimub 50S subühiku assambleerimise hilises faasis.

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PUBLICATIONS

CURRICULUM VITAE (English)

Name and surname: Kalle Kipper

Date of birth: 22.02.1981

Place of birth: Tallinn, Estonia

Marital status: single

Address: Insitute of Molecular and Cell Biology, Riia 23, 51010 Tartu, Estonia

E-mail: kalle.kipper@ut.ee

Education:

1987–1999	Tallinn 44. Secondary School
1999–2003	BSc in Gene Technology, Faculty of Biology and Geography, University of Tartu.
2003–2005	MSc in Biochemistry and Biophysics, Faculty of Biology and Geography, University of Tartu. Title of the thesis „Processivity of *Trichoderma reesei* cellobiohydrolase Cel7A in cellulose hydrolysis“
2005–...	Graduate studies in Molecular Biology, Faculty of Science and Technology, University of Tartu

Scientific activity:

I have been studying some aspects of protein biosynthesis in *Escherichia coli* using in vitro reconstituted systems

CURRICULUM VITAE (Estonian)

Nimi ja eesnimi:	Kalle Kipper
Sünniaeg:	22.02.1981
Sünnikoht:	Tallinn, Eesti
Perekonnaseis:	vallaline
Aadress:	TÜ Molekulaar- ja rakubioloogia Instituut, Riia 23, 51010 Tartu, Eesti Vabariik
E-mail:	kalle.kipper@ut.ee

Hariduskäik:

1987–1999	Tallinna 44. keskkool
1999–2003	BSc geenitehnoloogia erialal, Tartu Ülikool, Bioloogia-geograafia teaduskond
2003–2005	MSc biokeemia ja biofüüsika erialal, Tartu Ülikool, Bioloogia-geograafiateaduskond. Magistritöö pealkiri „Trichoderma reesei tsellobiohüdrolaas Cel7A protsessiivsus tselluloosi hüdrolüüsil“
2005–...	LO MRI doktorant molekulaarbioloogia erialal, Tartu Ülikool, Loodus- ja tehnoloogiateaduskond

Teaduslik tegevus:
Olen tegelenud mõnede *Escherichia coli* valgusünteesi aspektide uurimisega kasutades *in vitro* katsesüsteeme

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