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TUNING RIBOSOMAL ELONGATION CYCLE BY MUTAGENESIS OF 23S rRNA

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PRESS

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Dissertation is accepted for the commencement of the degree of Doctor Philosophy (in Molecular Biology) on October 17, 1997 by the Council of the Institute of Molecular and Cell Biology, University of Tartu

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Dr. Mart Speek (Institute of Molecular and Cell Biology, Tartu, Estonia)

Commencement: December 15, 1997

Publication of this dissertation is granted by the Estonian Science Foundation

Tartu Ülikooli Kirjastuse trükikoda
Tiigi 78, EE 2400 Tartu
Tellimus nr. 315

Vend Priidule

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LIST OF ORIGINAL PUBLICATIONS

Current dissertation is based on the following original publications referred to in the text by their Roman numerals:

I **Saarma, U.** & Remme, J. 1992. Novel mutants of 23S RNA: characterization of functional properties. Nucl. Acids Res **20**: 3147–3152.

II **Saarma, U.,** Lewicki, B. T. U., Margus, T., Nigul, S., and Remme, J. 1993. Analysis of mutations in the 23S rRNA. *In* The Translational Apparatus: structure, function, regulation, evolution. *Edited by* Nierhaus, K. H., Franchesci, F., Subramanian, A. R., Erdmann, V. A., and Wittmann-Liebold, B. Plenum Press, New York, pp. 445–454.

III **Saarma, U.,** Remme, J., Ehrenberg, M., and Bilgin, N. 1997. An A to U transversion at position 1067 of 23S rRNA from *Escherichia coli* impairs EF-Tu and EF-G function. J Mol Biol **272**: 327–335.

IV **Saarma, U.,** Spahn, C. M. T., Nierhaus, K., and Remme, J. 1997. Mutational analysis of the donor substrate binding site of the ribosomal peptidyltransferase centre. (Manuscript submitted to RNA).

LIST OF ABBREVATIONS

PTase	—	peptidyl transferase
PTC	—	peptidyltransferase centre
GTP	—	guanosine triphosphate
GDP	—	guanosine diphosphate
r-protein	—	ribosomal protein
EF-Tu	—	elongation factor Tu
EF-G	—	elongation factor G
aa-tRNA	—	aminoacyl-tRNA
pep-tRNA	—	peptidyl-tRNA

INTRODUCTION

Protein synthesis is a vital process for all living creatures on Earth. It is the last phase of gene expression that culminates with the translation of mRNA sequence into protein language. The responsibility for the efficiency and accuracy of this process lies on ribosomes. Ribosomes are large ribonucleoprotein particles that consist of one large and one small subunit. In eubacterium *Escherichia coli* these subunits have sedimentation coefficients 50S and 30S, respectively. Most important ribosomal enzymatic activity — peptidyl transferase (PTase), is an integral part of the large subunit (Monro, 1967). 50S subunit contains also another functionally significant region where ribosomal elongation factors bind — the factor centre (Fig. 1). The smaller subunit is accountable for the mRNA decoding process. Both subunits consist of a large number of ribosomal proteins and the following RNAs: in 30S subunits there is 16S rRNA, while 50S subunits contain two rRNA molecules, 23S and 5S rRNA. Remarkably, the primary and secondary structure of ribosomal RNAs are highly conserved throughout evolution. In contrast, ribosomal proteins exhibit a comparatively low level of sequence conservation.

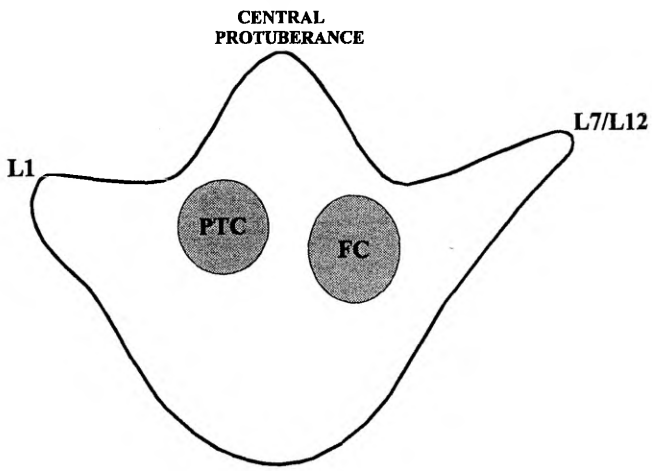


Figure 1. The outline of large ribosomal subunit with its attributes: L1 and L7/L12 arms plus the central protuberance which holds primarily 5S rRNA with its proteins. PTC is for peptidyltransferase centre and FC for factor centre where elongation factors Tu and G bind.

As soon as one realizes that the ribosome is an enzyme, the following question arises — why does it contain the RNA that is so highly conserved in evolution? Here the most likely explanation is that the remarkable uniformity of rRNA structure is a reflection of its functional conservation. Hence it could be

asked — are ribosomes ribozymes? Suggestions that in the development of life on Earth evolution based on RNA replication preceded the appearance of protein synthesis were made nearly 30 years ago (Crick, 1968; Orgel, 1968; Woese, 1967). Discovery of RNA catalysis approximately 15 years ago has injected a new vigor into the evolutionary theories of catalytic molecules (Guerrier-Takada *et al.,* 1983; Kruger *et al.,* 1982). From the RNA world viewpoint, the ribosome is an RNA catalytic machine that aligns the anticodons of tRNAs to the codons of messenger RNA in a complementary fashion and promotes a peptidyl transferase reaction between an aminoacyl tRNA and the adjacent peptidyl tRNA. During the decoding process and peptidyl transferase reaction, the regions of tRNA and rRNA that interact with each other are relatively compact. Codon recognition appears to involve no more than 15 nucleotides of the tRNA structure and a small subdomain of 16S rRNA. The peptidyl transferase centre involves only three nucleotides, the conserved CCA in 3' terminus of tRNA and, apparently, only a small region of 23S rRNA. Thus, these two functional domains are small enough to be within RNA world ribozyme size.

The biological role of RNA is limited by its reactivity. A single RNA molecule can catalyze chemical reactions through its ability to fold into complex three-dimensional structures and to bind specifically other molecules and metal ions. The reactivity of RNA is determined by its 2'-hydroxyl groups located in ribose moieties. The range of activities exhibited by natural ribozymes may not, however, reflect the true catalytic potential of RNA, and this possibility has triggered attempts to develop ribozymes with new catalytic activities. Bearing its potential for functioning, both as a genome and as a gene product, RNA is suitable for *in vitro* evolution experiments enabling the selection of molecules with new properties. The development of *in vitro* selection and *in vitro* evolution methodologies will provide a large number of RNAs with new and improved properties. Using these strategies, ribozymes have been selected that can cleave amide bond (Dai *et al.,* 1995), ligate RNA (Bartel & Szostak, 1993; Wright & Joyce, 1997), catalyze RNA polymerization (Ekland & Bartel, 1996), accelerate aminoacyl group transfer in a way similar to protein aa-tRNA synthetases (Illangasekare *et al.,* 1995) and bind CCdA-puromycin, a high-affinity ligand of ribosomal peptidyl transferase designed as a transition state analogue of peptide bond formation (Welch *et al.,* 1997). The *in vitro* selected RNA binding site for CCdA-puromycin shows an eight-base sequence similarity to the highly conserved nucleotides of the peptidyl transferase loop domain of 23S rRNA (positions 2448-2455 according to *E. coli* numbering). Although this structure is apparently not an absolute requirement for binding, it suggests that these 23S rRNA nucleotides might be part of the ancient peptidyl transferase. In principle, a step further, selection of RNA with the ability to catalyze peptidyl transfer would be a nice way to prove that with some probability, ancient peptidyl transferase could have been composed of RNA.

Several classes of ribozymes catalyze reactions not only at phosphorus centres but also at a carbon centre. For example, Cech and colleagues engineered a group I intron by changing its internal guide sequence to base-pair with the P-site peptidyl transferase substrate CAACCA(fMet). Essentially, an ester bond was placed into the active site of a group I intron by tethering an amino acid to an oligoribonucleotide sequence that would normally position a phosphodiester bond into the active site. The authors found that this ribozyme catalyzes the hydrolysis of the aminoacyl ester bond (Piccirilli *et al.,* 1992). Conversely, it has been shown that ribosomal peptidyl transferase can catalyze transfer of aminoacyl groups to a phosphate centre (Krayevsky & Kukhanova, 1979). The demonstration of catalysis at a carbon centre performed by an RNA enzyme suggests that prior to the involvement of proteins the RNA world could have expanded to include reactions of amino acids and other non-nucleic acid substrates. That is to say, RNA may have acted as an aminoacyl tRNA synthetase (Illangasekare *et al.,* 1995). With tRNAs, mRNA, and ribozymes as tRNA synthetases and peptidyl transferases, it is possible to imagine of information-driven protein synthesis being carried out solely by RNA.

Investigations of specifying functional domains of the ribosomal RNAs have given us an important information to understand how the large rRNA structures could have evolved from a world were the largest RNAs were most likely an order of magnitude smaller than contemporary ribosomal RNAs. The application of site-directed mutagenesis and other genetic approaches has strenghtened the idea of functional importance of rRNA. Mutations have been mapped that impair EF-Tu and EF-G functions, subunit association, initiation, antibiotic sensitivity, tRNA binding, translational accuracy and peptidyl transferase activity.

In this thesis I will concentrate on reviewing the current knowledge how 23S rRNA tunes ribosomal peptidyltransferase, translational accuracy and elongation factor dependent reactions and summarize our contribution to this field.

Roots of this dissertation can be traced back for more than 30 years, when Estonian molecular biology was founded by late Dr. Artur Lind and his colleagues. Their very first interests were structural and functional aspects of protein biosynthesis. This tradition has been successfully followed and kept alive in the laboratory of molecular biology.

1. LITERATURE OVERVIEW

1.1. Ribosomal elongation cycle

The first round of elongation is unique in that the aminoacyl-tRNA-IF-2•GTP ternary complex is bound to the ribosome with the initiator tRNA (in prokaryotes fMet-tRNA^{fMet}) at the P-site, while the E-site is empty. All the other cycles are beginning with a peptidyl-tRNA (pep-tRNA) bound to the ribosomal P-site and the deacylated tRNA to the ribosomal E-site. Aminoacyl-tRNA (aa-tRNA) molecule is delivered to the ribosomal A-site in an active complex with the protein elongation factor Tu•GTP complex, concurrent with the release of the E-site bound deacyl-tRNA. After the GTP hydrolysis on EF-Tu and the release of EF-Tu•GDP complex, aa-tRNA and pep-tRNA are ready for peptidyl transfer, which is the transfer of growing peptide chain from pep-tRNA in the ribosomal P-site to the amino acid of the aa-tRNA. The quality of decoding of the genetic information is warranted by selection of correct codon-anticodon interaction before and after GTP hydrolysis on EF-Tu. In addition, proper interaction of the CCA 3' end of aa-tRNA with the ribosomal peptidyl transferase centre plays also a role in tRNA selection process. Continuation of the ribosomal elongation cycle is guaranteed by the active protein translocase complex EF-G•GTP, which binds to the ribosome after peptide bond formation and translocates tRNA substrates together with mRNA by three nucleotides in respect of the ribosome. After the EF-G•GDP leaves the ribosome and tRNA's are positioned to the P- and E-sites, next round of peptide elongation can take place (Fig. 2).

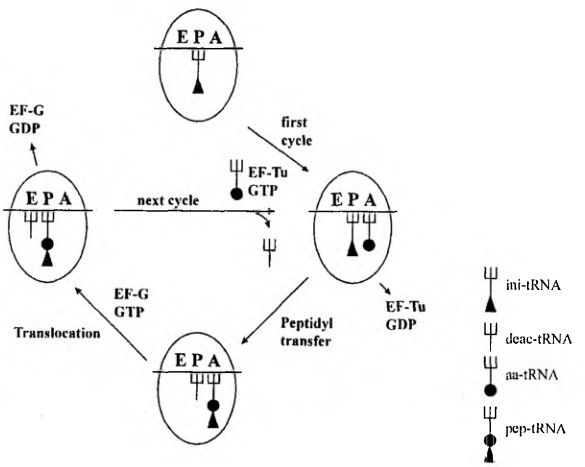


Figure 2. Schematic representation of the ribosomal elongation cycle. Ribosomes are depicted as ellipses. Letters E, P and A are for tRNA binding sites.

Up to this point, "ribosome community" has no principle disagreement, while the details of ribosomal elongation cycle are matter of ongoing discussions. Different experimental approaches have led to the establishment of "hybrid site model" and "α-ε model" of the ribosomal elongation cycle.

1.1.1. The "Hybrid site model" of ribosomal elongation cycle

The "hybrid site" concept is based on the chemical footprinting of 16S and 23S rRNA during different steps of elongation (Noller *et al.,* 1990). According to the footprinting data, tRNA anticodon loop shields a distinct set of nucleotides in 16S rRNA from chemical modificaton when bound either to A- or P-sites, whereas the CCA 3' end of the tRNA located in A-, P- or E-sites protects a distinct set of bases in the 23S rRNA. Beginning with a peptidyl-tRNA in the P/P state (first letter for the 30S and second for the 50S subunit), an aminoacyl-tRNA is brought in as a ternary complex and binds initially in the A/T state (T-site is for incoming aa-tRNA, where only the anticodon is interacting with ribosomal decoding centre). After departure of the EF-Tu•GDP complex, the peptidyl-tRNA and aminoacyl-tRNAs are in P/P and A/A states, respectively, ready for peptidyl transfer. When peptidyl transfer is over, tRNAs shift from P/P to P/E and from A/A to A/P state, respectively. Here the two tRNAs move independently on the two ribosomal subunits — maintain their locations on the small subunit but move with respect of the large subunit. At the end, translocase EF-G moves the anticodon ends of both tRNAs, together with mRNA, relative to small subunit (P/E and E states are established). According to this model, at least six distinguishable binding states for tRNA can be brought forth: A/T, A/A, A/P, P/P, P/E and E. Interestingly, E-site is located entirely in the 50S subunit.

The hybrid site model emphasizes the role of rRNA in protein synthesis and unlike the two-site ribosomal elongation cycle model it uncouples the tRNA movement on the large and small ribosomal subunit.

1.1.2. The "α-ε model" of ribosomal elongation cycle

When protection of the tRNA sugar-phosphate backbone by ribosomes was studied, it was found that only 6 of 64 investigated nucleotides of AcPhe-$tRNA^{Phe}$ and 1 of 63 investigated nucleotides of deacyl-tRNA show a significant change in the protection upon translocation, indicating that contacts between tRNA and ribosome are essentially the same in pre- and posttranslocational states (Nierhaus *et al.,* 1996). The "α-ε model" replaces the classical tRNA binding sites by positions, named α and ε. These are movable domains, α preferentially for peptidyl-tRNA and ε for deacylated tRNA. In the pretranslocational ribosome the α-ε domain is located at the A and P positions to create

the Aα and Pε sites. In the posttranslocational ribosome the α-ε domain is located at the P and E positions, creating the Pα and Eε sites. Nonmovable decoding domain is marked by a letter δ. When ribosomal elongation cycle begins, the peptidyl-tRNA is bound to the Pα and deacylated tRNA to the Eε site. Now the ternary complex enters the low-affmity A-site, the δ domain at the A position. If codon-anticodon interaction has occurred, it triggers the allosteric transition and α-ε domain moves without the bound tRNAs from the P and E positions to the A and P positions, deacylated tRNA from the Eε site is expelled and ternary complex binds with high affinity. GTP is hydrolysed on EF-Tu and EF-Tu•GDP complex rapidly leaves the ribosome. After the peptide bond is formed, the pretranslocational ribosome is the substrate for EF-G•GTP. During translocation, the α-ε domain is moving together with both tRNA's from the A and P positions to the P and E positions, GTP is hydrolysed and EF-G•GDP leaves the ribosome. Thus, according to the "α-ε model" ribosomal pre- and posttranslocational states alternate by simultaneous movements of domains α and ε together with the tRNAs, allowing proper positioning of the anticodon region, needed to maintain the reading frame and also a precise movement and docking of the CCA 3' ends, which is important for the peptidyl transfer.

Largely, the hybrid site model and the α-ε model are compatible with and also complement each other. The main discrepancy concerns the A/P hybrid state, which does not exist accordingly to the α-ε model.

1.2. Genesis of peptidyltransferase centre during ribosome biosynthesis

Ribosome formation in *Escherichia coli* involves coordinated synthesis and folding of three ribosomal RNAs and 54 ribosomal proteins, processing and modification of the rRNAs, and assembly of both rRNA and r-proteins into functional subunits. All these processes are tightly connected to each other and therefore, despite decades of studies, we are still far from drawing final conclusions, although significant portion of knowledge has already been accumulated (Condon *et al.*, 1995).

Escherichia coli rRNA genes are organized into seven independent operons that are under the control of two strong tandem promoters. A single transcription unit includes the rRNA coding and its leader, spacer, and trailer sequences. The latter have been demonstrated to possess the regulatory function necessary for efficient ribosome formation. The common order of rRNA genes in operons is 16S — spacer (containing either $tRNA^{Ala}$ and $tRNA^{Ile}$ or $tRNA^{Glu}$) — 23S — spacer — 5S. Although small differences in primary structure are known to exist between the genes in different transcriptional units, the sequences are virtually (>99%) identical. rrnB operon serves as a model for the ribosomal RNA

gene organisation (the first operon that was cloned and sequenced). Formation of functional ribosomes is strongly dependent of RNA polymerase type, implying the significance of cotranscriptional events during ribosome assembly (Lewicki *et al.,* 1993). The requirement for a highly coordinated synthesis of rRNA and r-proteins is particulary demanding under the fast growth conditions of bacteria in which ribosomes contribute significantly to the dry mass of the cell. In bacteria the ribosomes can amount to more than 30% of the dry mass (Tissieres *et al.,* 1959), whereas in eukaryotes they represent not more than 5% (Blobel & Potter, 1967). Transmission electron microscopy images of exponentially growing *E. coli* show that the cells are filled with ribosomes. According to some calculations, up to 40% of total energy production is consumed by ribosome biogenesis. It follows that coordinated synthesis is not only a prerequisite for an effective assembly, but also a necessity for an economical consumption of the cell energy.

1.2.1. Processing

Processing begins before transcription of an rrn operon is finished (Apirion & Gegenheimer, 1984). Thus, the 5'→3' directional processing is coupled with transcription. RNase III is a major nuclease responsible for cleaving the precursor RNA into pre-16S and pre-23S (unprocessed '30S precursor' is found in wild type cells in very small quantities ~ 1%). RNase E and RNase P are required to process 5S and tRNAs, respectively. Intriguingly, RNase III cleavages are not of vital importance — *E. coli* mutants lacking the enzyme can survive, albeit with significantly prolonged generation time (Srivastava & Schlessinger, 1990). 50S subunits containing pre-23S RNA are shown to be active in protein synthesis, though their activity is decreased compared to mature 50S subunits (Sirdesmukh & Schlessinger, 1985). Final trimming of 23S rRNA takes place in polysomes (Srivastava & Schlessinger, 1988). Enzymes involved in this step are not yet identified.

1.2.2. Posttranscriptional modification

rRNA modification starts during its transcription and completes in late stages of ribosome assembly. Prompted by their distribution in regions of rRNA implicated in the various functions of the ribosome, such as subunit association, tRNA binding and peptidyl transfer (Brimacombe *et al.,* 1993), posttranscriptional modifications are considered to be essential for ribosome functions. The importance of modifications is further emphasized by the fact that none of the 23S rRNA homologues have been found without posttranscriptional modifications. However, the degree of rRNA modification varies between different organisms. In general, the more ancient the organism is, the less modifications

its rRNAs contain. The least modified tend to be mitochondrial rRNA sequences (for example yeast mitochondrial 23S rRNA homologue has only three modifications, while *E. coli* 23S rRNA has 23). In boldest speculation, authors have attributed to the pseudouridine residues a direct role in catalysis of the peptidyltransferase reaction, clustered around the peptidyltransferase centre of the 50S subunit (Lane *et al.,* 1992). In addition, it has been proposed that dihydrouridines might function to enhance the conformational flexibility required for dynamic translation and methylated 2' hydroxyls might prevent undesired hydrogen bonding interactions (Kowalak *et al.,* 1995). However, these proposals are without experimental verification.

Current experimental evidence has given a ground to the belief that modifications are simply "fine tuning" of the ribosome functions. Active 50S subunits can be formed by using 50S proteins and *in vitro* transcribed 23S RNA lacking all the modifications, though their PTase activity is severely inhibited (Green & Noller, 1996). Similarly, active 30S subunits can be reconstituted from *in vitro* transcribed 16S RNA (Krzyzosiak *et al.,* 1987). tRNAs are rich in postranscriptional modifications; yet, *in vitro* transcripts of these molecules are readily aminoacylatable and are active in protein synthesis under *in vitro* conditions (Sampson & Uhlenbeck, 1988). Although the consequences of the absence of posttranscriptional modifications in rRNA and tRNA may be substantial *in vivo*, it appears that they are not fundamental. From evolutionary perspective, nonfundamental role of posttranscriptional modifications is consistent with the idea of their late acquisition during evolution and can be explained as "fine tuning" of an already functional translational apparatus (Noller & Woese, 1981).

To identify the region of 23S rRNA were the presence of modified nucleotides has substantial impact on ribosomal peptidyltransferase activity, an *in vitro* complementation analysis was carried out with ribosome-derived 23S rRNA fragments combined with the complementary *in vitro* transcript in a chimeric reconstitution reaction. Using this approach, segment of 23S rRNA extending from positions 2445 to 2523 and containing six posttranscriptional modifications was found to contribute most to the assembly of the ribosomal peptidyl transferase centre (Green & Noller, 1996).

1.2.3. Assembly

E. coli 50S subunit with a mass of *ca.* 1.85 million Da is composed of two RNAs, 5S (120 nt) and 23S (2904 nt) and 34 r-proteins. Assembly of 50S subunit from these components is a process of an ordered series of RNA-RNA, RNA-protein and protein-protein interactions. Even though the structure of the ribosome is very complex, functionally active ribosomes can be reconstituted from purified components *in vitro* demonstrating that all information necessary for correct assembly is contained in the structure of the mature ribosomal com-

ponents. Both, 50S reconstitution *in vitro* and 50S assembly *in vivo* have shown to occur via three precursor particles. Optimal 50S reconstitution from purified natural 23S rRNA, 5S rRNA and TP-50 requires two steps: (1) an initial incubation for 20 minutes at 44°C and 4mM Mg^{2+} followed by (2) 90 minutes incubation at 50°C and at 20mM Mg^{2+}. Under these conditions, intermediates with sedimentation coefficients 33S, 41S and 48S are consequtively formed. Similarly, the *in vivo* assembly has shown to occur via three precursor particles, sedimenting at 34S, 43S and 'near 50S'. In both cases, protein analysis revealed that the first and the second particle contained very similar complement of rRNA and r-proteins in spite of the drastic difference in their respective S-values. The 'near 50S' particles contained all the components of the active 50S subunit, but like the first two, they were found to be totally inactive. Thus, structural transitions in the final stage of assembly are crucial to activate ribosomes. It is likely that *in vitro* reconstitution of the 50S subunit reflects basic principles of the *in vivo* assembly. The only difference is considerably slower kinetics of the *in vitro* process. It has been speculated that *in vivo* assembly may involve some unique mechanisms which would help to decrease the kinetic barrier for the assembly reaction. For example, assembly may take place in association with membrane or some extraribosomal protein could be involved. Recently, several nonribosomal auxiliary factors SrmB, DeaD, DbpA and DnaK have been found to affect the cellular assembly of ribosomal subunits, most likely by facilitating rRNA folding (Fuller-Pace *et al.,* 1993; Nicol & Fuller-Pace, 1995; Nishi *et al.,* 1989; Toone *et al.,* 1991). Participation of nonribosomal proteins might be one of the reasons, why ribosome formation within *E. coli* cells occurs in few minutes at 37°C, while the succesfull reconstitution *in vitro* needs 1.5 hours incubation at 50°C under nonphysiological ionic condition. Another reason could be that the *in vivo* assembly is a highly ordered cooperative process, starting with a relatively short 5' sequence of the rRNA and proteins essential for the early assembly, whereas in reconstitution the mature 23S rRNA is exposed to all TP-50 proteins.

In spite of the large number of proteins (20) which can bind to the naked 23S rRNA *in vitro*, only two of them, L24 and L3 can initiate the assembly process (Nowotny & Nierhaus, 1982). Surprisingly, a temperature-sensitive mutant has been described which lacks r-protein L24 (Dabbs, 1986). L20 was identified as a protein able to replace L24 and initiate 50S subunit formation (Franceschi & Nierhaus, 1988). This finding serves as example of certain degree of flexibility during 50S assembly.

It would be significant to know whether any form of truncated 23S ribosomal RNA is capable of forming active particles? To study this possibility, Liiv and coauthors have made a series of overlapping deletions covering the entire 23S rRNA. They found that all deletions, except those lacking 5' or 3' end of the molecule, were able to form stable particles together with a specific set of ribosomal proteins, though none of them exhibited any peptidyltrans-

ferase activity (Liiv *et al.,* 1996). In a very recent paper, experimental evidence was provided that helix 1, formed between the 5' and 3' ends of 23S rRNA, is an important determinant for the ribosomal large subunit assembly (Liiv & Remme, 1997, submitted).

Taken together, it appears that the genesis of functional peptidyl transferase is a very late assembly event. Not only a complete set of ribosomal components guarantees the formation of functional subunits, but ordered series of structural transitions are of high significance. The final transition seems to be essential for the creation of active peptidyltransferase centre.

1.3. Molecular mechanism of peptide bond formation

Although we know the basic scheme of the ribosomal peptidyl transferase, transfer of the growing peptidyl group from the CCA terminus of one tRNA molecule to aminoacyl group attached to the CCA terminus of another tRNA molecule, the nature of catalysis is not clear. Is the PTase an enzymatic activity of a specific ribosomal component or does ribosome work as a template, bringing the ends of two tRNAs into proper juxtaposition to allow spontaneous peptide bond formation?

1.3.1. Ribosome as a 'template'

According to the 'template hypothesis', ribosome serves as a template for proper alignment of the substrates of the peptidyl transferase reaction, which in this case is simply the aminolysis of the activated ester bond of peptidyl-tRNA by the α-amino group of aa-tRNA via the formation of tetrahedral intermediate (Krayevsky *et al.,* 1975). The ribosome is not involved catalytically in the reaction. Nierhaus and coworkers argued that the required rate of peptide bond synthesis *in vivo* (15–20 amino acids per second) could be achieved by appropriately positioning the α-amino group of aminoacyl-tRNA for nucleophilic attack on the ester linkage of peptidyl-tRNA, excluding the functional groups of the ribosome in the catalysis of the PTase reaction (Nierhaus *et al.,* 1980a).

1.3.2. Ribosome as an enzyme

The second model attributes the catalytic properties of the PTase reaction to the ribosome. The idea was inspired by the mechanism of action of serine proteases. These enzymes operate via general nucleophilic or acid-base catalysis (Nierhaus *et al.,* 1980a). Three amino acid residues participate directly in the covalent events at the active centre: serine, histidine and aspartic acid. The same amino

acids have been found to be universally conserved in ribosomal protein L2. As a variation of the action of serine proteases, an ordered catalysis mechanism for the ribosome was proposed: deprotonation and activation of the nucleophilic α-amino group of the aa-tRNA by histidine-carboxyl system. The deprotonation of the α-amino group is considered here as the 'catalysed' reaction. Next steps are the stabilization of the tetrahedral intermediate, produced by the nucleophilic attack of the α-amino group on the ester carbonyl, and enhancement of breakdown of the tetrahedral intermediate by proton donation from the histidine-carboxyl system. Garrett and Woolley critisized some aspects of the model. The most important remark was that the catalytic group which enhances the nucleophilicity of the the α-amino group of the acceptor substrate does not necessarily have to be confined to ribosomal protein; it could be also provided by the phosphates of an RNA molecule (Garrett & Woolley, 1982).

None of the models are neither proved nor rejected.

1.4. Catalytic flexibility of the ribosomal peptidyltransferase centre

Under normal circumstances ribosomes are the catalysts of peptide bond synthesis, while under extrordinary conditions *in vitro*, they are capable of catalysing the formation of several types of chemical bonds. Generally, the peptide acceptor in the ribosome is the amino group of an amino acid residue in aminoacyl-tRNA, whereas the carbonyl group at the C-terminus of the peptidyl residue of the peptidyl-tRNA serves as the peptide donor. Chemical alterations in the nature of both substrates can be tolerated. *E. coli* ribosomes have been demonstrated to be able to catalyse the formation of amide, ester, thioester, thioamide and phosphinamide bonds (Krayevsky & Kukhanova, 1979). Such a high degree of flexibility could have resulted from the specific character of the PTase centre — it must adopt many different amino acids, attached to structurally different tRNAs.

1.5. Ionic conditions for peptidyl transferase

The PTC of *E. coli* ribosomes can exist in active or inactive state. One of the requirements for activity is the presence of monovalent and divalent ions. Removal of NH_4^+ ions from *E. coli* ribosomes completely inactivates them. One K^+ or NH_4^+ ion per ribosome must be bound for efficient catalysis of PTase reaction. Eukaryotic ribosomes use K^+ or Na^+ ions (Na^+ is known to inhibit PTase in prokaryotes) rather than K^+ or NH_4^+. Inactivated ribosomes can be reactivated by heating them at 40°C in the presence of necessary ions. Antibiotics like streptomycin, neomycin, erythromycin, lincomycin and chloramphenicol

inhibit both inactivation and activation of the ribosomes. Kasugamycin prevents only inactivation and kanamycin only activation (for review, see Krayevsky and Kukhanova, 1979). Divalent ions are also required. The presence of Mg^{2+} ions is a prerequisite for efficient acceptor and donor substrate binding. The role of ions is most probably to maintain the structure of rRNA. If PTase activity indeed belongs to rRNA, there is a possibility that the reaction is catalysed by metal ions. All RNA catalysed reactions, characterized so far, involve metal ions in their catalytic mechanism (Pan *et al.,* 1993). It has been proposed that by coordinating RNA, the divalent metal ions are involved in the catalysis of peptidyl transfer (Winter *et al.,* 1997). Three possibilities of how metal ions may aid peptide bond formation have brought out: 1) the developing negative charge on the leaving group (tRNA ribose) could be stabilized by a directly coordinated metal ion; 2) or metal ion coordination to the oxygen of the carbonyl group of the amino acid may render the carbon centre more susceptible to nucleophilic attack; 3) metal ion which coordinates hydroxyl group could act as a general base on the amino group, enhancing the nucleophilicity of the amine nitrogen. Pb^{2+} induced cleavage of the sugar-phosphate backbone has been used to probe the metal binding sites in rRNA. Two prominent cleavage sites were found in 23S rRNA: A505 and C2347. At high lead concentration, cleavage at position C2440 was observed. All cleavages occur in the regions that are unpaired in the secondary structure model of 23S rRNA and can be competed for by high concentrations of Mg^{2+}, Mn^{2+}, Ca^{2+}ans Zn^{2+} ions, suggesting that lead is bound to the general metal binding sites (Winter *et al.,* 1997). Similar results have been obtained for group I introns (Streicher *et al.,* 1993) and RNase P (Zito *et al.,* 1993).

1.6. 23S rRNA topography

To understand the nature of an enzyme is to understand its functional structure. An ultimate goal would be to know all structural transitions and movements of active groups during the action of an enzyme. The most powerful physico-chemical technique that gives a detailed atomic resolution picture is the x-ray crystallography. The evolution of our understanding of how the ribosome does its job is currently in a 'low-resolution phase'. At the moment, when we do not have an atomic map of the structure and we are nowhere near to the functional structure of the ribosome (the 'high-resolution phase'), our knowledge is limited to data created with a number of low-resolution methods. These methods can describe three-dimensional folding of rRNAs, rRNA-protein and protein-protein interactions in the ribosome. High-resolution structures are available for several ribosomal proteins and segments of rRNA (Uhlenbeck *et al.,* 1997).

Ribosomes are big enough (250Å in diameter) to obtain some useful information about them from electron microscopy images. In 1970s and 1980s, the

approximate shape of the ribosomal subunits was deduced. Using antibodies against specific ribosomal components and several staining strategies, the locations of several landmarks on the surface of the ribosome were mapped (Oakes *et al.,* 1986; Stöffler & Stöffler-Meilicke, 1986). These investigations have established the overall morphology of the 50S ribosomal subunit, which exhibits three distinct features: L1 protuberance, central protuberance, L7/L12 stalk and the body of 50S (Fig. 1). In 1990s, Frank's and van Heel-Brimacombe's groups took one step further and developed the strategy called "image reconstitution". Two independent 20–25Å resolution reconstitutions of the *E. coli* 70S ribosome (Franck *et al.,* 1995; Stark *et al.,* 1995). The results of both groups were very similar, elucidating many structural details, such as extensive system of channels within the 50S subunit and an intersubunit gap ideally shaped to accommodate two transfer RNA molecules. Agrawal *et al.,* (1996) and Stark *et al.,* (1997), extended their work to analyze the movements of ribosome-bound tRNAs during protein synthesis. The first group imaged ribosomes with deacylated tRNAs bound simultaneously to all three sites of the ribosome, while the other visualised two tRNAs bound to both pre- and posttranslocational ribosomes. Earlier data have positioned the decoding region as well as the peptidyltransferase centre to the interface of the two ribosomal subunits. Electron microscopy studies confirmed these observations. Both groups detected an extra density, attributed to tRNAs. From van Heel's group images, it is clearly seen that tRNAs move relative to each other during translocation. The direction of tRNA movements during translocation from A to P and from P to E sites is from L7/L12 side towards L1 binding region. Interestingly, the shapes of the pre- and posttranslocational ribosomes were indistinguishable at this resolution (20Å).

1.6.1. Primary structure

Determination of the primary structure of 23S rRNA and cloning the whole *E. coli* rrnB operon by Brosius and colleagues (Brosius *et al.,* 1980) has been a landmark of the new era in ribosomology, because many useful methods to study the ribosome and ribosomal RNA are not applicable without the possibility to manipulate with rRNA genes. *E. coli* 23S rRNA consists of 2904 nucleotides and has multiple posttranscriptional modifications. Analysis of phylogenetic conservation of 23S rRNA sequences has brought forth functionally important regions of the molecule. The occurrence of posttranscriptional modifications in regions with highly conserved features suggests that they might be also functionally important (see also section 1.2.2).

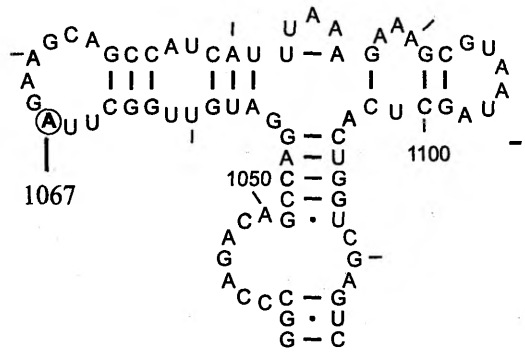
1.6.2. Secondary structure

With sufficiently extensive collection of homologous sequences, one can through comparing them, define the range of their similarities and differences, and the patterns that characterize evolutionary changes in the molecules primary structure. By means of phylogenetic sequence comparisons the secondary structure model of 23S rRNA has been proposed (Noller *et al.,* 1981). The secondary structure of 23S rRNA (Fig. 3A and 3B) is composed of six large domains, radiating out from a central ring and formed by helices, defined by their respective long-range interactions: domain I (16-25/515-524), domain II (579-585/1255-1216), domain III (1295-1298/1642-1645), domain IV (1656-1664/1997-2004), domain V (2043-2054/2615-2625), and domain VI (2630-2637/2781-2788). The most conserved region in the secondary structure is the central ring of domain V and its surrounding (Fig. 4). This region could be considered as the 'heart' of the 50S subunit — changes in this region often lead to inhibition or block of the central ribosomal reaction, the peptidyl transfer (Table 1; section 1.10). Evidence for the existence of each helix was derived also from structure probing analysis (Egebjerg *et al.,* 1990). About 40% of the 23S rRNA is single-stranded and as discussed below, a vast majority of experimental data points lie in single-stranded regions. It is noteworthy that long, regular helices do not exist in the 23S rRNA. Instead, the structure is formed by joining together many short helices, the junctions of which tend to create a variety of structural irregularities.The reason for this kind of architecture has been rationalized in two ways (Noller & Woese, 1981). First, multiple small helices allow a much more complex three-dimensional structure and second, the long and stable helices would create kinetic 'traps', interfering with ribosome assembly.

1.6.3. High-order structure

Comparisons among 23S rRNA sequences reveal a number of positions that covary in composition, suggestive of high-order structural elements. A number of long-range tertiary structural interactions have been predicted, bringing together areas that are distant in the secondary structural diagram (Gutell & Fox, 1988).

Based on various types of topographical information, mostly such as intra-RNA crosslinking, RNA-protein cross-linking, protein footprinting as well as the classical binding-site data and computational analysis, preliminary models for three-dimensional folding of 23S rRNA have been proposed (Brimacombe, 1995; Mitchell *et al.,* 1990; Nagano *et al.,* 1988). The most comprehensive 23S rRNA model is the one established by Brimacombe and coworkers. It summarizes a majority of structural data currently available that are relevant to



- THIOSTREPTON BINDING STRUCTURE (TBS)

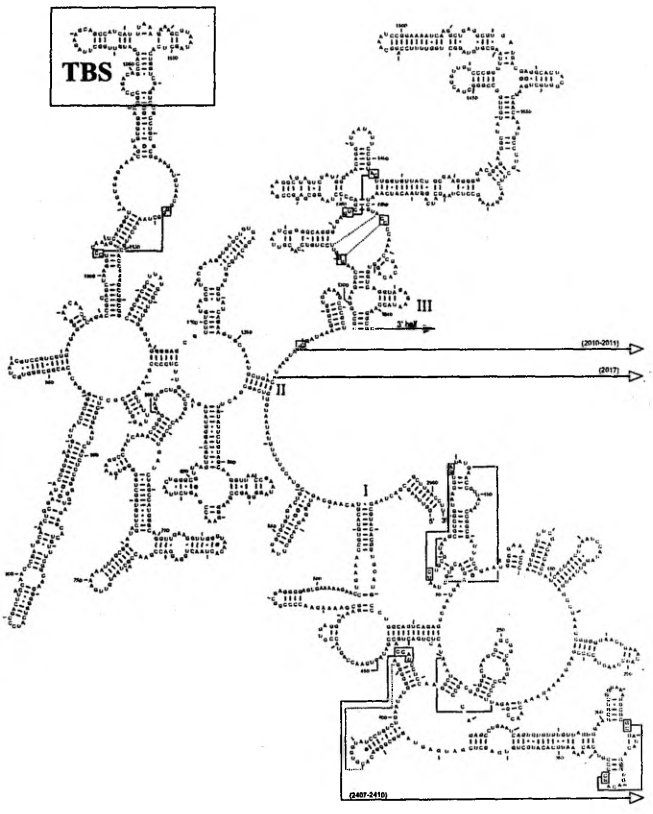


Figure 3A. 5' half secondary structure diagram for *Escherichia coli* 23S rRNA (adapted from Gutell, 1993). Tertiary interactions are connected by thicker solid lines. Every 10th position is labeled by a tick mark, every 50th is numbered. Thiostrepton binding structure (TBS) of 23S rRNA is rectangled and magnified above. Position 1067 is encircled.

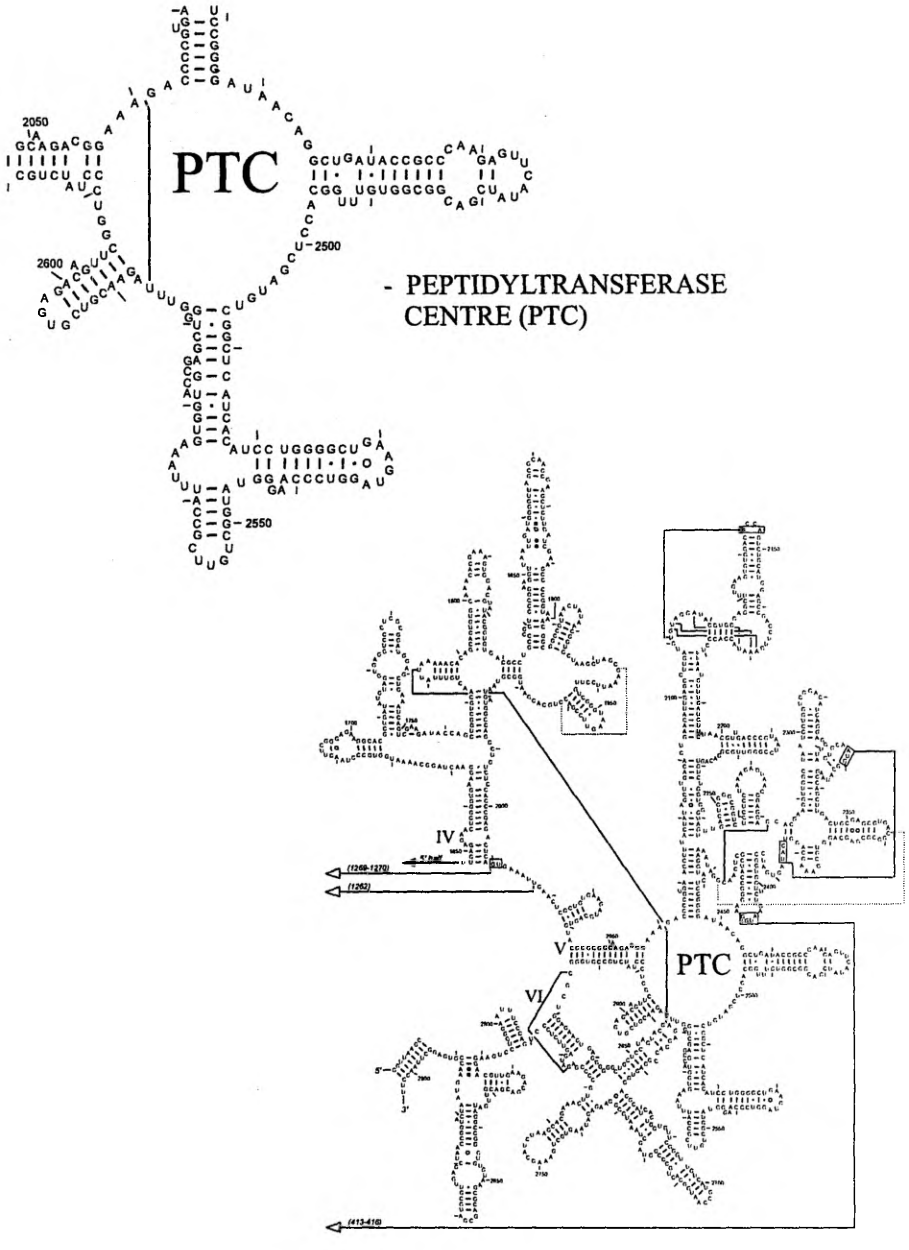


Figure 3B. 3' half secondary structure diagram for for *Escherichia coli* 23S rRNA (adapted from Gutell, 1993). Tertiary interactions and numbering are the same as in Figure 3A. Peptidyltransferase centre (PTC) of 23S rRNA is magnified above.

three-dimensional interactions of 23S rRNA. Taking into account that the RNA component is two-third of the mass of the ribosome, autors believe that the overall shape of the 23S rRNA should at least approximately correspond to the morphological shape of the ribosomal large subunit, observed by electron microscopy. The three-dimensional model of 16S rRNA serves as a good example to endorse this expectation — it resembles very well the image of 30S subunit (Mueller *et al.*, 1995). The starting point of the models is the phylogenetically established secondary structure of the 23S rRNA. Some features of the three-dimensional model are supported with experimental data while some are highly speculative. Relatively large number of structural elements are implicated in beeing close to, or part of the functional core, being consistent with the obseravation that the PTC is rich in RNA (Picking *et al.*, 1992).

1.7. Functional centres of the 50S subunit

A cascade of methods has been developed to determine functionally significant regions of rRNA. Three regions of 23S rRNA have shown to be involved in ribosome functions. The thiostrepton binding sequence in domain II (Fig. 3A, bases 1052-1108) and α-sarcin stem loop structure in domain VI (Fig. 3B, bases 2647-2673) are implicated in elongation factor EF-Tu and EF-G dependent reactions (for details, see section 1.13). Ribosomal peptidyltransferase centre involves primarily nucleotides in domain V (Fig. 4).

A few classical methods are outlined below that have aided to get insight into the ribosomal peptidyl transferase centre.

1.7.1. Cross-linking

In the site-directed cross-linking approach, a photoreactive group is introduced at a certain position within a functional ligand, which is bound to the ribosome under specific conditions. The following photoactivation of the reactive group leads to crosslink(s) between ribosomal component(s) and the functional ligand. The crosslinked complex could be isolated and the site of cross-link determined, providing us information about the proximity of the functional ligand to a specific functional entity of the ribosome. The approach has demonstrated its value in a series of experiments were the bound ligands have retained their functional activity (see below). Photo-reactive groups could be attached to different places in tRNA, mRNA or the nascent peptide, enabling to gain insight into a variety of contacts between ribosomal ligands, rRNA or ribosomal proteins.

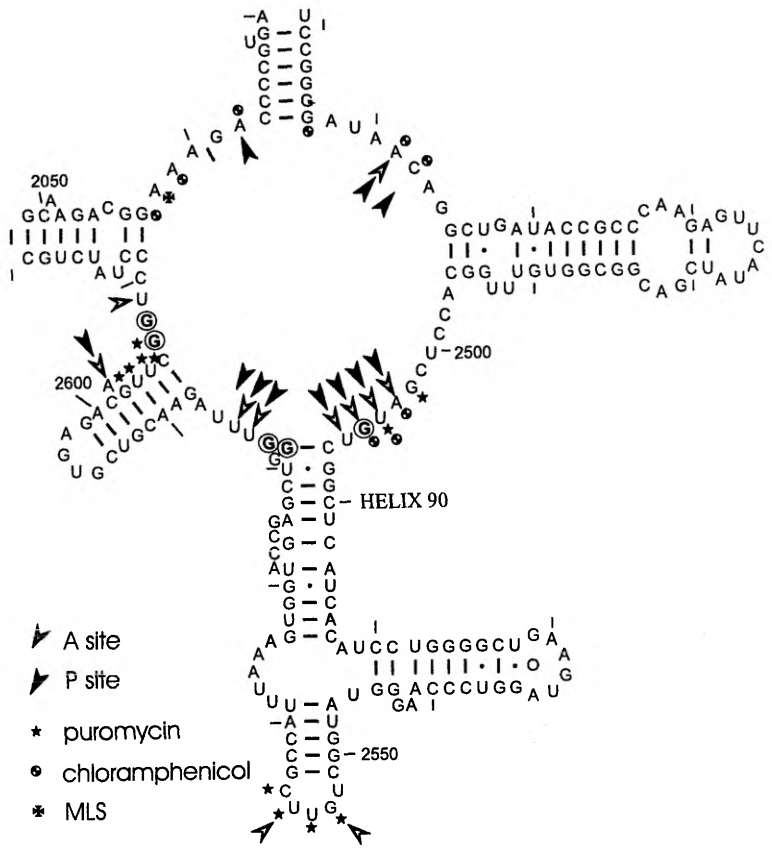


Figure 4. Central ring of domain V of *Escherichia coli* 23S rRNA. Mutations characterized in these thesis are encircled. tRNA protections and crosslinks are represented by arrowheads; antibiotic protections, crosslinks and resistance mutations by smaller symbols (for review see Spahn & Prescott, 1996). MLS is for macrolide-lincosamide-streptogramin type B antibiotics.

Introduction of the photoreactive base analogues like 2-azido- or 8-azido-adenosine (photoactivated azidoadenosines form very short cross-links, about 2–4 Å) into 3' end of tRNA at positions 73 or 76 of $tRNA^{Phe}$ gives additional information about the structure of the PTC. 2-azido-A73 derivatives reacted only with the protein L27, but both the 2-azido-A76-tRNA and 8-azido-A73-tRNA reacted with several proteins (mainly L27, with minor cross-links to L2, L15 and L16) and with 23S rRNA. G1945 of 23S rRNA was cross-linked by P-site bound $[2N_3A76]tRNA^{Phe}$ (Wower *et al.*, 1989), but the data were questioned later and the authors suggested that the labeling of G1945 takes place in the ribosomal A-site. 2-azido-A76-tRNAs bound to ribosomal A-, P- and E-site

label five segments whithin domains IV and V of 23S rRNA (Wower *et al.*, 1995). p-azido derivative of puromycin (an analogue of the 3' end of aa-tRNA) has been cross-linked to the universally conserved residues G2502 and U2504 (Hall *et al.*, 1988).

Upon irradiation with UV light at 320 nm, BP-Phe-tRNA^{Phe} (BP — bensoyl-propionyl) bound to poly(U)-programmed ribosomes, cross-links exclusively to 23S rRNA (despite of the potential reactivity of BP moiety, attached to the amino group of the Phe towards both protein and RNA). The fact that the cross-linked Phe-tRNA derivative retains the ability to form a peptide bond is an indication that the cross-linked region of the 23S rRNA can be an integral part of PTC (Barta & Kuechler, 1983). When RNA was isolated from the ribosomes specifically labeled with BP-[^{3}H]Phe-tRNA^{Phe} from either A- or P-site, and was used as a template for the primer extension analysis with reverse transcriptase, three labeled regions of domain V of 23S rRNA were identified (Steiner *et al.*, 1988). A-site bound tRNA cross-links to nucleotides U2584 and U2585 as the major affinity labeling sites plus A2503 and U2504 as the minor sites. For P-site bound tRNA, nucleotides C2452 and A2453 became labeled. Similarly with A-site experiments, some minor sites were also observed: U2504, G2505 and U2506. However, when BP-Phe-tRNA^{Phe} was bound to ribosomes in which P-site binding was blocked by the excess of deacylated tRNA, the cross-links obtained were across the loop at residues U2584 and U2585. Here, the results seem to be in controversy, which could be eliminated by the 'hybrid site' model — under the excess of deacylated tRNA, the tRNA is probably bound in the A/P-state, where it is simultaneously in the 30S A-site and 50S P-site. This is a puromycin unreactive state and has been previously thought to represent A-site binding. When bound in the P/P-state, the BP-tRNA crosslinks mainly to A2451 and C2452, coinciding with the observed protection of the 3' CCA terminus of tRNA at A2451 (see also section 1.1.1).

When an aromatic azide is attached to the amino acid of Phe-tRNA^{Phe}, it labels nucleotide A2493 from both A- and P-sites (Mitchell *et al.*, 1993). More recently, the study has been extended to map the cross-linking sites of peptides with various lenght, synthesized *in situ* on the ribosome in the presence of a synthetic mRNA analogue coding for methionine, lysine, phenylalanine and glutamic acid (Stade *et al.*, 1994). The peptides, carrying diazirino derivatives attached either to the α-amino group of the N-terminal methionine residue, or to the ε-amino group of an immediately adjacent lysine residue, were cross-linked to 23S rRNA positions 2062, 2506, 2585 and 2609. These positions are probably very close to the PTC. However, when the peptide was three or four residues long, a new cross-link was observed at position 1781 (helix 65) which is several hundreds of nucleotides away in another secondary structural domain. The data show that the exiting peptides are directed towards helix 65 in domain IV of 23S rRNA and it is noteworthy that nucleotides 1782 and 2609 are among several pairs of nucleotides which have been identified to connect domains IV and V (Mitchell *et al.*, 1990). Using intra-RNA cross-linking method, helices

35, 40, 72 of 23S rRNA and helix IV of the 5S rRNA have been found to be adjacent to the central ring of domain V (Döring *et al.,* 1991). With progressively longer peptides, cross-links to nucleotide 750 at the loop end of helix 65 and to nucleotide 1614 at the loop end of helix 59A have been observed (Stade *et al.,* 1995). The first occured from peptides of 6 residues or longer, whereas the second revealed onself when the peptide reached a lenght of 25 amino acids. Again, the proximity of the position 750 in helix 35 to the PTC is corroborated by earlier data of direct UV-cross-link between positions 746 and 2614 (Mitchell *et al.,* 1990) as well as by the protection of position 752 by antibiotic vernamycin B (Moazed & Noller, 1987). Helix 59A, the cross-link site from 25-26 amino acid residues long peptides is close to the binding site for protein L23 (Vester & Garrett, 1984). L23 together with L29 have been identified to contact the 23S rRNA region embracing nucleotides between positions 60 and 140 in domain I. Immuno-electron microscopy studies have located this site at the rear of the 50S subunit (Bernabeu & Lake, 1982). Peptides of 29-33 amino acids that are the longest peptides that should approach the peptide exit site, cross-link to the position 91 (helix 7) of 23S rRNA (Stade *et al.,* 1995). Taking the data together it becomes evident that the growing peptide makes contacts with remarkably widely separated regions of 23S rRNA. The peptide is directed from the PTase ring in domain V to domain IV (helix 65), then to domain II (helix 35), and to domain III (helix 59A), and finally to domain I (helix 7). Due to the absence of experimental data for peptides with lenght of 15-25 amino acids, some significant points on the pathway for the nascent peptide are likely to be missing. Another interesting phenomenon has been emphasized by Brimacombe and coworkers — during the peptide growth in the ribosome, the specific cross-links for the very first amino acids disappear at some lenght of the peptide and appear again when the peptide has become longer. It is a very likely indication of the folding events that occur already within the ribosome and preclude the existence of a tunnel, wide enough to allow the peptide to fold back on itself and reach a sub-set of the 'earlier' cross-links. The role of the 50S subunit in protein folding has also brought into prominence by Hardesty and colleagues (Hardesty *et al.,* 1993) and by Dasgupta's team (Das *et al.,* 1996). The latter work has demonstrated a protein-folding activity *in vitro* in ribosomes from bacterial, plant and animal sources: ribosomes can refold a number of enzymes which are denatured with guanidine/HCl. After closer inspection, the protein-folding activity was attributed to the 50S subunits and the active site for folding of denatured protein to the 23S rRNA. Disruption of the tertiary structure of 23S rRNA inhibited its protein-folding activity. Neither the 30S particle nor the 16S rRNA were shown to be involved.

1.7.2. Footprinting

The method is based on an idea that binding of functional ligand to the ribosome must limit or prevent the access of chemical or enzymatic probes to the rRNA if the ligand and rRNA interact. Earlier, mostly single- and double-strand specific nucleases have been exploited to follow the interactions between ribosome and its substrates. Nowadays, the most successful method has been the use of chemical probes that attack the bases, ribose, or phosphate groups in the rRNA, allowing identification of the sites of ligand interaction by primer extension technique. Importantly, the changes in reactivities could be induced also by conformational changes in the ribosome due to the ligand attachment (referred in literature as class III sites), giving us the major shortcoming of the method and making interpretation of the data difficult. Both, the RNA-ligand contacts and ligand induced conformational changes, would be valuable to know. Unfortunately, the method does not allow to distinguish one from another.

Small chemical probes are especially good due to their high specificity, permitting localization of individual atoms in the rRNA: diethyl pyrocarbonate (N7 of A), kethoxal (N1 and N2 of G), dimethyl sulfate (N1 of A, N3 of C and N7 of G), CMCT (N3 of U and N1 of G), hydroxyl radical generated by Fe^{2+} (C4' of ribose) and ethylnitrosourea (phosphate oxygen). The precise positions of the modifications as well as the relative extent of modification can be identified by primer extension analysis. Reverse transcriptase either stops or pauses at modified nucleotides. In case of chain scission (hydroxyl radical attack of ribose, ethylnitrosourea attack at phosphate and dimethyl sulfate modification of G at N7) reverse transcription terminates.

One of the first indications of the importance of rRNA in translation came from modification studies with kethoxal, showing that modificaton of a few guanines in 16S rRNA results in a loss of tRNA binding to 30S subunits (Noller & Chaires, 1972). Later on, this approach has been extensively used to map the binding sites of tRNAs, and for various type of antibiotics. tRNAs bound to the ribosomal A-, P- and E-sites were found to protect specific set of bases in the rRNA. A- and P-site bound tRNAs protect bases of 16S and 23S rRNA. When tRNA is in the E-site, it is shown to shield bases in 23S rRNA, exclusively.

Most of the bases protected by tRNA in 23S rRNA are found in domain V, but few are also found in domains II and IV (Moazed & Noller, 1989).

A-site specific bases are A2254, A2439, A2451, G2553, U2555, A2602 and U2609 in domain V, C1941 in domain IV and G1041, G1068 and G1071 in domain II. Among those, A2439, A2451, U2555, A2602 and U2609 depend on acyl moiety, whereas protection of G2553 requires 3' terminal A.

P-site bound tRNA gives specific footprints at bases A1916, A1918 and U1926 in domain IV, G2252, G2253, A2439, A2451, G2505, U2506, U2584, U2585 and A2602 in domain V. Acyl moiety is necessary to protect bases

A2439, A2451 and A2602. U2506, U2584 and U2585 depend on 3' terminal A and G2552 and G2553 on 3' CA.

The protection of G2112, G2116, A2169 and C2934 can be assigned to the binding of deacylated tRNA to the E-site. All these protections depend on the 3' terminal A.

The only overlap between A- and P-sites occurs at nucleotides A2439 and A2451, both protections were demonstrated to be dependent on the presence of acyl group. A2451 is one of the nucleotides within the selected eight base-pair sequence that interacts with CCdApPuromycin, a high-affinity ligand of peptidyl transferase (Welch *et al.,* 1997). Puromycin is a structural and functional analogue of the 3' aminoacyl-A of aa-tRNA. Thus, the region around A2451 may be involved in interaction with puromycin or 3' terminus of aa-tRNA. The protection experiments by Moazed and Noller lead to another important conclusion — the integrity of the universally conserved 3' CCA terminus of tRNA is responsible for most of the contacts between tRNA and 23S rRNA. Progressive removal of 3' terminal A and penultimate C eliminates most of the protections observed. The role of tRNA 3' end in establishing necessary contacts with 23S rRNA was further emphasised by a complementary study, where the protection pattern of tRNA 3' oligonucleotide fragments (CACCA-AcPhe, UACCA-AcLeu and CAACCA-fMet) was elucidated. All three oligonucleotides protect 23S rRNA bases G2552, G2553, A2439, A2451, U2506 and U2585 (Moazed & Noller, 1991). These account for most of the bases that are protected also by the full-lenght P-site specific tRNA and correspond precisely to those bases whose protection is abolished by the changes in the 3' terminal CCA of the bound tRNA.

Correlating the tRNA "footprints" with the state of ribosomal elongation cycle the binding mimics, Moazed and Noller suggested the 'hybrid site model', which serves to explain the mutual movements of tRNA and ribosomal RNA in the ribosome. The model emphasizes the kinetic aspect of tRNA and rRNA interactions and firms the functional role of rRNA during elongation (see also section 1.1.1).

A. Mankin and colleagues have taken a step further and identified 23S rRNA nucleotides that are necessary for tRNA binding to the ribosomal P-site by using so-called 'damage selection' approach. By binding biotinylated tRNA substrates to randomly modified large ribosomal subunits and capturing resulting complexes on the avidin resin, four nucleotides of 23S rRNA: G2252, A2451, U2506, and U2585 were identified whose modifications prevent binding of pep-tRNA analog to the ribosomal P-site (A. Mankin, personal communication). This finding is in good agreement with RNA footprinting and affinity labeling results mentioned above, which also suggest that these four residues reside in the vicinity of the pep-tRNA 3' terminus.

1.8. Ribosomal substrate binding sites

Every working ribosome binds simultaneously two tRNAs, either in A- and P-sites or in P- and E-sites. A- and P-sites, or alternatively acceptor and donor substrate sites, are of fundamental importance for PTase, while E, alias exit site is for departuring tRNA.

During the course of translation, the ribosome recognizes and adopts at its acceptor and donor site 3' ends of over 60 different tRNAs with their attached amino acids. Free α-amino group of the aa-tRNA is a common determinant for acceptor substrates in binding to the A'- site (acceptor site in the PTC). The binding of substrates to the P'-site (donor site in the PTC) requires the presence of the carbonyl group of the first C-terminal peptide bond. Common determinants in both the A' and P' substrate sites may include the aminoacyl ester linkage, the very similar but by no means identical three-dimensional shape of the invariant CCA 3' sequence of tRNAs.

The acceptor activity of aa-tRNA depends on the nature of attched amino acid. Phe and Tyr are the best acceptors, while Gly and Trp are with the lowest reactivity. For efficient activity, amino acid must be located at the 3' hydroxyl, it has to be in L-configuration and in form of α-amino acid. It is likely that there is a hydrophobic pocket in the acceptor substrate site, since aromatic amino acids (except tryptophan) bind more strongly than most of their aliphatic equivalents (Harris & Symons, 1973). Footprinting experiments provide additional support to the hydrophobic pocket hypothesis, showing that specific nucleotides in rRNA are protected by aromatic amino acids (see also Section 1.11). The relatively strong binding of pA-Lys suggests that there might exist also an adjacent hydrophilic centre, possibly involving phosphate groups where the side chains of lysine and arginine bind (Harris & Symons, 1973; Rychlik *et al.,* 1970). However, these binding differences could reflect conformational changes induced by amino acid at the acceptor site rather than the existence of specific centres (Krayevsky *et al.,* 1975).

All known mature tRNAs have in common a 3' single stranded CCA end that interacts with acceptor and donor sites of peptidyltransferase during the peptide chain elongation process on the ribosome (Sprinzl *et al.,* 1989). During the course of protein synthesis, the 3' end of a tRNA must interact with several components of the translational machinery. The universal 3' CCA is essential for aminoacylation of tRNA, though the selection of which amino acid to incorporate is made by features of tRNA other than the universal CCA. None of the changes in CCA sequence could abolish the aminoacylation, only the reaction efficiency was severely reduced for G76 and G75 mutant tRNAs (Liu & Horowitz, 1994). The integrity of the 3' CCA end of the tRNA appears to be crucial for peptide bond formation — 3' ends of two adjacent tRNAs must come into close proximity to allow the peptidyl transfer. Chemical and nuclease protection studies indicate that the 3' CCA sequence also functions in formation

of ternary complex between aa-tRNA, EF-Tu and GTP, and in the binding of tRNA to 23S rRNA at the ribosomal A-, P- and E-sites of the 50S ribosomal subunit during the elongation phase of translation. Thus, interactions between the 3' end of the tRNA and the ribosome may occur at all stages of the translation cycle (Noller *et al.,* 1990). Although peptide bond formation is normally integrated in the sequence of reactions during protein synthesis, there is evidence that only the terminal moieties of tRNA interact with the PTC. By using substrate analogs of the tRNA that lack certain functional parts but retain others, it is possible to separate the peptidyl transfer reaction from the other reactions. An aminoacylated CCA trinucleotide alone can interact with the large ribosomal subunit and donate its attached amino acid to an acceptor molecule located in the ribosomal A-site (Monro & Marcker, 1967). Even more truncated versions like aminoacylated A and CA can participate in PTase reaction *in vitro*, albeit with reduced efficiency.

Compounds such as puromycin (analog of the terminal aminoacyl adenosine), and other modified nucleosides (like ethenoadenosine-Phe or ethenocytidine-Phe) that cannot undergo Watson-Crick base pairing, bind effectively to the acceptor site (Chladek *et al.,* 1976). The 3' terminal adenosine of tRNA can also be replaced by formycin without loosing the binding to the acceptor site (Bakhst *et al.,* 1976). These results suggest that base pairing between ultimate A and rRNA is not is required for acceptor activity. Interaction of ribosome with cytosines of the CCA end of tRNA is demonstrated to be of importance for acceptor activity. When A-Gly and UpA-Gly do not react with polylysyl-tRNA in the P-site, CpA-Gly was found to be reactive, albeit with reduced efficiency compared to puromycin (Rychlik *et al.,* 1967). It has also been observed that replacement of the Cp moiety in the position C75 with other nucleotides has more profound negative effects than that of analogous replacements in the third position. It has been demonstrated that UCA-Phe is more active than CUA-Phe, or analogically, GCA-Phe is better acceptor than CGA-Phe or GGA-Phe. Similarly to the ultimate A in the 3' end of tRNA, universal cytosines at positions 75 and 74 of tRNA are unlikely to make Watson-Crick base pairing with rRNA in the ribosomal A'-site. Replacement of these natural cytidylic acid residues in CCA-Phe with guanylic acid moieties resulted only a small decrease of acceptor binding activity (Tezuka & Chladek, 1990). It appears that the stacked ordered structure of the CCA terminus is important for recognition by the A'-site. Disruption of the stacking geometry, coincides with the loss of acceptor activity (Chladek & Sprinzl, 1985). The nucleotides beyond the CCA sequence most probably do not interact with the 50S subunit. When binding and acceptor activities increase in order A-Phe<CA-Phe<CCA-Phe, pentanucleotide fragments exhibit no further improvement compared to trinucleotide fragments. This suggests that the CCA trinucleotide of the tRNA contains most, if not all, of the structural elements required for the interaction with the PTC.

Most of the donor site specificity has been investigated by means of the 'fragment reaction'. Monro and coworkers have found that the 3' terminal frag-

ments of fMet-tRNA^{fMet} (from CAACCA- to pA-fMet) bound to the ribosomal P'-site undergo a ribosome-catalysed reaction with the ribosomal A-site placed acceptor puromycin to yield fMet-puromycin (Monro & Marcker, 1967). The reaction takes place in the presence of limited number of translational components: the large ribosomal subunit, A- and P-site substrates and alcohol plus magnesium and potassium ions. No mRNA, 30S subunit, translation factors or cofactors, or even intact tRNA is required. Instead, 3' end fragments of tRNA or its analogs that interact directly with the PTC could be used as acceptor or donor substrates. The authenticity of the 'fragment reaction' as a true model for PTase was verified by demonstrating, that PTase inhibitors such as chloramphenicol, lincomycin, carbomycin, amicetin inhibit the 'fragment reaction' as well (Celma *et al.,* 1967). The 'fragment reaction', with its limited number of components and truncated tRNAs, refers to a small structural region of the large ribosomal subunit that is necessary for the PTase activity.

The formyl group of fMet-tRNA, which could be replaced by an acetyl group, provides strong specificity for binding to the donor and not to the acceptor substrate site (Monro *et al.,* 1968). pA-fMet is known as the smallest donor substrate, though not the efficient one (Cerna *et al.,* 1973b). The presence of cytidines increases its donor activity significantly, pA-fMet<CA-fMet<CCA-fMet. Similarly to acceptor activity, there is no further increase in donor activity comparing trinucleotide and pentanucleotide derivatives (Chladek & Sprinzl, 1985). The donor activity of pA-fMet could be significantly enhanced by pC (Cerna, 1975). However, pC fails to stimulate the donor activity of CpA-fMet. It has been suggested that pC can stack on the A and pair to adjacent G on the rRNA, thereby stabilizing the complex (Krayevsky *et al.,* 1976). CN(AcMet) that is unable to undergo Watson-Crick base pairing has found to be completely inactive (Quiggle *et al.,* 1981). It can be concluded that CCA terminus of the tRNA plays a crucial role in the binding of peptidyl-tRNA. The peptidyl-transferase P'-site requires also the L configuration of the amino acid portion of the substrate and the integrity of the 2'-OH group (Quiggle *et al.,* 1981). The donor activity varies, depending on the amino acid bound to the tRNA, in order Met>Leu>Phe...>Gly (Krayevsky & Kukhanova, 1979).

To examine the role of the universal CCA terminal sequence of tRNA in the process of peptidyl transfer, all possible CCA mutants of valine tRNA were constructed. PTase activity monitored in the 'fragment reaction' was severely repressed by majority of tRNA mutations. Three mutations UCA, CUA and CCG have shown to retain low level activity (Tamura, 1994).

Ribosomal acceptor and donor substrate sites are conformationally interdependent. Peptidyl-tRNA bound to the P-site stimulates the binding of aa-tRNA to the A-site and *vice versa* (Chladek & Sprinzl, 1985). Peptidyl-tRNA has stimulatory effect only as the 3' isomer, 2' isomer is inactive (Bakhst *et al.,* 1976). Binding of an acceptor substrate to the A'-site is also stimulated by P'-site bound tRNA (Ulbrich *et al.,* 1978). Antibiotics sparsomycin and chloramphenicol, known to inhibit acceptor substrate binding, have an opposite

effect on donor substrate docking (Lazaro *et al.,* 1991; Ulbrich *et al.,* 1978). fMet-tRNA^{fMet} bound in the P-site undergoes alcoholysis only when the acceptor substrate site is filled (Scolnick *et al.,* 1970). The ribosomal acceptor site could be largely dependent on r-proteins. Ribosomes, treated with RNase T1 retain their acceptor substrate binding activity, whereas donor activity is completely abolished (Cerna *et al.,* 1973a). Thus, despite of the closeness between CCA ends of both acceptor and donor site tRNAs in the PTC, architecture of the sites seems to be rather different.

1.9. Probing ribosomal peptidyl transferase by mutagenesis of 23S rRNA

Mutagenesis has proven a valuable technique to put functional capacities of biologically important molecules on the test. The methodology is best applied when the model system is sufficiently well developed. A majority of ribosomal studies have been performed on *Escherichia coli*, which is genetically and biochemically one of the best understood organisms. *E. coli* has seven rRNA operons, presenting a major challenge for studying rRNA by mutagenesis — expression of mutant RNAs from a plasmid is accompanied by host genome encoded homologs. Thus, both mutant rRNAs and wild type rRNAs are assembled into ribosomes. To evaluate the influence of mutation on ribosome functions, wild type ribosomes have to be repressed. Recently, one operon model system, based on *Halobacterium halobium*, has become available (Mankin *et al.,* 1992). However, it is limited from the aspect that phenotypically lethal ribosomal mutations cannot be subjected to further biochemical analysis. As it has been expected and demonstrated even before the appearance of one operon system, cells that bear mutations affecting ribosomal peptidyl transferase function are usually not viable (Table 1). In order to isolate ribosomes with poisonous mutant rRNAs, a methodology based on multioperon organism like *E. coli* has been developed. It uses expression plasmid, where ribosomal 23S RNA gene is under the control of an inducible promoter. For the isolation of 'poisonous' ribosomes, cells are grown to a certain density allowing to express only the wild type rRNAs. Then, synthesis of mutant rRNA molecules is induced and the culture is grown further for a certain time period. Next, cells are harvested and ribosomes isolated. As the result, we have a mixed population of mutant and wild type ribosomes (the ratio is usually 1:1, if P_L-c*Its*857 system is used). Several experimental 'tricks' have been developed to distinguish between activities of mutant and wild type ribosomes. Mutations, conferring resistance to antibiotic thiostrepton, base substitution A1067U (Paper I) and to erythromycin/clindamycin, base change A2058G (Leviev *et al.,* 1995) have been exploited to discriminate wild type ribosomes. Recently, additional opportunity has emerged, called 'chimaeric reconstitution'. A RNase H fragment of mature

23S rRNA is combined with complementary *in vitro* transcript in reconstitution reaction with large subunit proteins and 5S rRNA to yield catalytically active 50S subunits (Samaha *et al.,* 1995). However, none of these methods is capable to yield the results with 100% unambiguity. Despite of the fact that A1067U mutation has no influence on the ribosomal PTase, it is shown to increase the generation time of bacteria, possibly due to a perturbed EF-Tu cycle (Paper III). There is strong evidence indicating that functional connection might exist between domains II and V (Douthwaite, 1992a; Mankin *et al.,* 1994). Position A2058 lies in the peptidyl transferase ring at the secondary structure model and the mutation at A2058 could interfere with the influence of PTase mutation. Moreover, erythromycin is thought to block the entrance of the channel for the growing peptide. 'Chimaeric reconstitution' analysis suffers from excluding several posttranscriptional modifications that might be important for PTase, though they have been shown not to be fundamental (Green & Noller, 1996).

Based on the results of structure probing experiments with peptidyl transferase substrates and inhibitors (for details, see Section 1.5.4 and 1.11), 23S rRNA domains V and in a much lesser extent domain IV have been subjected to site directed or random mutagenesis with a hope to specify 23S rRNA nucleotides involved in peptide bond formation. The effects of the first mutations in the domain V were published by Vester and Garrett in 1988. Cells with A2060C and A2450C mutations were shown to be nonviable and the mutant rRNA was strongly underrepresented in 70S ribosomes, but not in 50S subunits. In addition, G2502C and G2503C transversions caused a decreased growth rate and the latter mutation was found to confer resistance against chloramphenicol. Most surprisingly, A2503C RNA was demonstrated to be preferred over chromosome encoded natural 23S rRNA (more than 90% ribosomes contained mutant RNA), implying to the importance of this region for 50S assembly. Furthermore, in an attempt to gain insight into the structural alterations induced in the domain V, part of the domain (from position A2051 to G2603) was probed with various chemical and enzymatic probes. As a result, only minor structural changes were observed (Vester & Garrett, 1988). However, no connection was made in this work between the growth phenotypes and ribosomal peptidyl transfer function.

The first 23S rRNA mutations demonstrated to affect PTase were base substitutions at positions G2505 and G2583. Using thiostrepton to discriminate between wild type and mutant ribosomes under *in vitro* conditions (puromycin reaction), all three substitutions at position G2583 and G to C replacement at position 2505 were demonstrated to inhibit NAcPhe-puromycin formation (Paper I; see also Section 2).

Recently, Porse and colleagues have performed an extensive mutagenesis of 23S rRNA domain V and analysed the mutants with respect to their influence on cell growth, ribosome assembly and PTase. Secondary mutation A2058G was introduced and the ability of mutant ribosomes to carry out peptidyl transfer reaction was measured in the presence of clindamycin (Porse & Garrett,

1995; Porse *et al.*, 1996). Extreme effects on cell growth were observed for mutants U2506A, U2506C, U2506G, Ψ2580C, G2583U, U2584G, U2585A, U2585C and U2585G, which all exhibited dominant lethal phenotype (they were not able to grow even in the presence of 60 to 70% wild type ribosomes encoded by host chromosome). In general, dominant lethality correlates with severely affected PTase activity *in vitro*. A decrease in activity in the 'fragment reaction' could be a consequence of weakened binding of either PTase substrates: puromycin or peptidyl-tRNA fragment or impairment of peptide bond formation. 23S rRNA mutations U2506C, U2584G and U2585G that are dominant lethal, express significant activity in forming peptide bonds. Therefore, severe growth deficiency of these mutants can not be explained solely by impaired peptidyl transferase, but some other translational process(es) must be affected. Mutants G2583A and U2584C were extraordinary in that their growth rate was close to wild type but their ribosomes exhibited low activities. When Ac-Phe-tRNA^{Phe} was used as a donor instead of the fragment, ribosomes from mutants U2584A, U2584C and to a lesser extent U2585C and U2585G displayed enhanced peptidyl transferase activity, suggesting that the whole tRNA helps to compensate for defective interaction between the CCA end of the tRNA and the ribosome. In general, mutant RNAs incorporated readily into 50S and 70S particles, implying to unaltered 50S subunit assembly and subunit association. There are few exceptions: G2505C, G2550A, Ψ2580G, U2584A and U2584G mutants were severely reduced in 70S ribosomes.

Two mutations, namely G2550A and A2565U, are rather peculiar — they incorporate extensively into 50S subunits, but are underrepresented in 70S monosomes. Incorporation into ribosomal particles was found to be adversely correlated with the PTase activity. 50S particles from these mutants are with very low PTase activity, but the activity is drastically elevated after association with 30S subunits. Thus, G2550A and A2565U mutations seem to weaken the subunit-subunit interaction. In a single case, for mutant U2528C, the authors have noticed an increased PTase reaction rate for 50S subunits; 70S ribosomes with the same mutation were not different from wild type ribosomes.

Two phylogenetically invariant sequences, UGG2251 and ΨGG2582, can be found in 23S rRNAs that could have the potential to interact via Watson-Crick base pairing with the universal CCA 3' end of the tRNA at the ribosomal PTC (Gutell, 1993). When base changes were introduced into the first two positions of these sequences, no assembly or polysome formation defects were detected. While the mutations on the helix-loop 80 region (UG2250) hardly affected bacterial growth and peptide bond formation, the helix 90 mutants (ΨG2581) caused severe growth effects and were seriously inhibited in poly(Phe) synthesis (Spahn *et al.*, 1996a) and in PTase assay (Spahn *et al.*, 1996b). Cells with G2581A mutation in their 70S ribosomes were unable to grow and exhibited no PTase activity. The influence of Ψ2580C mutation was rather mild on bacterial growth. Notably, ribosomes with the same mutation were shown to have lost

about half of their efficiency in the PTase assay. Intriguingly, ribosomes with G2581A and Ψ2580C mutants did not response to addition of CMP in reaction between pA-fMet and Phe-tRNA^{Phe} (the same was also observed for G2582A and C, see Section 2.1.1). CMP is known to stimulate this reaction by a factor of four, but has no stimulatory effect with the CA-fMet. The result has been interpreted that the universal C75 of tRNA is essential for the correct docking of the donor substrate to the PTC (see above). Accordingly, the whole ΨGG2582 sequence could be, either directly or indirectly, involved in binding of the C75 of the peptidyl-tRNA to the donor site.

Among the bases of 23S rRNA that are protected from chemical probes by P-site tRNA and by CCA containing oligonucleotide analogues of P-site tRNA are the universally conserved nucleotides G2251, G2252 and G2253. Therefore, these nucleotides are possible candidates to interact with the tRNA C74C75 at the ribosomal donor site. Lieberman and Dahlberg were the first to analyze this region. Double mutant G2252C/G2253C was found to severely compromise cell growth. Mutant RNA assembled into 50S and 70S particles but was discriminated against in polysomes. The PTase activity, assayed in the 'puromycin reaction', was decreased, but the effect was rather moderate (influence of this double mutation on peptide bond formation could be underestimated, since the formation of AcPhe-puromycin was judged in the presence of wild type ribosomes). To test whether this effect derives from disruption of base pairing with the invariant cytidines of tRNA 3' end, a mutant tRNA gene was constructed with the GGA sequence instead of CCA. Mutant tRNA was ready to accept amino acid but failed to react as a P-site substrate, with either wild type or mutant ribosomes.Although these results support a role for G2252 and G2253 of 23S rRNA in the peptide bond formation, they fail to provide evidence supporting canonical base pairing between these residues and the 3' end of peptidyl-tRNA (Lieberman & Dahlberg, 1994). However, recent work of Samaha and coworkers disproves this observation to some extent. Using chimaeric reconstitution strategy to obtain pure population of mutant 50S subunits in combination with highly sensitive 'fragment reaction' they were able to demonstrate that compensatory mutation at position 74 (C to G) of tRNA restores the PTase activity of G2252C mutant ribosomes. Thus, interaction of tRNA in the 50S donor site involves Watson-Crick base pairing between C74 of tRNA and G2252 of 23S rRNA. The role of this interaction is probably to position the acceptor end of peptidyl-tRNA in the PTase site. Additionally, it was found that G75 mutation of tRNA cannot undergo Watson-Crick base pairing with G2253 in the same experimental system (Samaha *et al.,* 1995). The results are in good correlation with the observed effects of these mutations: G2252 mutations were dominant lethal, while G2253A and U mutants were only slightly affected in growth rate and PTase activity. G2253C mutation was found to confer recessive slow growth phenotype and was deficient in PTase assay *in vitro* (Porse *et al.,* 1996). The G2252 mutants were determined to be underrepresented in polysomes, which could be the consequence of impaired

PTase activity that impedes the rate of movement of ribosomes along mRNA (Samaha *et al.,* 1995). The requirement for canonical base pairing between tRNA and 23S rRNA that is so important for peptide bond formation under *in vitro* conditions, seems to be less stringent *in vivo*, at least with the $tRNA_1^{Val}$. Substitution of the $tRNA_1^{Val}$ 3' terminal CCA sequence to UCA had little or no effect on tRNA-ribosome interaction while the GCA and ACA mutations significantly increased the level of frame shifting and stop codon readthrough (O'Connor *et al.,* 1993). It seems therefore that Watson-Crick base pairing between C74 of tRNA and G2252 of 23S rRNA is not fundamental for peptide bond formation but is required for maintaining the accuracy of translation.

When another candidate for the interaction with C75 of tRNA, G2251 of 23S rRNA, was subjected to analysis, it was found that mutations G2251A and U confer dominant lethal phenotype and are severely deficient in PTase activity. No suppression of this decrease was observed using C75 mutant tRNA fragments (Green *et al.,* 1997). The absence of Watson-Crick suppression does not rule out the possibility of existence of non-canonical base pairing (Porse *et al.,* 1996) or that other moieties like phosphate or ribose of the RNA backbone are involved in direct interaction between 23S rRNA and tRNA (Hüttenhoffer & Noller, 1992).

1.10. Antibiotics and peptide bond formation

The majority of antibiotics inhibit cell growth by binding to functionally essential regions of the ribosome. Correlating antibiotic binding site with its mode of action provides a valuable source of information to understand the structure-function relationship of the ribosome. In general, antibiotics are produced by micro-organisms to inhibit the growth of others. One of the earliest indications that rRNA could be target for antibiotic action came from studies on *Staphylococcus aureus*. This organism was shown to protect itself from the attack of MLS antibiotics (macrolides, lincosamides and streptogramin type B compaunds) by methylating its 23S rRNA (Lai *et al.,* 1973). Later, several *Streptomyces* species being producers of many known antibiotics have been demonstrated to use similar strategy. The producer of erythromycin, *Streptomyces erythraeus* dimethylates A2058 of its own 23S rRNA (Skinner *et al.,* 1983) and *Streptomyces azureus*, the producer of thiostrepton, exploits similar self-defence by methylating nucleotide A1067 of its 23S rRNA (Thompson *et al.,* 1982). Thus, antibiotics seem to use rRNA as the primary target. The only example of antibiotic interaction with r-proteins is erythromycin, that binds weakly in solution to the ribosomal protein L15 (Teraoka & Nierhaus, 1978). The fact that rRNA-antibiotic contacts are the basis for the act of inhibition is reinforced by the observations, that group I self-splicing introns and hammer-

head ribozymes are inhibited by aminoglycoside antibiotics (Stage *et al.,* 1995; von Ahsen *et al.,* 1991).

Using the chemical footprinting method, a large proportion of antibiotics that interfere with PTase reaction have been shown to protect specific bases in 23S rRNA (Spahn & Prescott, 1996). Chloramphenicol is a classical inhibitor of PTase that inhibits peptide bond synthesis both *in vivo* and *in vitro*. It is known to affect A'-site binding, while P'-site binding remains unaffected (Harris & Pestka, 1977). The drug protects 23S rRNA nucleotides A2059, A2062, A2451, G2505 and enhances the reactivity of A2058 in the central ring of domain V (Moazed & Noller, 1987). Mutations at positions 2032, 2057, 2058, 2447, 2451, 2452, 2503 and 2504 confer resistance to chloramphenicol (Garrett & Rodriguez-Fonseca, 1995). Alterations of 23S rRNA nucleotides G2505 to C and G2583 to A or U lead ribosomes to hypersensitivity towards chloramphenicol (Paper I). Proteins L16 and L27 yielded cross-links to chloramphenicol (Cooperman *et al.,* 1990) and L16 enhances binding of the drug to the ribosome (Nierhaus & Nierhaus, 1973).

Lincosamide antibiotics (lincomycin and its derivative clindamycin) together with macrolides (like erythromycin and carbomycin) are known to compete with chloramphenicol for the ribosome binding. They all, except erythromycin, inhibit the 'puromycin reaction'. Erythromycin appears to block entrance of the tunnel for the growing peptide (Arevalo *et al.,* 1988), thereby stimulating premature dissociation of the peptidyl-tRNA from the ribosome (Menninger & Otto, 1982). In the same paper, carbomycin and spiramycin, and quite recently also lincosamide antibiotics were shown to have the same effect (Menninger & Coleman, 1993). Chloramphenicol instead, prevents the displacement of peptidyl-tRNA (Menninger, 1976). Lincomycin and clindamycin protect 23S rRNA bases A2058, G2061, A2451 and G2505 from chemical modifications and the patterns of modification differ in that A2059 is protected by clindamycin but not by lincomycin (Douthwaite, 1992b). Both erythromycin and carbomycin alter chemical reactivities of bases A2058, A2059 and G2505, while only carbomycin protects also A2062. Mutations G2505C, G2583U and G2583C led to increased sensitivity to lincomycin in 'puromycin assay' (Paper I; see also Section 2). Base changes at positions G2032, A2058 and A2059 render ribosomes resistant to lincomycin. Clindamycin resistance involves rather close set of bases, positions G2032, G2057 and A2058. Deletion of bases 1219-1230 in the domain II as well as mutations at positions G2057 and A2058 give resistance to erythromycin, but alterations of nucleotide G2032 make ribosomes hypersensitive to erythromycin (Garrett & Rodriguez-Fonseca, 1995). Thus, the binding sites for macrolides, lincosamides and chloramphenicol are largely overlapping, while their mode of action is not identical.

Puromycin is a structural analogue of the 3' end of Tyr-tRNA^{Tyr}. It is known as an effective A-site competitor and while bound, it can accept the growing peptidyl residue from the ribosomal donor site (Vazquez, 1979). After the complex between peptidyl-tRNA and puromycin has been formed, no further

peptidyl transfer can take place, since the amide bridge cannot be cleaved by the ribosome and peptidyl-tRNA-puromycin complex falls off the ribosome. Azido-puromycin has been cross-linked to nucleotides G2502 and G2504 of 23S rRNA and to ribosomal proteins L15, L18 and L23 (Cooperman *et al.,* 1990). As a minimized substrate of the ribosomal A-site, puromycin has been exploited as a classic tool for investigating ribosomal peptidyltransferase reaction.

Sparsomycin and amicetin are also powerful inhibitors of PTase. Sparsomycin enhances binding of the donor substrates to the ribosomal P-site and competitively inhibits the acceptor substrate binding to the ribosomal A-site (Vazquez, 1979). Chloramphenicol and lincomycin have been shown to compete with sparsomycin for binding to the ribosomes, suggesting that the binding takes place close to the ribosomal PTC. In contrast to most of the PTase inhibitors, sparsomycin and amicetin do not 'leave any strong footprints behind'. However, mutants of *Halobacterium halobium* and *Halobacterium salinarium* were isolated that exhibit resistance to sparsomycin. Mutations at positions C2471, C2518, and U2519 (C2452, C2499, and U2500 accordingly to *E. coli* numbering) for the first, and the absense of modification at U2603 for the second archeon, have demonstrated to confer resistance against the drug (Lazaro *et al.,* 1996; Tan *et al.,* 1996). In a similar manner, amicetin resistant mutant, carrying U to C mutation at position corresponding to 2438 in *E. coli* 23S rRNA has been isolated (Leviev *et al.,* 1994).

Integrating protection patterns of PTase antibiotics on 23S rRNA with the knowledge of their structure and mode of action, Garrett and coworkers have constructed a map for the ribosomal PTC (Porse *et al.,* 1995; Rodriguez-Fonseca *et al.,* 1995). The region A2451-C2452 has been implicated as a hydrophobic site for the side chains of aromatic amino acids. The photoreactive benzoylphenyl derivative, linked to the 3' end of the P/P site bound tRNA, crosslinks to A2451-C2452 (Steiner *et al.,* 1988). A2451 is deprotected on excision of amino acid residues from both A/A- and P/P-site bound tRNAs (Moazed & Noller, 1989). Single site mutations at these positions in archeal rRNAs produce resistance to celesticetin, anisomycin and chloramphenicol (Hummel & Böck, 1987; Mankin & Garrett, 1991). Narciclascine, anisomycin and chloramphenicol alter reactivities of this region (Moazed & Noller, 1987; Rodriguez-Fonseca *et al.,* 1995). All these antibiotics contain aromatic groups which are important for their inhibitory action (Vazquez, 1979).

Donor substrate site is proposed to involve nucleotides U2506, U2585, A1787 and G2252. Reactivity of nucleotides A2506 and U2585 is shown to be enhanced on removal of the 3'-terminal adenosine of P-site bound tRNA. Peptidyl-tRNA analogues protect bases G2252-G2253 from chemical modifications (Moazed & Noller, 1989). Recently, a possibility to form a basepair between G2252 of 23S rRNA and C74 of P-site bound peptidyl-tRNA has demonstrated (Samaha *et al.,* 1995). Protection effects were observed for griseoviridin and virginamycin M, both thought to interfere with P-site substrate binding (Vazquez, 1979). A nucleotide covariance study predicted that nuc-

leotides U1782 and U2586 interact (Larsen, 1992) and cross-link was identified between nucleotides U1782 and G2608/G2609 (Mitchell *et al.*, 1990). Griseoviridin induced an enhanced chemical reactivity of A1787 in addition to protecting U2585 (Rodriguez-Fonseca *et al.*, 1995).

C2394 is one of the three sites that is protected by E-site bound deacylated tRNA (Moazed & Noller, 1989) but protection by virginamycin M and toxin T2 was observed exclusively on eukaryotic 28S rRNA at this position. Macrolides yield footprints at A2508, A2509 and U2609 (Rodriguez-Fonseca *et al.*, 1995). Nucleotides at the A2058-A2062 region and U2609 supposedly constitute part of an entrance of the nascent peptide channel. The A2058 to A2062 region is also easily accessible to chemical probes in the ribosome (Egebjerg *et al.*, 1990). Methylation of A2058 has been demonstrated to give resistance against erythromycin in its producing organism (Skinner *et al.*, 1983). Recently, Tenson and coworkers have found that erythromycin resistance could be mediated by a specific interaction of the 23S rRNA-encoded pentapeptide (E-peptide) with the ribosome (Tenson *et al.*, 1996). E-peptide is assumed to interact with the large ribosomal subunit in the vicinity of the peptidyltransferase centre at the site of erythromycin binding (Tenson *et al.*, 1997).

1.11. Translational accuracy

Translation is a relatively rapid and accurate process, proceeding at a rate of about 20 amino acids per second with an average of 5×10^{-4} missense events per codon. The process is well balanced in respect to speed and accuracy. For example, if the ribosome selects tRNA with maximum accuracy, then translation is exceedingly inefficient and *vice versa* (Kurland & Ehrenberg, 1987). Therefore, deviations in tRNA selection could be very costly or even lethal for the cell.

How is the translational accuracy 'kept in frame' and what are the components and mechanisms involved in this process?

Ribosomal mutations and universal inhibitors of protein synthesis that alter ribosome functioning have been useful tools to answer these questions. In 1961 Gorini with colleagues noticed the peculiarity that an arginine auxotroph that contained also mutation rendering it resistant to streptomycin could be restored to prototrophy by addition of streptomycin to the growth medium (Gorini *et al.*, 1961). After careful inspection, evidence was presented that streptomycin was altering the specificity of translation via an interaction with the ribosome (Gorini, 1964). This was the first time when the importance of ribosomal structure was recognized to influence reading of the code during translation — up until that time, it was thought that all the specificity of translation of the genetic code depends on the interaction between transfer RNA and messenger RNA.

Streptomycin and other aminoglycoside antibiotics have been demonstrated to induce misreading of the genetic code, whereas kasugamycin appears to increase the accuracy of translation. Many fidelity mutants of tRNA have been isolated (Murgola, 1985). Mutations in ribosomal proteins S4, S5, L7/L12, elongation factor Tu and release factor 1 and 2 decrease the accuracy of protein synthesis, while mutant r-proteins S12, S17 and L6 increase it implying that accuracy tuning depends on components other than codons and anticodons (Yarus & Thompson, 1983).

During the course of translation, tRNAs contact a number of bases in 16S and 23S rRNA. The anticodon end of tRNA and mRNA associated to it interact with the decoding centre of 30S subunit and the CCA 3′end of the tRNA interacts with the PTC of the 50S subunit. Residues in both 16S and 23S rRNAs have the potential to influence the interaction between tRNAs and the ribosome and, consequently, the selection of correct tRNA. Base changes in 16S rRNA were demonstrated to suppress nonsense mutations, frameshift mutations, and cause increased translational misreading (Noller, 1991; Lieberman & Dahlberg, 1996).

To date, 23S rRNA is also included into the list of components that control the precision of translation. At first, it would seem to be a little surprising, since the codon-anticodon interaction on 30S subunit is known to be responsible for choosing the right aa-tRNA during the decoding process. However, according to the current knowledge, establishment of a codon-anticodon interaction is not the final stage for correct docking of the whole tRNA that would be properly decoded and ready for peptidyl transfer. After the ternary complex enters the ribosomal A-site, the anticodon part of the tRNA makes contact with mRNA and the decoding centre of the 30S subunit. This is the moment for initial selection. The ternary complex, cognate or non-cognate, may either establish the codon-anticodon interaction by hydrolyzing GTP or dissociate from the ribosome. When the initial selection is over and tRNA-mRNA interaction is firm, EF-Tu-GDP leaves the ribosome and aa-tRNA is positioned into the proof-reading site. At this site, the CCA 3' end of the tRNA is not yet fixed. Aminoacyl-tRNA may now be discarded from the ribosome or become docked into the peptidyl transfer site of the 50S subunit. When docking of the CCA end occurs, then the selection is over and peptidyl transfer can take place. For efficient peptide bond formation, the CCA ends of both aa-tRNA and pep-tRNA need to be properly positioned. 23S rRNA and several r-proteins have been shown to be involved in binding of both substrates (see Section 1.9). Thus, experimental evidence indicates that 23S rRNA could actually participate in tuning translational accuracy.

The first 23S rRNA mutants that affect translational accuracy were discovered in our laboratory. We checked the misreading properties of mutant ribosomes and found that mutations at position G2583 (to C and U) lead to increased translational accuracy *in vitro* (Paper I). The same mutations attributed

error restrictive character to the ribosomes also *in vivo* (Nigul *et al.*, unpublished; Paper II; for details, see Section 2).

Another 23S rRNA mutant with a G to C transversion at position 2661 in the universally conserved α-sarcin loop that interacts during ribosomal elongation cycle with elongation factors Tu and G, has been shown to confer ribosomes an error restrictive phenotype *in vitro* (Bilgin & Ehrenberg, 1994). The gain in accuracy was attributed to the improved initial selection rather than proof-reading, correlating with a loss in k_{cat}/K_M for the ternary complex-ribosome interaction. This agrees with the expectation that when the accuracy in a certain step approaches its maximum then the k_{cat}/K_M must drop considerably. Combining the 23S rRNA mutation with error restrictive mutation of r-protein S12, even lower error rate was observed. The G2661C mutation causes a loss of viability in combination with a hyperaccurate rpsL mutation (Tapprich & Dahlberg, 1990). Viability can be restored by introduction of a tufA mutation (Tapio & Isaksson, 1991) or by addition of antibiotic streptomycin to the media (Bilgin & Ehrenberg, 1994).

In addition to hyperaccurate mutants, multiple error prone 23S rRNA mutants have been obtained. The fidelity of decoding is decreased by mutations at position U2555, a base protected by the aminoacyl residue of A-site bound tRNA (O'Connor & Dahlberg, 1993) and by mutations at positions G2252/G2253 (Gregory *et al.*, 1994), bases protected by the CCA end of peptRNA (Moazed & Noller, 1989). Moreover, G2252 and C74 of tRNA have shown to base-pair (Samaha *et al.*, 1995). Eight different 23S rRNA mutants (and none of 16S rRNA) were recovered by isolating rRNA mutants that increase frameshifting (O'Connor & Dahlberg, 1995). One group of mutations (U2460G, U2492C, U2493C and deletion of U2493) was located in helix 89, close to the central loop of domain V. No tRNA or antibiotic dependent protection was observed in helix 89 in chemical footprinting experiments. In eukaryotic 28S rRNA, bases in the region equivalent to the loop end of helix 89 became hypersensitive to chemical modification reagents upon association of subunits into 80S ribosomes (Holmberg *et al.*, 1994). These data indicate that this 23S rRNA region undergoes structural rearrangements during subunit association. Mutations at position U2493 result in a drastic decrease in peptidyltransferase activity (Porse & Garrett, 1995). The other group of mutations lies in a small single-stranded loop in domain IV: C1914U, insertion of an extra A between A1916 and U1917 and deletion of A1916. Bases A1916, A1918 and U1926 are protected from chemical modification by the 30S subunit (Noller, 1991) and several intra-RNA cross-links have been obtained between domain IV and V (Mitchell *et al.*, 1990). Mutations at several positions in domain IV, close to position 1916, greatly reduce the peptidyltransferase activity of the mutant ribosomes (Leviev *et al.*, 1995). These data place the CCA end of the tRNA close to both the 1916 region and the peptidyltransferase loop in domain V. Another mutant isolated during these experiments contains substitution of

C2666 to U in 23S rRNA. Position 2666 is close to position 2661 mentioned above that is involved in tRNA selection. All the mutations that change the accuracy of translation are in highly conserved or invariant elements of ribosomal RNA.

Conclusively, changes in the interaction of the CCA end of the tRNA due to mutations in the putative peptidyltransferase centre have serious impact on overall accuracy of translation (Table1).

1.12. Ribosomal proteins and peptidyl transferase

The question 'Which protein is the peptidyl transferase?' has been asked for more than two decades. In early days, isopycnic CsCl gradient and high magnesium concentration was used to "strip" the 50S subunit, and three discrete particles with increasing protein content were found. The transition coincided with the loss of both a small group of proteins and peptidyltransferase activity. The activity was restored by replacing the proteins L6, L11 and L16 (Staehelin *et al.*, 1969). Similar results were obtained by other groups. Stripping proteins with LiCl (Moore *et al.*, 1975; Nierhaus & Montejo, 1973), a single split protein L16 was found to restore the catalytic activity (as discussed below, L16 is not crucial for PTase). Single omission tests, where 50S subunits lacking one ribosomal protein were reconstituted and tested in the fragment reaction for peptidyltransferase activity, have pointed to a larger set of proteins necessary and sufficient for the activity: L2, L3, L4, L15 and L16 (Schulze & Nierhaus, 1982). Although the reconstitution technique does not answer, whether the ribosomal component is essential for the assembly of the whole active centre or part of it, it certainly defines the group of possible candidates. Since the peptidyltransferase takes place in close proximity to acceptor ends of A- and P-site bound tRNAs, the crosslinks between 3' terminals of such tRNAs and ribosomal components must refer to the closeness to the catalytic centre. Ribosomal components identified by this approach are the proteins L2, L15, L16, L27 and L33, plus 23S rRNA (Wower *et al.*, 1993). Photoaffinity labeling by antibiotics that inhibit peptidyl transferase provided evidence for the presence of L2, L15, L16, L18, L22, L23 and L27 as well as the central loop of domain V of *E. coli* 23S rRNA at or near the peptidyltransferase centre (Cooperman *et al.*, 1990). L15, L16 and L27 were later exluded from the list of possible candidates — viable *E. coli* mutants lacking L15 and L27 could be isolated (Dabbs, 1986; Wower *et al.*, 1995). L16 was found dispensable for both catalytic activity and poly(Phe) synthesis (Francheschi & Nierhaus, 1990; Remme *et al.*, 1983; Tate *et al.*, 1987). Hence, proteins L2, L3, L4 together with 23S rRNA are the main suspects for being crucial for the ribosomal PTase.

It has been proposed that peptidyl transferase operates via a charge-relay system similarly to serine proteases, though the serine proteases cleave the pep-

tide bond, while ribosome forms it (Nierhaus *et al.,* 1980b; Rychlik & Cerna, 1980). The catalytic core of serine proteases consists of three amino acids: serine, histidine and aspartic acid. The involvement of histidine in peptide bond formation is supported by several observations: the pH dependence of peptidyl transferase correlates with the involvement of an imidazole residue of histidine, or the N-terminal a-amino group of peptide (Maden & Monro, 1968); histidine-modifying agents inhibit the peptidyltransferase activity of the 50S subunit (Rychlik & Cerna, 1980). An involvement of serine or aspartic acid in the peptidyltransferase reaction has never been demonstrated. Among r-protein candidates for peptidyltransferase, evolutionally the most highly conserved protein is L2. Similarly to serine proteases, L2 has universally conserved histidine, serine and aspartic acid. It has been shown that the modification of one histidine residue of L2 is sufficient for the loss of PTase activity (Wan *et al.,* 1975). In addition, antibodies raised against L2 block the fragment reaction (Nag *et al.,* 1986; Olsen *et al.,* 1991). Furthermore, L2 is capable of binding to a portion of domain IV of 23S rRNA (Beauclerk & Cundliffe, 1988) that is structurally and functionally linked to the central loop of domain V. The latter region of 23S rRNA was clearly identified as a part of the peptidyltransferase centre (Cooperman *et al.,* 1990; Garrett & Rodriguez-Fonseca, 1995; Noller, 1993). 50S subunits depleted of L2 were shown to be inactive in puromycin reaction as well as in dipeptide formation assay (Dietrich, G., personal communication). The results indicate that at least one of the following reactions is dependent on L2: binding of A-site acceptor tRNA and/or P-site donor tRNA or the PTase. Yet another likely explanation is that protein L2 might be necessary for the structural stability of the catalytic centre. Interestingly, L2 has also a significant affinity to tRNA (Remme *et al.,* 1985). Ribosomes with His229Arg mutation in protein L2 have shown to be inhibited (five-fold) in the 'puromycin reaction' (Dietrich, G., personal communication). In another study, when the same histidine was mutagenized to Gln a complete loss of PTase activity was observed (Cooperman *et al.,* 1995).

In addition to L2, another 50S subunit protein, namely L27, has been made responsible of modulating ribosomal PTase (Wower *et al.,* 1995). In this study, a constructed minus L27 mutant exhibited an impaired PTase activity, as tested by 'puromycin reaction'. As noted above, L27 has thought to reside in close proximity to PTase substrates or the PTase inhibitors.

The importance of L3 and L4 in peptidyltransferase reaction remaines enigmatic.

As a potential catalyst, RNA has its own apparent deficiencies when compared with proteins. The common amino acids provide a much wider array of functional groups than the four major RNA nucleotides. Therefore, proteins seem to be much more adaptable to substrate binding and chemical catalysis than RNA. The inherent versatility of proteins as enzymes is shown by their ability to catalyze more comprehensive range of chemical processes that is known for ribozymes.

Using aa-tRNA and pep-tRNA in mRNA directed polypeptide formation, Nitta and coworkers found that the PTase reaction is promoted by a tertiary amine, pyridine. Tertiary amines appear to accelerate the PTase by means of its catalytic character on the nucleophilic substitution reaction. In 1995 M. Shimizu published a paper, where the catalysis of peptidyl transfer reaction was shown to occur without the aid of ribosome. A dipeptide, alanylhistidine, was demonstrated to act as a catalyst for the peptidyl transfer reaction between the amino acid moieties of phenylalanyl, lysyl, prolyl and glycyl tRNAs in the absence of ribosomes. This reaction was strictly dependent on their model templates: poly U, poly A, poly C and poly G, respectively. Histidine, that is tertiary amine like pyridine has no catalytic activity unless complexed with alanine. Interestingly, amongst 18 different dipeptides, only alanylhistidine exhibited significant catalytic properties, while histidylalanine did not (Shimizu, 1996).

1.13. Other modulators of peptide bond formation

A nonribosomal protein factor termed as EF-P is known to enhance both the affinity of acceptor substrate binding and peptide bond formation (Chung *et al.*, 1990). EF-P does its job by an unknown mechanism, possibly by 'fine-tuning' the catalytic centre.

Both, 5S rRNA and 30S subunit can be neglected when PTase is considered from the fundamental point of view. However, for efficient protein biosynthesis both 5S rRNA and 30S subuinit are required. 5S rRNA is a component of the large ribosomal subunit of cytoplasmic ribosomes from all living organisms, with the exception of fungi and animal mitochondria. Particles lacking 5S rRNA possess only 8–10% of the activity of mature 50S subunit as judged by puromycin reaction. The same particles were unable to bind aa-tRNA-EF-Tu•GTP ternary complex (Dohme & Nierhaus, 1976).

As already mentioned above, the PTase reaction occurs at the subunit interface. 30S subunit shields the catalytic centre against ribonuclease and protease attack and both subunits protect most of the P-site bound tRNA from hydroxyl radical attack (Hüttenhoffer & Noller, 1992). Ribosomal protein L2, that is known to modulate the rate of PTase reaction in *E. coli*, crosslinks to several proteins of the 30S subunit (Lambert & Traut, 1981). 30S subunit may indirectly participate in peptide bond formation by influencing the conformation of both tRNAs and the 50S subunit. In the fragment reaction between C-A-C-C-A-fMet and puromycin, which can occur without 30S subunit, the rate of fMet-puromycin formation is increased by a factor of two when 70S ribosomes are used (Monro, 1967).

1.14. Elongation factor cycles and 23S rRNA

During each round of ribosomal elongation cycle, elongation factors Tu (EF-Tu) and G (EF-G) interact alternately with the ribosome at overlapping, mutually exclusive sites. Both factors are members of the family of G-proteins, the activity of which is regulated by the binding of GTP or GDP (Bourne *et al.*, 1991). Binding of GTP activates both proteins, while GDP bound factors are in their inactive state. Like most essential processes during protein synthesis, the cyclic performance of *Escherichia coli* translational elongation factors is linked with specific regions in the rRNA. Two structural motifs of *E. coli* 23S rRNA have been established that are in control of the fluent action of elongation factors during ribosomal elongation cycle. One such motif has been situated in the domain II on the secondary structure model of *E. coli* 23S rRNA (Fig. 3A). According to the site of binding for thiopeptide antibiotic thiostrepton it is termed as "thiostrepton binding structure", TBS (nucleotides between positions 1051 to 1108). Another structural element, known as the site of action of cytotoxin α-sarcin (the "α-sarcin stem-loop structure") is lying in the domain VI (Czworkowski & Moore, 1996; Thompson, 1996). These two regions are distant from each other on the primary and secondary structure of *E. coli* 23S rRNA. However, on a higher structural level in the ribosome, they might reside in close vicinity. Immuno electron microscopy, footprinting and cross-linking experiments have demonstrated, that both elongation factors share partially overlapping binding sites on the ribosome (Thompson, 1996). EF-G has been cross-linked to the TBS (Sköld, 1983). It also shields several nucleotides against chemical modification in the same domain, as well as in the α-sarcin loop. EF-Tu protects nucleotides in the α-sarcin loop, but no shielding was detected in the TBS (Moazed *et al.*, 1988). Mutational analysis has shown that *E. coli* ribosomes with G to C transversion at position 2661 in 23S rRNA have EF-Tu and EF-G cycles kinetically impaired (Bilgin & Ehrenberg, 1994). Similarly, an A to U transversion at position 1067 impairs the cyclic action of both elongation factors (Paper III, see also Section 2). These data are implying to the orchestrated performance between the TBS and α-sarcin loop during ribosomal elongation cycle.

Computer simulated secondary structures of the TBS from organisms representing different kingdoms are universally conserved (de Rijk *et al.*, 1996). In support to phylogenetic data, experimental evidence has demonstrated the possibility of heterologous interaction between *E. coli* protein L11 and the TBS from archaebacteria, yeast and mouse (Beauclerk *et al.*, 1985; El-Baradi *et al.*, 1987; Stark *et al.*, 1980). Successful exchange of the TBS between *E. coli* and yeast without the loss of ribosome functions (Musters *et al.*, 1991; Thompson *et al.*, 1993) provides an evidence for functional conservation of this rRNA region.

Originally, the importance of the TBS in the ribosome function was deduced from experiments, where the mode of action of two antibiotics, thiostrepton and

micrococcin, was studied. These antibiotics inhibit a variety of reactions in the ribosome cycle, among them are EF-Tu and EF-G dependent activities (Vazquez, 1979). For both antibiotics, the only established binding site lies in the TBS (Rosendahl & Douthwaite, 1995). Thiostrepton producing organism *Streptomyces azureus* has been shown to protect himself from the action of the antibiotic by specifically methylating the ribose of nucleotide A at position 1067 (*E. coli* numbering) of 23S rRNA (Thompson *et al.*, 1982). The modification simply restricts the binding of thiostrepton to the ribosome. Micrococcin and thiostrepton, although structurally related to each other, have diametric influence on EF-G dependent GTPase: micrococcin stimulates this reaction, while thiostrepton inhibits it (Cundliffe & Thompson, 1981). This phenomenon correlates with their different binding to the ribosome — structural probing of 23S rRNA has revealed, that binding of micrococcin enhances and thiostrepton reduces the accessibility of the chemical modificator DMS to N1 position of the base A1067 (Egebjerg *et al.*, 1989). When A at position 1067 in 23S rRNA was replaced by U or C, ribosomes became highly resistant to both antibiotics (Thompson *et al.*, 1988). Structural probing data suggest that direct interaction may exist between A1067 and thiostrepton or micrococcin (Rosendahl & Douthwaite, 1995). Nucleotide A at position 1067 of 23S rRNA is conserved among prokaryotic, archaebacterial and chloroplast 23S rRNAs, but eukaryotes have mostly G at this position (de Rijk *et al.*, 1996). The fact is coinciding with the observation, that prokaryotic ribosomes are sensitive to thiostrepton, while their eukaryotic counterparts remain totally insensitive. However, the difference in the antibiotic binding is only partially attributable to the nucleotide change. Eukaryotes are thiostrepton resistant possibly due to the exchange of protein L11 with the eukaryotic homologue (Thompson *et al.*, 1993; Uchiumi *et al.*, 1995). Thiostrepton binding is modulated by the ribosomal protein L11 (Thompson *et al.*, 1979), which also binds to the TBS (Ryan & Draper, 1989). Comprehensive analysis of the interaction between L11 and its binding region has demonstrated, that L11 stabilizes tertiary structure of the TBS and is therefore important factor for accurate folding of this structure (Draper, 1996). L11 and ribosomal protein complex $L10(L7/L12)_4$, often named as L8, have been shown to promote EF-Tu and EF-G dependent functions (Beauclerk *et al.*, 1984). Importance of the TBS has also become evident in etiology of human diseases. An anti-28S autoantibody was found in sera of patients with systemic lupus erythematosus (Uchiumi *et al.*, 1991). This antibody binds to the eukaryotic 28S rRNA (but not to its eubacterial homologue) and inhibits EF-2 dependent GTP hydrolysis. Specificity of the antibody binding is dependent on the nature of position 1159 (1067 by *E. coli* numbering). G1159 has the binding ability, but when substituted to A, the binding is lost. Substitution of A1067 to G in *E. coli* 23S rRNA confers the antibody binding (Uchiumi & Kominami, 1994). Thus, various types of data imply to the central role of position 1067 for ribosome performance.

Taking together, all the date establish that the TBS together with the α-sarcin stem-loop structure of 23S rRNA, several r-proteins and elongation factors are involved in sophisticated interplay to implement the ribosome cycle.

2. RESULTS AND DISCUSSION

2.1. Functional analysis of *E. coli* 23S rRNA mutants

2.1.1. Peptidyl transferase

For a long period, when enzymatic activity was considered to be solely the property of proteins, the search for the ribosomal peptidyltransferase concentrated mainly on ribosomal proteins of the 50S subunit. The other and more objective reason was the absence of adequate methods for analysis of rRNA and existence of powerful techniques for detection of ribosomal proteins (like two-dimensional electrophoresis and immunoassays, allowing a straightforward identification of affinity-labeled proteins). By using derivatives of aa-tRNAs and antibiotics that inhibit ribosomal peptidyl transferase, proteins like L2, L15, L16 and L27 were most often found to be affinity labeled. Mapping by immunoelectron microscopy has shown that L2, L15 and L27 are clustered at the subunit interface, where the interaction of peptidyl transferase substrates is thought to occur. However, no single ribosomal protein has been found to possess the peptidyltransferase activity.

Our interest has been to specify the components of the ribosomal peptidyltransferase centre and to reveal the role of 23S rRNA in this reaction. As judged from the review of previously published papers, several nucleotides in domain V of 23S rRNA were already suspected to be part of or located in the close neighbourhood of the peptidyltransferase centre. Importantly, some of them are also universally conserved throughout evolution. As a further step toward the understanding of 23S rRNA functional role in PTase reaction, we chose the site-directed mutagenesis approach to change the nucleotide sequence in the putative PTase centre of *E. coli* 23S rRNA. In order to determine the effects of base substitutions on peptidyl transfer reaction, 23S rRNA nucleotides G2505, G2582, G2583, G2607 and G2608 were subjected to mutagenesis and further analysis. So far, the absence of methods which could discriminate between wild-type (chromosome born) and mutant ribosomes (plasmid born) was a major obstacle in scoring the effect of 23S rRNA mutations to the peptidyltransferase reaction. Therefore, the first task was to find a way to repress selectively the influence of wild-type ribosomes. For this purpose, we introduced the second mutation, an A to U transversion at position 1067 into the gene of 23S rRNA (Paper I). This base substitution renders ribosomes resistant to antibiotic thiostrepton (a powerful inhibitor of translocation) under *in vitro* translation conditions.

Mutant ribosomes were analyzed in respect of their influence on bacterial growth and in the following series of *in vitro* reactions: for their ability to direct

poly(U) dependent translation, sensitivity to peptidyltransferase inhibitors and impact on missense error frequency.

By using thiostrepton based selection between wild type and mutant ribosomes in puromycin reaction (Fig. 5), we demonstrated that all three possible substitutions of G2583 and G to C replacement at position 2505 inhibit poly(U) translation and the NAcPhe-puromycin formation. 23S rRNA mutants at positions G2505 and G2583 are the first rRNA mutants demonstrated to affect ribosomal peptidyl transferase. These mutants exhibited also an increased sensitivity to peptidyl transferase inhibitors such as chloramphenicol and lincomycin. While enhanced chloramphenicol sensitivity was observed in poly(U) translation, the effect was not seen in puromycin reaction (Paper I). In the same paper, an error restrictive phenotype was attributed to the mutations at the position G2583 (see below). Because chloramphenicol and lincomycin are shown to affect primarily the acceptor substrate binding, correlation between hypersensitivity to these antibiotics and altered translational accuracy of G2583 mutants could be described in terms of the improperly positioned aa-tRNA to the ribosomal A-site.

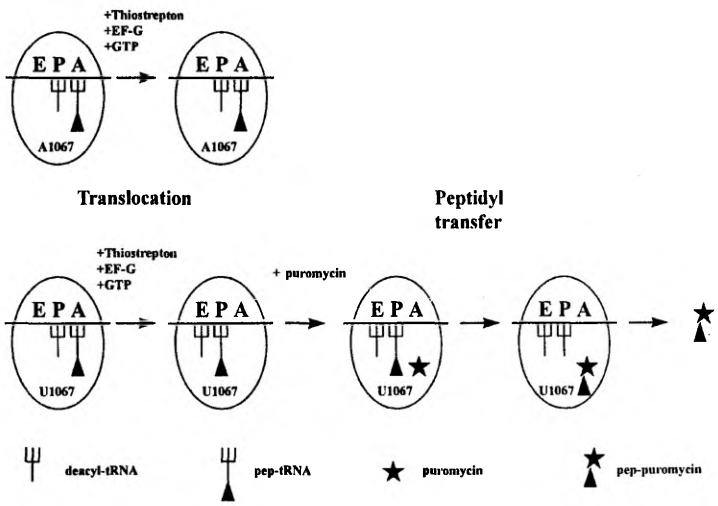


Figure 5. Schematic representation of the "selective transpeptidation strategy". Ribosomes and tRNA binding sites are as in Figure 2. A1067 ribosomes are with wild type 23S rRNA, whereas U1067 ribosomes are carrying mutation (A1067U) in the same molecule. Base change at position A1067 to U renders ribosomes resistant to antibiotic thiostrepton. By using thiostrepton under *in vitro* conditions, puromycin reaction kinetics can be measured exclusively on plasmid borne ribosomes that carry U1067 mutation in 23S rRNA (Paper I and Spahn *et al.*, 1996b).

In the next series of experiments a more advanced experimental system was used (heteropolymeric mRNA instead of poly(U) and codon specific tRNAs). The mutations mentioned above and additional mutations G2582A and G2582U were found to be severely deficient in peptide bond formation. These results are in accordance with the observation that all these mutations were deleterious to bacterial growth (Paper I). At the same time, the mutations were shown to have no effect on ribosome assembly. The mutant 23S rRNAs distributed equally in the polysomal fraction, in the 70S ribosomes and in 50S subunits (Paper IV).

Furthermore, the role of 23S rRNA nucleotides in helix 90 region for peptidyl transferase substrate (pep-tRNA) binding was assessed by CMP stimulation assay. In this experiment pA-fMet is the minimal donor substrate for the ribosomal peptidyltransferase reaction but its donor activity is significantly lower than that of pCA-fMet. Phe-tRNA^{Phe} has been used as an acceptor substrate. CMP is known to stimulate the PTase reaction between pA-fMet and acceptor substrate by a factor of four, but has no stimulatory effect with the pCA-fMet (Cerna *et al.*, 1973b). This result suggested that the universal C75 of tRNA 3' end is essential for the correct docking of the donor substrate at the PTC and that CMP stimulates the donor activity of pA-fMet by taking the place of C75 (Cerna, 1975). In a recent paper, Spahn and coworkers have shown that the pA-fMet reaction of ribosomes carrying mutations Ψ2580C and G2581A cannot be stimulated by CMP (Spahn *et al.*, 1996b). The same assay was performed with the ribosomes containing mutations at positions G2505, G2582 and G2583. The mutations at G2582A and G2582C were demonstrated to have lost the CMP stimulation, while the adjacent G2583C and G2505C mutations responded fully to CMP (Paper IV). Thus, the loss of CMP stimulation seems to be restricted to mutations within the ΨGG2582 sequence, indicating that ΨGG2582 could form a binding pocket for C75 of tRNA. C74 of pep-tRNA has already been shown to base-pair with G2252 from the helix 80 region of 23S rRNA (Porse *et al.*, 1996; Samaha *et al.*, 1995). Using the "damage selection" approach, nucleotides at positions U2506 and U2585 were identified to be important in interaction with 3' A76 of pep-tRNA (A. Mankin, personal communication). Additionally, a functional coupling between nucleotides of helixes 80 and 90 has been shown (Green *et al.*, 1997; Samaha *et al.*, 1995), making these helices the most likely candidates for the interaction with the 3' end of pep-tRNA.

2.1.2. Translational Accuracy

The very first 23S rRNA mutants that affect translational accuracy were discovered in our laboratory and the finding was rather fortuitous. Occasioned by a few experimental hints and by a theoretical proposition that peptidyl transferase could be a limiting and irreversible step for aa-tRNA selection

(Remme, 1993; Remme & Villems, 1985), we checked the misreading properties of mutant ribosomes and found that mutations G2583C and G2583U lead to increased translational accuracy *in vitro*. Accuracy increased in the order G<A<U<C (Paper I). The same mutations attributed error restrictive character to the ribosomes also *in vivo* (Nigul *et al.*, unpublished; Paper II). Cells with such hyperaccurate ribosomes were not viable. There are examples available were the viability of cells was restored by introducing an extra mutation or agent (like antibiotic) that could change the accuracy level back to normal (Bilgin & Ehrenberg, 1994). Attempts to restore the growth of G2583C mutant with variety of ram mutants (mutations in r-protein and EF-Tu) was not successful (Fisher, M., unpublished). G2583C, G2582U and G2582A ribosomes have considerably lower processivity both *in vitro* and *in vivo* and exhibit enhanced pep-tRNA drop-off rate (Paper I; Maiväli *et al.*, manuscript in preparation). These results are in accordance with theoretical considerations of Kurland and Ehrenberg who have proposed that high accuracy requires comparatively low affinity between tRNA and the ribosome, while high processivity requires a high affinity between ribosome and tRNA (Kurland & Ehrenberg, 1987). What could be the reason why G2583C, G2583U and G2583A mutations are lethal? One possible explanation is an enhanced drop-off rate of pep-tRNA. Excessive accumulation of pep-tRNA is shown to be lethal (Menninger, 1976). Alternatively, the main reason to cause such a severe growth phenotype could lie in paralysed peptidyl transferase (Paper IV). Obviously, mutations at position 2583 affect the ribosomal PTC in the way that the CCA 3' end of the tRNA becomes improperly oriented in the PTC, leading to increased time for peptidyl transfer. This means longer period for tRNA selection and, since the binding of the CCA end is affected, pep-tRNA tends to fall off from the ribosome. Thus, peptidyl transfer and translational accuracy seem to be tightly dependent on each other and the severe growth inhibition could be induced by both factors. The finding that mutations at the 3' end of $tRNA_1^{Val}$ (bound to the P- or E-sites of the ribosome) increased frameshifting and readthrough of downstream stop codons, provides additional link between peptide bond formation and the fidelity of decoding (O'Connor *et al.*, 1993).

Base changes G2607C, G2608A, G2608C and G2608U had no effect on poly(U) translation. Neither they changed the sensitivity towards chloramphenicol and lincomycin nor affected translational accuracy (Paper II).

Taking together the results of translational accuracy and transpeptidation experiments one can stress the importance of nucleotides in helix 90 region on ribosomal peptidyl transferase and to the binding/selection of its substrates. Although the experiments with 23S rRNA are strongly suggestive of rRNA involvement in the peptidyltransferase reaction, circumstancial evidence still exist for the possible involvement of ribosomal protein(s).

Table 1.

	Mutant	Second mutation	Growth	Peptidyl transfer	Accuracy	Publication
1.	C1914A		ok		ok	9
2.	C1914U		m		ram	9
3.	delA1916		m		ram	9
4.	A1916+AA		st		ram	9
5.	G1921C		m		ok	9
6.	U1926C	A2058G	R, L	st		15
7.	U1940A	A2058G	R,L	st		15
8.	U1946C	A2058G	R, L	st		15
9.	U1951C	A2058G	R, L	ok		15
10.	U1955G	A2058G	R, L	st		15
11.	U1956A	A2058G	R, L	ok		15
12.	U1979C	A2058G	R, L	st		15
13.	U1982A	A2058G	R, L	st		15
14.	G1984A	A2058G	R, L	st		15
15.	A2060C		L			10
16.	U2249C	A1067U	ok			1
17.	G2250A	A1067U	ok	ok		1, 2
18.	G2250A/C2254U	A1067U	ok			1
19.	G2251A	TAG	R, L	st		3
20.	G2251U	TAG	R, L	st		3
21.	G2252A	TAG	D, L	st		4
22.	G2252A		L			6
23.	G2252C	A2058G	R, L	st		5
24.	G2252C		L			6
25.	G2252U	TAG	D, L	st		4
26.	G2252U	A2058G	R, L	st		5
27.	G2252U		L		ram	6
28.	G2252C/G2253C		m	m		7
29.	G2253A	A2058G	ok	m		5
30.	G2253A	TAG	R, m			4
31.	G2253A		ok		ok	6
32.	G2253C	A2058G	R, m	st		5
33.	G2253C		m		ram	6
34.	G2253U	A2058G	ok	m		5
35.	G2253U	TAG	R, m			4
36.	G2253U		ok		ram	6
37.	C2254U	A1067U	ok			1
38.	A2450C		L			10
39.	U2457+G		m		ram	9
40.	U2457+A		m		ok	9
41.	U2457+C		m		ok	9

Continue (table 1)

Mutant	Second mutation	Growth	Peptidyl transfer	Accuracy	Publication
42. U2457+U		m		ok	9
43. G2458A		ok		ok	9
44. G2458A/U2493C		m		ram	9
45. G2458C/U2493C		m		ram	9
46. U2460A		m		ram	9
47. U2460C		m		ram	9
48. U2460G		m		ram	9
49. U2460C/G2490C		ok		ram	9
50. U2460G/G2490A		m		ram	9
51. U2460G/G2490U		m		ram	9
52. U2460G/G2490C		m		ram	9
53. U2477A		ok		ok	9
54. U2477C		ok		ok	9
55. G2490A		m		ram	9
56. G2490C		ok		ok	9
57. G2490U		ok		ram	9
58. U2492A		m		ram	9
59. U2492C		m		ram	9
60. U2492G		m		ram	9
61. U2493A	A2058G	R, L	m		8
62. U2493A		m		ram	9
63. U2493C	A2058G	R, L	m		8
64. U2493C		m		ram	9
65. delU2493		m		ram	9
66. A2497G	A2058G	m	m		8
67. G2502A		m			10
68. A2503C		m			10
69. A2503G	A2058G	R, m	st		8
70. G2505A	A2058G	R, m	m		5
71. G2505C	A2058G	R, m	m		5
72. G2505C	A1067U	L	m, st	ok	12, 13
73. G2505U	A2058G	R, L	st		5
74. U2506A	A2058G	D, L	st		5
75. U2506C	A2058G	D, L	m		5
76. U2506G	A2058G	D, L	st		5
77. C2507U/G2581A	A1067U	L			1
78. C2507del/G2581A	A1067U	L			1
79. G2508U	A2058G	ok	ok		5
80. U2514C	A2058G	ok	ok		8
81. A2516U	A2058G	ok	ok		8
82. U2528A	A2058G	m	ok		8
83. U2528C	A2058G	ok	ok		8

Continue (table 1)

Mutant	Second mutation	Growth	Peptidyl transfer	Accuracy	Publication
84. A2530G	A2058G	ok	m		8
85. U2546C	A2058G	ok	ok		8
86. G2550A	A2058G	R, m	m		8
87. U2552A	A2058G	R, m	m		8
88. U2552C	A2058G	R, m	m		8
89. U2555A		m		ram	11
90. U2555C		ok		ok	11
91. U2555C	A2058G	ok	ok		8
92. U2555G		m		ram	11
93. G2557A	A2058G	m	m		8
94. U2561C	A2058G	ok	ok		8
95. A2565U	A2058G	R, m	m		8
96. Ψ2580A	A2058G	R, L	st		5
97. Ψ2580C	A1067U	m	st		1, 2
98. Ψ2580C	A2058G	D, L	m		5
99. Ψ2580C	A2058G	R, L	st		8
100. Ψ2580G	A2058G	R, L	st		5
101. G2581A	A2058G	R, m	m		5
102. G2581A	A1067U	L	st		1, 2
103. G2581C	A2058G	R, L	m		5
104. G2581U	A2058G	R, L	m		5
105. G2582A	A2058G	R, L	st		5
106. G2582A	A1067U	L			16
107. G2582C	A2058G	R, L	st		5
108. G2582U	A2058G	R, L	st		5
109. G2582U	A1067U	m			16
110. G2583A	A2058G	R, m	st		5
111. G2583A	A1067U	L	m, st		12, 13
112. G2583C	A2058G	R, L	st		5
113. G2583C	A1067U	L	m, st	ER	12, 13
114. G2583U	A2058G	D, L	st		5
115. G2583U	A1067U	L	m	ER	12
116. U2584A	A2058G	R, L	m		5
117. U2584C	A2058G	R, m	m		5
118. U2584C	A1067U	ok			16
119. U2584G	A2058G	D, L	m		5
120. U2584G	A2058G	D, L	st		8
121. U2585A	A2058G	D, L	st		5
122. U2585A	TAG	D, L	st		3
123. U2585C	A2058G	D, L	st		5
124. U2585C	TAG	D, L	m		3
125. U2585G	A2058G	D, L	m		5

Continue (table 1)

	Mutant	Second mutation	Growth	Peptidyl transfer	Accuracy	Publication
126.	U2585G	TAG	D, L	m		3
127.	A2589G	A2058G	R, m	m		8
128.	A2600U	A2058G	ok	ok		8
129.	G2607C	A1067U	ok		ok	14
130.	G2608A	A1067U	ok		ok	14
131.	G2608C	A1067U	ok		ok	14
132.	G2608U	A1067U	ok		ok	14

1. Spahn *et al.*, 1996a
2. Spahn *et al.*, 1996b
3. Green *et al.*, 1997
4. Samaha *et al.*, 1995
5. Porse *etal.*, 1996
6. Gregory *et al.*, 1994
7. Lieberman & Dahlberg, 1994
8. Porse & Garrett, 1995
9. O'Connor & Dahlberg, 1995
10. Vester & Garrett, 1988
11. O'Connor & Dahlberg, 1993
12. Paper I
13. Paper II
14. Paper IV
15. Leviev *et al.*, 1995
16. Maiväli *et al.*, manuscr. in preparation

TAG — sequence tag
ok — no inhibition
m — moderate inhibition
st — strong inhibition
R — resessive phenotype
D — dominant phenotype
L — lethal
ram — error prone
ER — error restrictive

2.1.3. Ribosomal elongation factor cycles

The aim of this study was to specify the involvement of the TBS (nucleotides 1051-1108) in translational elongation cycle. For this purpose, we have analyzed kinetic properties of ribosomes with A to U transversion at position 1067 in *E. coli* 23S rRNA.

To date, the TBS has been considered mainly as an important structure for EF-G binding and for EF-G dependent GTPase activity. The consideration is based on the observations that this 23S rRNA region is a binding site for ribosomal protein complex L10-L7/L12, L11, antibiotics thiostrepton and micrococcin which are all known either to promote or inhibit EF-G dependent GTPase reaction (Cundliffe, 1990; Thompson, 1996). A supposition, that the TBS might be involved in EF-G dependent GTPase (the TBS has been often called as ribosomal GTPase centre) has remained so far without direct experimental

verification. Among other sites EF-G crosslinks to A1067 (Sköld, 1983) and protects nucleotides at position A1067 and A1069 from chemical modification (Moazed *et al.*, 1988). Data presented here confirm the interaction between EF-G and the TBS (Paper III). As seen from EF-G cycle time experiment (Paper III), a decrease in k_{cat}/K_M value for U1067 ribosomes refers to a reduced binding efficiency between EF-G and mutant ribosomes. At the same time, there is no change in k_{cat} limit, indicating to an unaltered translocation rate for mutant ribosomes. As shown earlier, an uncoupled EF-G dependent GTP hydrolysis proceeds normally for U1067 ribosomes (Thompson *et al.*, 1988). This notion leaves the question of whether the TBS is EF-G dependent GTPase centre still open for discussions. Thus, only modest deviations in elongation factor G cycle appeared for the mutant ribosomes, which is attributable to weaker interaction between EF-G and mutant ribosomes.

There is no evidence that demonstrates a direct interaction between EF-Tu and the TBS. Results in paper III are referring to functional interaction between EF-Tu and the TBS. Reduced k_{cat}/K_M value for U1067 ribosomes in EF-Tu cycle time experiment reflects an inefficient ternary complex binding to the mutant ribosomes. A decreased k_{cat} value implies that the maximal cycling rate for EF-Tu is inhibited due to the U1067 mutation. That is to say, EF-Tu stays longer in contact with the mutant ribosomes during elongation cycle. This phenomenon could be explained by another finding — the rate of GTP hydrolysis on EF-Tu is inhibited on U1067 ribosomes (Paper III). Once bound to the ribosome, EF-Tu cannot be released before GTP hydrolysis is completed. Thus, for the mutant ribosomes, GTP hydrolysis has become rate limiting for EF-Tu cycle.

Hence, the mutant 23S rRNA has an effect on elongation factors Tu and G binding to the ribosome and on EF-Tu dependent GTPase. We infer, that integrity of the TBS is crucial for EF-Tu to carry out its functions during ribosome cycle. Thompson *et al.*, (1988) have found that U1067 mutation in 23S rRNA increases the doubling time of bacteria 1.5 fold. The growth impairment could be attributed to the perturbed EF-Tu cycle of U1067 ribosomes. This postulation receives a support from *in vivo* experiments, were it was demonstrated that the rate of filling the ribosomal A-site is correlating with the speed of elongating ribosomes, which in turn determines the growth rate of bacteria (Pedersen, 1984). The alterations in kinetic efficiencies for U1067 ribosomes in EF-Tu cycle demonstrate the importance of A1067 in constituting proper functional conformation for ternary complex binding to the ribosomal A-site. The assumption that the TBS might constitute a part of the ribosomal A-site receives support from earlier findings, where modest protections against chemical modifications at positions G1068 and G1071 appeared after the tRNA has been bound to the ribosomal A-site in an EF-Tu•GTP dependent manner (Moazed & Noller, 1989) and from the pattern of binding and action for antibiotics thiostrepton and micrococcin, both known to inhibit ribosomal A-site related processes. Thiostrepton and micrococcin can bind to the TBS, primarily

to nucleotides A1067 and A1095 of *E. coli* 23S rRNA and base changes at these positions are rendering ribosomes resistant or less sensitive to thiostrepton or micrococcin (Rosendahl & Douthwaite, 1995; Thompson *et al.,* 1988). The structure for thiostrepton binding is complex, depending, beside few direct interactions, on integrity of the tertiary structure of the TBS and the presence of ribosomal protein L11 (Ryan & Draper, 1991). Xing and Draper have suggested, that thiostrepton mimics some ribosomal component or factor which normally interacts with the L11 N-terminal domain (Xing & Draper, 1996). Thiostrepton inhibits (p)ppGpp formation, peptide chain termination and binding of ribosomal factors IF-2, EF-Tu and EF-G to the ribosome (Gale *et al.,* 1981). Interestingly, depending on experimental conditions, thiostrepton can stimulate ribosomal peptidyltrasferase (Kutay *et al.,* 1990). All these events are ribosomal A-site dependent. Therefore, based on current data together with earlier findings, we conclude, that TBS forms a structure within the ribosome, which determines a part of the ribosomal A-site.

Are the deviations in ribosome cycle of the mutant ribosomes dependent only on the base change of A to U at position 1067? Or does the mutation alter the high-order structure of the TBS? Alternatively, the binding of ribosomal protein L11 could be affected by the mutation. When the structure of domain II of 23S U1067 RNA was probed, no structural alterations were detected in comparison to wild-type 23S rRNA. In addition, L11 has retained its binding ability to U1067 ribosomes (Rosendahl & Douthwaite, 1995), as well as to 23S rRNA fragment (1051-1108) containing this mutation (Ryan & Draper, 1991). Thus, U1067 mutation in 23S rRNA seems to have no influence neither to the structure of 23S rRNA nor to the binding of L11 and at a glance, all the changes in the EF-Tu cycle can be attributed simply to the base change. However, the data derived from the structure probing experiments describe only static state of the ribosome. Taking into account dynamic nature of the ribosome and the fact, that we have observed several perturbations in the EF-Tu cycle we infer, that the U1067 mutation could hinder to some extent the course of proper structural rearrangements within the ribosome during ribosomal elongation cycle. E. Cundliffe has made already long ago a suggestion, that the role of L11 is to promote specific conformational changes in the TBS during translation and that thiostrepton inhibits protein synthesis by trapping ribosomal RNA, not allowing these changes to occur (Cundliffe, 1990). This idea receives a support from a very recent experiments, where structural changes were demonstrated to occur in 16S rRNA during translation (Lodmell & Dahlberg, 1997). Likewise, "*in vitro* evolution" experiments have pointed to the possibility of existence of two different NH_4^+ dependent conformations of the TBS (Lu & Draper, 1995). Additional information about structural movements of rRNA during translation comes from the statement, that ribosomes are oscillating between two conformations, known as pre- and posttranslocational stages, at the course of the ribosome cycle (Hausner *et al.,* 1988). Pretranslocational stage is recognized by EF-G and posttranslocational stage by EF-Tu. Both elongation factors promote

the transition from one translocational stage to the other: EF-Tu induces transition from post- to pretranslocational and EF-G from pre- to posttranslational stage (Mesters *et al.,* 1994). Recently, crystal structure of the ternary complex of Phe-tRNA^{Phe} in its complex with EF-Tu · GDPNP (a GTP analog) has been published (Nissen *et al.,* 1994), showing a striking similarity with the structure of EF-G · GDP (Czworkowski *et al.,* 1994). These data are suggesting, that binding of one of the elongation factors causes a structural rearrangement, i.e. transition from one translocational state to another within the ribosome and determines binding site for another elongation factor. After the elongation factor has departured, the ribosome becomes fixed into a state, recognizeable by the other active elongation factor. Thus, structural changes within the ribosome are forcing ribosome cycle to go on and elongation factors promote this process. Therefore, it seems thoughtful to consider the impact of rRNA mutations on structural transitions during translation. Whilst no direct interactions between the TBS and EF-Tu has been found, and the binding of L11 as well as local structure of 23S rRNA have proved to remain unchanged at the time when EF-Tu and EF-G cycles are perturbed, we suggest, that the A to U transversion at position 1067 of *E. coli* 23S rRNA interferes with the functional structure of the ribosome. Most plausible explanation to the U1067 phenotype is that the mutation affects performance of elongation factor Tu by inhibiting the mutant ribosomes to switch from post- to pretranslocational state during ribosomal elongation cycle, resembling in some extent to the mode of inhibition of thiostrepton. Alternatively, the base change at position 1067 interferes directly with EF-Tu and ribosome interaction.

We have not found any kinetic deviations for U1067 ribosomes in the "puromycin reaction" (data not shown). Therefore, the increase in time to synthesize dipeptide might be explained by slower release of EF-Tu · GDP from U1067 ribosomes. An increased processing time of EF-Tu on mutant ribosomes (Paper III) also favours the latter explanation. However, it has to be mentioned that functional interactions (based on studies with antibiotics) occur between 23S rRNA domains II and V (Mankin *et al.,* 1994).

As a summary of this work, we have presented the results (Paper III) that affirm the involvement of the TBS in translational elongation factors Tu and G dependent reactions. We conclude that integrity of the TBS in domain II of Escherichia coli 23S rRNA is vital for fluent action of translational elongation factors Tu and G during ribosome cycle and that nucleotide at position 1067 within the TBS seems to be a significant determinant in this process.

CONCLUSIONS

The present study can be summarized as follows:

1. Development of experimental strategy:
A common denominator for all known experimental strategies to analyse 23S rRNA mutations has been the insufficiency to discriminate between wild type (chromosome derived) and mutant (plasmid borne) ribosomes. Bacteria with single rRNA operon cannot be utilized due to a simple reason — mutations at crucial positions af 23S rRNA gene are often deleterious for cell growth. We have developed an experimental strategy by taking advantage of the mutation A1067U of 23S rRNA that renders ribosomes resistant to antibiotic thiostrepton — an efficient inhibitor of ribosomal translocation reaction. Adjoining the thiostrepton resistance mutation to the mutation of interest in the same 23S rRNA gene makes all the plasmid borne ribosomes resistant to the antibiotic. Subsequently, the presence of thiostrepton in a suitable *in vitro* assay enables to measure the activities of the mutant ribosomes, exclusively (Fig. 5).

2. Analysis of *E. coli* 23S rRNA mutations:
a) mutations in domain V:
To evaluate the importance of evolutionally highly conserved nucleotides in domain V of 23S rRNA for ribosomal functions, we have introduced several base substitutions into the 23S rRNA gene: G2505C, G2582A, G2582U, G2583A, G2583C, G2583U, G2607C, G2608A, G2608C and G2608U (Fig. 3B and 4). Our study has shown that base changes within the domain V are often perilous to bacteria, leading to retarded growth or even cell death (mutations G2505C, G2582A, G2582U, G2583A, G2583C and G2583U). *In vitro* experiments have revealed correlation between growth phenotype and peptide bond formation — 23S rRNA mutants with growth deflections have been found to suffer from deficient peptidyl transferase, as judged in 'puromycin reaction'. Mutations in domain V have demonstrated to be hypersensitive to antibiotics known to block ribosomal peptide bond formation, providing an additional link between domain V and ribosomal peptidyl transferase function. Furthermore, CMP stimulation experiments with pA-fMet as minimal donor of the PTase suggest that G2582 in addition to Ψ2580 and G2581 might interact with nucleotide C75 of P-site located tRNA.

Translational accuracy and ribosomal peptidyl transferase have supposed to be bound. We have proven it experimentally by showing that decresed missense error rate could be attributed to 'peptidyl transferase' mutants G2583C and G2583U.

b) mutation in domain II:

An A to U transversion at position 1067 of 23S rRNA has been previously characterized as to confer resistance against antibiotic thiostrepton, a powerful inhibitor of ribosomal translocation reaction. This region of 23S rRNA (Fig. 3A) has also been connected to elongation factor EF-G dependent functions. By using an advanced fast kinetic methodology we have evaluated kinetic characteristics of U1067 ribosomes. We could show that the mutant ribosomes are impaired in their effective association rate constants for both EF-G and EF-Tu binding. In addition, the times that EF-G and EF-Tu spend on the ribosome during elongation are significantly increased by the A to U transversion. On a contrary to earlier assessments, we claim that the U1067 mutation affects EF-Tu function more than EF-G function. The rates of EF-Tu dependent GTP hydrolysis and subsequent release of EF-Tu·GDP are severely inhibited, while the EF-G dependent translocation rate was not impaired. We infer that nucleotide U1067 of 23S rRNA domain II is important determinant for both elongation factors during the elongation cycle.

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MUUTUSED RIBOSOOMI ELONGATSIOONITSÜKLIS MUTATSIOONIDE TÕTTU 23S rRNAs

KOKKUVÕTE

Rakuline valgusüntees on elutähtis protsess kõigis tuntud elusorganismides. Valkude süntees toimub ribosoomidel (eestikeelse vastena võib leida ka terminit pihukeha). Mudelorganismina kasutatava *Escherichia coli* ribosoomid moodustuvad kahest allühikust, sedimentatsioonikoefitsientidega 30S ja 50S. 50S allühik (jn. 1), mis omakorda koosneb 34 ribosoomivalgust ja kahest ribosomaalsest RNAst, 5S ja 23S rRNA, vastutab keskse ribosoomi poolt läbiviidava reaktsiooni, peptidüüljäägi ülekande ehk peptidüüli transferaasi, eest. Seni ei ole leitud ühtegi ribosoomivalku, mis suudaks katalüüsida peptiidliite moodustumist. Üksnes kaudsed eksperimentaalsed andmed on viidanud 23S rRNA V domääni (jn. 3B ja 4) võimalikule osalusele ribosoomi peptidüülitransferaasses reaktsioonis. Ka hiljutine katalüütiliste RNAde avastamine viitab võimalusele, et hoopis rRNA võib olla peamiseks komponendiks peptiidsideme sünteesil.

Käesolev doktoritöö ongi ajendatud huvist teada saada, milline on 23S rRNA osa peptiidsideme sünteesi katalüüsil ja milline 23S rRNA piirkond võiks selle protsessi eest vastutada. Eksperimentaalse strateegiana valisime 23S rRNA suunatud mutageneesi ja saadud mutantsete ribosoomide kineetilise analüüsi *in vitro*.

On tuntud tõde, et teaduslike uuringute kvaliteet sõltub otsese kasutatavast eksperimentaalsest süsteemist. Senini ei olnud võimalik määrata mutantsete ribosoomide kineetilisi parameetreid, nende hulgas peptiidsideme moodustumise kiirust. Põhjus on lihtne, mutantsete 23S rRNA geenide ekspressiooniga plasmiidilt kaasneb alati ka kromosomaalsete 23S rRNA geenide (*E. coli*'s on seitse rRNA operoni) süntees, mis tähendab, et eraldades rakust ribosoome, saame alati mutantsete ja metsikuttüüpi ribosoomide segapopulatsiooni. Selline olukord ei võimalda aga adekvaatselt hinnata mutantsete ribosoomide mõju uuritavale ribosoomi funktsioonile. Esmaseks ülesandeks oligi välja töötada meetod, mis võimaldaks *in vitro* tingimustes alla suruda metsikuttüüpi ribosoomide valgusünteesi võime. Oli teada, et A1067U transversioon muudab ribosoomid resistentseks antibiootikumi tiostreptooni suhtes. Tiostreptoon on tuntud kui efektiivne ribosoomi translokatsioonireaktsiooni blokaator. Viies samasse 23S rRNA geeni nii A1067U mutatsiooni kui ka meid huvitava mutatsiooni, saame ekspressiooni tulemusena ribosoomid, mis suudavad *in vitro* tingimustes transleerida tiostreptooni juuresolekul. Samal ajal surub antibiootikum alla metsikuttüüpi ribosoomide aktiivsuse (põhimõtteline skeem on kujutatud joonisel 5).

Muteerisime mitmed evolutsiooniliselt kõrgeltkonserveerunud nukleotiidid 23S rRNA domäänis V: G2505C, G2582A, G2582U, G2583A, G2583C,

G2583U, G2607C, G2608A, G2608C ja G2608U. Leidsime, et enamiku mutantide kasv on kas tugevalt alla surutud või ei suuda mutantse 23S rRNAga bakter üldse kasvada (välja arvatud mutandid G2607C, G2608A, G2608C ja G2608U). Bakteri kasvufenotüüp korreleerus ribosoomide võimega sünteesida peptiidsidet: tugevalt allasurutud kasvufenotüübiga mutantide ribosoomid ei olnud võimelised sünteesima peptiidsidet. 23S rRNA mutandid G2505C, G2583C ja G2583U osutusid võrreldes metsikuttüüpi ribosoomidega tundlikumaks peptidüüli transferaasi inhibeerivate antibiootikumide klooramfenikooli ja linkomütsiini suhtes, mis viitab veel kord 23S rRNA V domääni seotusele peptiidsideme moodustumisega (Publikatsioonid I, II ja IV).

Katsed ribosoomi minimaalse doonorsubstraadiga pA-fMet osutavad võimalusele, et 23S rRNA V domääni nukleotiidid Ψ2580, G2581 ja G2582 moodustavad ribosoomi peptidüültransferaasses tsentris seondumiskoha tRNA nukleotiidile C75 (Publikatsioon IV).

Teatav valgusünteesi täpsuse tase on rakule samavõrra elutähtis kui peptiidsideme sünteesi võime. Mõõtes 23S rRNA mutantide valgusünteesi täpsust, leidsime, et mutatsioonid G2583C ja G2583U muudavad ribosoomid võrreldes metsikuttüüpi ribosoomidega täpsemaks, st. nad lülitavad valesid aminohappeid sünteesitava peptiidi koosseisu oluliselt harvem. Kirjandusest võib leida hüpoteese peptidüüli transferaasi seotusest translatsiooni täpsusega. Meie eksperimentaalsed andmed kinnitavad toodud seose olemasolu — mutatsioonid G2583C ja G2583U 23S rRNAs suurendavad nii valgusünteesi täpsust kui ka inhibeerivad peptiidsideme sünteesi (Publikatsioonid I ja IV). Saadud andmed on originaalsed. Kasutades analoogilist eksperimentaalset lähenemist ribosomaalse 23S RNA mutantide uurimisel on mitmed laborid hiljem leidnud sarnaseid ribosoomianomaaliaid.

23S rRNA domääni II (jn. 3A) on seni peetud oluliseks elongatsioonifaktorist G (EF-G) sõltuvates reaktsioonides. Uurisime 23S rRNA II domääni mutatsiooni A1067U transversiooni mõju ribosoomi elongatsioonitsüklile. Kasutasime metoodikat, mis võimaldab ribosoomist sõltuvate reaktsioonide kineetikat mõõta väga lühikeste ajavahemike jooksul (nn. *quench-flow*-meetod). Tuvastasime, et antud mutatsioon mõjutab elongatsioonifaktorist Tu (EF-Tu) sõltuvaid protsesse hoopis suuremal määral kui EF-G-st sõltuvaid. Mutatsioon A1067U inhibeeris mõlema elongatsioonifaktori ribosoomile sidumise kineetikat, samas näitasime, et EF-Tu-st sõltuva GTP hüdrolüüsi kiirus ning EF-Tu·GDP vabanemine ribosoomilt on olulisel määral aeglustunud. EF-G-st suunatav translokatsioon toimis aga samaväärselt kui metsikuttüüpi ribosoomidel. Publikatsioonis III näitasime esmakordselt, et lisaks EF-G sidumisele on 23S rRNA domään II seotud ka EF-Tu sidumise ja tema poolt läbiviidavate reaktsioonidega.

ACKNOWLEDGEMENTS

I wish to express my gratitude to all the people at Estonian Biocentre and Institute of Molecular and Cell Biology (University of Tartu) for creating a pleasant and stimulating working environment. In particular, I wish to thank:

My supervisor Dr. Jaanus Remme for his generous support, advice and patience through the years;

My co-workers from past and present, especially the 'old guard of 414-era';

Dr.'s Tõnis Örd and Tanel Tenson for efficient linguistic proof-reading;

Dr.'s Nese Bilgin, Måns Ehrenberg and their colleagues at the University of Uppsala, Department of Molecular Biology for creative atmosphere and excellent guidance through the 'kinetic window';

Dr.'s Knud Nierhaus, Cristian Spahn and their colleagues at Max Planck Institute (Berlin) for fruitful scientific cooperation, advice and support.

My friends and relatives for encouragement and positive thinking;

Most of all, I am indebted to my wife Kadri and our daughters for their love.

PUBLICATIONS

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Saarma, U. & Remme, J. 1992. Novel mutants of 23S RNA: characterization of functional properties. Nucl. Acids Res 20: 3147–3152.

Nucleic Acids Research, Vol. 20, No. 12 3147–3152

Novel mutants of 23S RNA: characterization of functional properties

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Received February 14, 1992; Revised and Accepted May 13, 1992

ABSTRACT

Single point mutations corresponding to the positions G2505 and G2583 have been constructed in the gene encoding *E.coli* 23S rRNA. These mutations were linked to the second mutation A1067 to T, known to confer resistance to thiostrepton (1). Mutant ribosomes were analyzed *in vitro* for their ability to direct poly(U) dependent translation, their missence error frequency and in addition their sensitivity to peptidyltransferase inhibitors. It was evident that the mutated ribosomes had an altered dependence on [Mg^{2+}] and an increased sensitivity to chloramphenicol during poly(U) directed poly(Phe) synthesis. In a transpeptidation assay mutated ribosomes were as sensitive to chloramphenicol as wild-type ribosomes. However, the mutant ribosomes exhibited an increased sensitivity to lincomycin. An increase in translational accuracy was attributed to the mutations at the position 2583: accuracy increased in the order G<A<U<C.

INTRODUCTION

A solid body of evidence has been published on the role of 23S RNA in the peptidyltransferase reaction, including crosslinking of photoreactive analogs of aa-tRNA, peptidyl-tRNA and puromycin (2,3), protection of rRNA against chemical modification by various ribosome ligands (4–6), inhibition of substrate binding by complementary oligodeoxynucleotides (7) and analysis of antibiotic resistant mutations in rRNA (for review 8). Based on these data, the central ring of domain V in the 23S RNA was defined as being part of peptidyltransferase centre. The importance of nucleotides within this region for cell viability and antibiotic resistance has been shown by site-directed mutagenesis (9–10). Protection of A2438, A2451 and A2602 are shown to be dependent on acyl moiety, U2506, U2584, U2585 on the 3′ terminal A and G2505 is protected by tRNA, lacking 3′ terminal CA (4, see fig.4).

In this paper the effects of mutations at the peptidyltransferase region of 23S RNA on the ribosomal functions, observed *in vitro*, are described. The mutant species of the gene for 23S RNA were expressed *in vivo*. In order to discriminate between the activities of plasmid encoded mutant ribosomes and wild-type ribosomes a second mutation in the gene of 23S RNA, corresponding to the position A1067 of 23S RNA, was used. Transversion A to T confers a high level resistance of *in vitro* translation to thiostrepton (1). In the presence of the drug it is possible to selectively inhibit wild-type ribosomes. By comparing the activities of the mixed ribosomal populations in the presence and absence of thiostrepton, it was possible to detect alterations caused by mutations in 23S RNA.

MATERIALS AND METHODS

Plasmids and strains

Plasmid pLST1067T, containing the rrnB operon with single point mutation A to T at the position 1067 was a kind gift from Dr J. Thompson (1). Plasmid pNO2680 contains the *E.coli* rrnB operon under control of the lambda leftward promoter (11) and plasmid ptBsB contains the *E.coli* 23S RNA structural gene under control of the tac promoter (12). *E.coli* CJ236 (dut ung thi relA) was used for the preparation of single-stranded DNA template for site-directed mutagenesis. JM109 containing the $lacI^q$ gene was the host for ptBsB based plasmids and pop2136, which contains the chromosomally located gene encoding for the temperature-sensitive lambda repressor c*Its*857, was used for expression of mutant rRNAs from the pNO2680 based plasmids. Plasmid pSP-65 (Pharmacia) containing the β-lactamase gene was used as a control during the cotransformation assays.

Construction of mutants

The single point mutations at the position 2505 (G to C) and at the position 2583 (G to A, U and C) of the 23S rRNA gene were constructed by oligonucleotide directed mutagenesis (13) using the SalI-BamHI fragment from rrnB cloned into m13 mp19. Following mutagenesis the Eco52I-BamHI fragments were cloned into expression vector ptBsB (12). XbaI-BamHI fragments from each ptBsB mutant were subcloned into pNO2680, carrying A1067 T transversion. The mutations were confirmed by DNA sequencing of the final expression vectors, as well as by sequencing the cellular 23S rRNA moiety, using the primer directed reverse transcriptase reaction (12). Manipulations with DNA were accordingly to (14). The resulting plasmids were designated as follows: single mutants A1067 to T—ptBsB1067T,

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pNO1067T; in the case of the double mutants, all of which contained the 1067T mutation and the mutation at either position 2505 or 2583, these were indicated only by the latter mutation.

Preparation of ribosomes

Transformants of *E.coli* JM109 carrying the plasmid ptBsB, were induced by 1 mM IPTG at A_{600}=0.5 and cells were harvested after 1 hour. *E.coli* pop2136 harboring pNO was grown at 30°C up until A_{600}=0.2 and subsequently induced for 2 hours at 42°C. The cells were lysed by lysozyme (0.5 mg/ml) in buffer A (60 mM NH_4Cl; 60 mM KCl; 12 mM $MgCl_2$; 20 mM Tris-HCl pH−8; 6 mM β-mercatoethanol) containing 16% (w/v) sucrose, by three freeze-thaw cycles. Cell debris was removed by centrifugation 16000 rpm, 15 min. Sorvall SS-34 rotor. Lysates were diluted three times with buffer A and loaded onto 1 ml sucrose cushion (20% sucrose in 0.5 M NH_4Cl; 12 mM $MgCl_2$; 20 mM Tris-HCl pH−7,6; 6 mM β-mercaptoethanol) in a Sw-60 (Beckman) rotor and the ribosomes were pelleted by centrifugation 33000 rpm, 12 hours. Ribosomes were redissolved in buffer A and stored in aliquots at −70°C. 70S ribosomes were prepared from the total ribosome fraction by sucrose gradient centrifugation (10-30% sucrose in buffer A) 20000 rpm 16 hours in a Beckman Sw-28 rotor. RNA was extracted from 70S fraction by phenol extraction and mutated regions were sequenced by dideoxy sequencing, using end-labeled primer (complementary to bases 2598−2617 in 23S RNA) (12).

Cell-free translation

Poly(U) translation was performed essentially as described in (15). 0.1 ml assay contained in buffer A 0.5 A_{260} units ribosomes, 0.01 mg bulk tRNA, 0.02 mg poly(U) (Reanal, Hungary), 2 mM ATP, 0.5 mM GTP, 8 mM phosphoenolpyruvate (PEP), 2 μg pyruvate kinase, 0.05 mM [^{3}H]Phe (40 cpm/pmol) (Amersham), 0.02 mM [^{14}C]Leu (700 cpm/pmol) (UVVVR, Czechoslovakia) and 0.2 mg S-100 enzyme. After 30 min. incubation at 30°C, reactions were stopped by addition 1 ml 5% trichloracetic acid (TCA), heated for 15 min. at 95°C. Precipitates were collected onto GF/A filters (Whatman) and counted for radioactivity in a Rackbeta 1219 (LKB) scincillation spectrometer. Thiostrepton (Calbiochem) was dissolved in 10% dimethylsulfoxide (DMSO) at concentration 0.05 mM. Therefore all assays in the presence of thiostrepton contained also 0.02−0.1% DMSO. Minus drug controls were made in the presence of DMSO.

Missence error frequency

The missence error frequency was determined by measuring the misreading of UUU codons by near-cognate $tRNA_4Leu$ competing with $tRNA^{Phe}$. The experiments were done in buffer A essentially as described by Ehrenberg *etal* (16). Source for elongation factors and synthetases was DEAE-fractionated S-100 supernatant (20 mg/ml protein). Two mixes were prepared: ribosome mix contained in 50 μl 0.5 A_{260} units of ribosomes, 0.2 mg/ml polyuridylic acid, 20pmoles $tRNA^{Phe}$ (1100 pmoles/A_{260}unit, Sigma) and 15 pmoles of thiostrepton if indicated; factor mix containing 60 pmoles $tRNA^{Phe}$, 4 mM ATP, 1 mM GTP, 16 mM phosphenolpyruvate, 7 μg pyruvate kinase, 0.12 mM [^{3}H]Phe (5.2 cpm/pmole), 0,06 mM [^{14}C] Leu (700 cpm/pmole), saturating amount of S-100 enzyme and between 0−150 pmoles $tRNA_4Leu$ (1520 pmoles/A_{260} unit, Subraiden). Ribosome mix was preincubated for 10 min. at 37°C, factor mix was preincubated for 5 min. at the same temperature. Reaction was started by mixing 50 μl of ribosome mix by the same amount of factor mix. Incubation was at 37°C for 1 min. Reaction was stopped by addition 1 ml of 5% TCA, heated at 95°C for 15 min. and filtrated through GF/A filter (Whatman). Filters were processed as described in previous section.

Puromycin assay

Dipeptide synthesis was studied essentially as described (15). Ribosomes (0.5 A_{260} units per 50 μl assay) were preincubated with poly(U) (0.01 mg) and bulk tRNA (0.017 mg) in buffer A for 10 min. at 37°C in the presence of thiostrepton (0.37 μM). After addition 3 pmoles of N-ac[^{3}H]Phe-$tRNA^{Phe}$ (1.5×10^5 cpm/pmol, charging efficiency 1000 pmol/A_{260} unit) incubation was continued for 20 min. at 37°C. Trasnslocation was carried out at 0°C for 30 min. by adding EF-G (3 μg) and GTP (0.2 mM) followed by puromycin reaction (0.5 mM puromycin) 30 min. at 0°C. Reactions were stopped by 200 μl 1 M potassium phosphate (pH−7.0); 10mM EDTA. N-Ac[^{3}H]Phe-puromycin was extracted by 1 ml ethylacetate, 0.5 ml of organic layer was counted in 10 ml of scincillation cocktail (High-Safe, LKB). Background values were obtained from minus puromycin experiments.

RESULTS

The effect of mutations on the cell growth

The mutation A1067 to T in the 23S RNA is known to reduce bacterial growth rate 1,5 fold (1). The effect of double mutations on the cell viability was studied using derivatives of the pNO vector transformed into the *E.coli* strain pop2136. At 30°C the lambda promoter is repressed and mutant RNAs are not significantly expressed. At 42°C the temperature-sensitive repressor cI_{857} is inactive and rRNA genes are transcribed from the plasmid. The plasmid pSP-65 was used as a control in the cotransformation assay. The number of colonies were the same at 30°C and 42°C for both pSP-65 alone and in combination with pNO 1067T, indicating that transformed bacteria could grow equally well at both temperatures. When derivatives of the pNO containing the double mutant 23S RNA gene were used for transformation, no colonies were found at 42°C. In a cotransformation assay at 30°C there were 10−20 times more colonies than at 42°C. Four colonies from each temperature group were analyzed for plasmid content and it was found that at 42°C the transformants exclusively contained plasmids pSP-65. From these data we concluded that mutations C2505 and all base substitutions at position 2583 were lethal at 42°C.

Cell-free translation by mutant ribosomes

For *in vitro* assays the ribosomes were isolated from *E.coli* JM109 carrying one of ptBsB plasmids and pop2136 transformed with one of pNO plasmids. RNA from 70S fraction was sequenced using end-labeled primers directed towards the 2500−2600 region and showed that ribosomes encoded by the pNO plasmids constituted 40−50% of the total 70S population, following 2 hours induction. By contrast, ribosomes encoded by ptBsB plasmids represented approximately 30% following 1 hour induction (see also 12). Further induction did not yield a higher percentage of mutant ribosomes.

In the presence of 5-fold molar excess of thiostrepton poly(U) translation on the ribosomes from uninduced cells (wild-type) were inhibited 97−99%. The ribosomes derived from pNO1067U were inhibited 50%, while the ribosomes from

Table I. Poly(U) dependent protein synthesis in the presence and absence of thiostrepton. Phe incorporation into peptides is in pmoles. Ratio of protein synthesis in the presence and absence of the drug is given as resistance. A-ribosomes were derived from ptBsB plasmids in *E.coli* JM109. B-ribosomes encoded by pNO plasmids in pop2136.

Ribosomes	10 mM $MgCl_2$ −thio	10 mM $MgCl_2$ +thio	resistance	25 mM $MgCl_2$ −thio	25 mM $MgCl_2$ +thio	resistance
A						
wild-type	54	1.62	0.03	26	0.26	0.01
U1067	52	9.36	0.18	27	4.86	0.18
C2505	33	4.29	0.12	38	8.36	0.23
C2583	29	3.48	0.12	39	9.36	0.24
B						
wild-type	52	0.78	0.015	32	0.32	0.01
U1067	49	20.6	0.42	31	13.7	0.44
C2505	41	13.5	0.33	32	16.9	0.51
C2583	36	10.4	0.29	35	18.9	0.54

Table II. Poly(U) dependent incorporation of Phe and Leu into peptides in the presence of thiostrepton. Ribosomes were isolated from *E.coli* pop2136 carrying one of the pNO plasmids.

Ribosomes	10 mM $MgCl_2$ pm Phe	pm Leu	Leu/Phe+Leu	15 mM $MgCl_2$ pm Phe	pm Leu	Leu.Phe+Leu
U1067	17.7	0.24	0.013	20.9	3.03	0.127
A2583	16.9	0.16	0.0094	23	2.52	0.099
C2583	11.5	0.056	0.0048	15.4	0.73	0.045
U2583	10.4	0.096	0.0091	12.2	0.99	0.075

ptBsB1067U were inhibited 82% (Table I). Drug resistance on double mutant ribosomes U1067/C2505 and U1067/C2583 was in both strains dependent on Mg^{2+} concentration: at 25 mM the resistance was nearly 2 times higher than at 10 mM (Table I). It is clear from table I that in all cases the resistance to thiostrepton was higher on the ribosomes derived from *E.coli* pop2136 strain. Thus, the data from poly(U) translation in the presence and absence of thiostrepton are in good correlation with the RNA sequencing data. It should be noted that the ribosomes containing the U1067 mutation are also inhibited in the presence of excess of thiostrepton 10−20%.

The effect of magnesium ions concentration on the translational activity was tested during poly(U) dependent poly(Phe) synthesis on the ribosomes derived from pNO plasmids in the presence of thiostrepton. The data are presented on the fig. 1. It was evident that the ribosomes containing the single mutation U1067 had an optimum at 12 mM $MgCl_2$ and at higher concentration of Mg^{2+}, processivity of poly(Phe) synthesis decreased. Ribosomes containing the double mutation clearly had a different dependence on $[Mg^{2+}]$. At 10−12 mM $MgCl_2$ the processivity was lower for the double mutants as compared with the ribosomes containing the U1067 mutation alone. In contrast, at 25 mM $MgCl_2$ all the double mutants exhibited higher activity during poly(U) translation than single mutant ribosomes. It is interesting to note that the double mutants C2505 and C2583 had virtually the same dependence on $[Mg^{2+}]$. The double mutant with U2583 showed the best processivity at high magnesium concentration and the U1067/A2583 mutation reflected the smallest difference on the Mg^{++} concentration as compared with ribosomes containing the single mutation U1067.

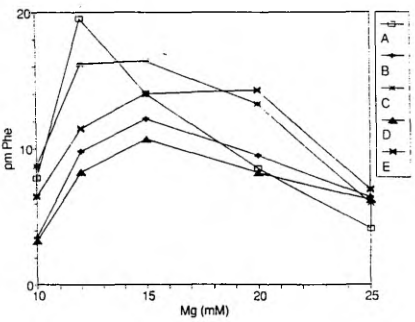

Fig. 1. Poly(U) directed translation by mutant ribosomes. Dependence on Mg^{2+} concentration. 0.5 A_{260} units of ribosomes were tested for activity in poly(Phe) synthesis in the presence of thiostrepton (1 μM) at different magnesium concentrations: A-single mutant U1067; B−E double mutants: C2505 (B); A2583 (C); C2583 (D); U2583(E).

The effect of mutations on the translational accuracy

The effect of mutations in 23S rRNA on the fidelity of translation was analyzed in conventional poly(U) translation assay and accordingly to the method of Ehrenberg (16). Table II shows the incorporation of Phe (correct) and Leu (incorrect) by mutant ribosomes at two magnesium ion concentrations in a conventional poly(U) translation system. In order to reduce the activity of wild-type ribosomes, the assays were carried out in the presence of thiostrepton. Incorporation of both amino acids into peptides was affected by increasing Mg^{2+} concentration, the effect on Leu incorporation was more dramatic. As it can be seen on table II the incorporation of Leu is lower on double mutant ribosomes as compared to single mutant ones at both concentrations of $MgCl_2$.

In order to determine whether or not the effect on Leu incorporation on mutant ribosomes is due to reduced mistranslation, we have used the method of Ehrenberg (16) for determination of missence error frequencies. This method is based on titration of ribosomes by miscognate $tRNA_4Leu$ in the presence of a constant amount of $tRNA^{Phe}$. The missence error frequencies were detected on mutant ribosomes in the presence and absence of thiostrepton; wild-type ribosomes were analyzed in the absence of drug. In this assay, equimolar concentrations of the drug were sufficient to almost completely inhibit wild-type ribosomes. The active fraction of ribosomes in the presence of thiostrepton was 1−5 pmoles per one A_{260} unit, as determined by Nac-Phe incorporation into poly(Phe) during 20 seconds (data not shown). This value did not differ significantly for the different mutants at optimal magnesium ion concentrations, but activity was preparation dependent. The missence error frequencies are presented on table III. Leucine misincorporation by single mutant U1067 ribosomes was nearly the same as that observed in the presence or absence of the drug. The same value was obtained on the wild-type ribosomes in the absence of the drug, indicating that under conditions used the A1067 to T transversion had no effect on the accuracy of translation. The same was true for the G2505 to C transversion (table III). On the other hand it implied that the presence of thiostrepton did not affect the selection aa-tRNA by the resistant ribosomes. By contrast it was evident that

Table III. Missence error frequencies of poly(U) translation in the presence and absence of thiostrepton. The wild-type ribosomes were isolated from *E.coli* pop2136 without plasmid, mutant ribosomes were isolated from the same strain carrying the pNO plasmid with mutated 23S RNA gene.

Ribosomes	−thiostrepton	+thiostrepton
wild-type	0.0119	0.0122
U1067	0.0121	0.0122
C2505	0.0120	0.0118
A2583	0.0109	0.0101
C2583	0.0082	0.0039
U2583	0.0098	0.0057

Table IV. The effect of lincomycin on transpeptidation with mutant ribosomes in the presence of thiostrepton. Backgrounds were detected in the absence of puromycin and subtracted.

	[^{3}H]NacPhe-puromycin (cpm).				
Ribosomes: Lincomycin (mM)	U1067	C2505	A2583	U2583	C2583
0	10121	6578	7459	8387	6121
0.05	8418	3649	4885	5187	3830
0.1	7637	3032	4319	4212	3162
0.3	6297	2305	3243	3335	2263
0.5	4887	1735	2366	2408	1756
1	3099	1068	1620	1603	1101
5	446	239	301	287	

both in the presence and absence of thiostrepton, the misincorporation of Leu was lower on ribosomes mutated at the position 2583 (table III). In the absence of the drug both, wild-type and mutant ribosomes participated in the polymerization reaction. In the presence of thiostrepton, wild-type ribosomes contributed less than 5% and nearly all amino acids incorporated into hot TCA insoluble material were due to the mutant ribosomes (see table I). As compared the missence error frequencies in the presence and absence of the drug, the single mutant U1067 and the double mutant U1067/C2505 ribosomes exhibited the same translational accuracy, while the ribosomes mutated at the position G2583 were more accurate in the presence of the drug. Taking into account that in the absence of thiostrepton mutated ribosomes contributed 40−50% in translation (percentage of mutant 23S RNA in the 70S fraction) it is evident from table III that mutant ribosomes translated poly(U) with the same accuracy both, in the presence and absence of the drug. The effect on the translational accuracy was biggest on C, intermediate on U and smallest on A at the position 2583.

The effect of mutations on antibiotic sensitivity

The sensitivity of mutant ribosomes to antibiotics which inhibit the peptidyltransferase reaction, were analyzed during poly(U) translation and during a 'puromycin assay'. No differences were detected between wild-type, single mutant U1067 and double mutant ribosomes with respect to their sensitivity to erythromycin (data not shown). Interestingly, notable differences were found with chloramphenicol. Data is presented on poly(U) translation as a function of chloramphenicol concentration, in the presence of thiostrepton (fig.2). The single mutant U1067 and double mutant U1067/A2583 were only slightly inhibited by chloramphenicol, using concentrations up to 3 mM. By contrast, the double mutants containing C2505, U2583 and C2583 were more sensitive than the wild-type ribosomes.

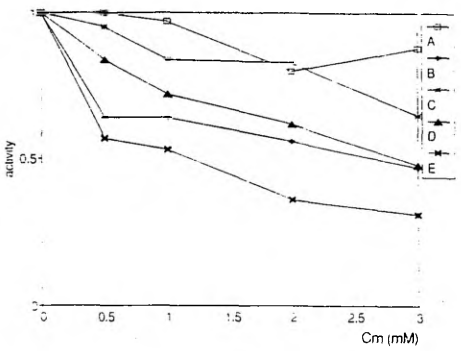

Fig. 2. Poly(U) directed translation by mutant ribosomes. Dependence on chloramphenicol (Cm) concentration in the presence of thiostrepton (1 μM). Activities of mutant ribosomes are expressed as ratio to 0 concentration of chloramphenicol. A-single mutant U1067; B−E double mutants: C2505 (B); A2583 (C); C2583 (D); U2583(E).

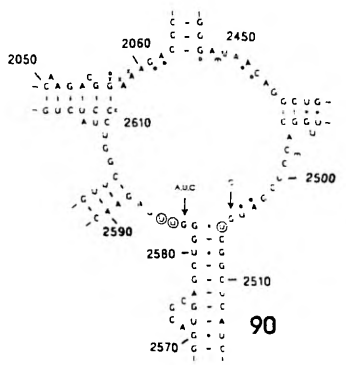

Fig. 3. Central ring of domain V of *E.coli* 23S RNA. Mutations described in this study are indicated by arrows. Protection of encircled bases are dependent upon the presence of the 3' terminal A (4). Nucleotides whose alteration can confer resistance to chloramphenicol (o) and to macrolides or lincomycin (x) (8).

In order to study the effects of antibiotics that interfere with peptidyltransferase on the mutant ribosomes, the ability of thiostrepton to block translocation on wild-type ribosomes was employed. NacPhe-tRNA was bound to the ribosomal A-site following occupation of the P-site by deacylated tRNA. After EF-G promoted translocation, peptidyl-tRNA becomes competent to puromycin. When this reaction is carried out in the presence of thiostrepton translocation occurs only on the U1067 containing ribosomes. For the peptide bond synthesis assay, 4 μM chloramphenicol inhibited the transfer reaction by 50% with both wild-type and mutant ribosomes indicating that the mutations in 23S RNA had no effect during this particular assay. Small differences between mutant ribosomes were detected in the inhibition pattern by lincomycin (see table IV). 50% inhibition

of the reaction on the mutant ribosomes was obtained with the following concentrations of lincomycin: single mutant U1067 0,35 mM and double mutants: C2505 0,07 mM; A2583 0,15 mM; U2583 and C2583 at 0,1 mM. Thus mutations C2505, U2583 and C2583 led to increased sensitivity to lincomycin during peptidyltransferase.

DISCUSSION

The effects of mutations at position G2505 (to C) and G2583, (all three substitutions) have been studied *in vitro*. These universally conserved nucleotides are located in the central ring of domain V (see fig.3). Substitution of one of these nucleotides by C could lead to an extension of helix 90 (17) by one base pair. The mutant genes were expressed under the control of two different inducible promoters: lambda p_L and tac. In these systems high levels of expression of plasmid encoded rRNA is possible.

Mutations at the positions 2505 and 2583 did not prevent processing and assembly of the 23S RNA into 50S particles. The resulting subunits were able to associate with 30S subunits in amount comparable to the wild-type 50S subunits. The mutated ribosomes were able to translate poly(U) *in vitro*. However, *in vivo* expression of these mutations were shown to be lethal at 42°C.

The mutated regions were linked to the mutation U1067, which is known to confer resistance to thiostrepton *in vitro* (1). By comparing the results plus and minus thiostrepton, no differences were detected with single mutant U1067 ribosomes during poly(U) translation, in the ribosomal accuracy and in the sensitivity to peptidyltransferase inhibitors.

The magnesium ion concentration has multiple effects on ribosomal structure and function. It is known to stimulate tRNA binding (18) and inhibit translocation (over 15 mM) (19). At higher magnesium concentrations translational accuracy is decreased (16). The mutations C2505 and C2583, which favor base-pairing with juxtaposed G reduces significantly reduced the processivity of poly(U) translation at low Mg^{2+} concentration. In contrast at higher concentration of magnesium ions, all double mutants exhibited higher processivity as compared to the single mutant U1067 ribosomes. Both the reduced processivity at low Mg^{2+} concentration and the resistance to the inhibitory effect of high Mg^{2+} concentrations indicated that tRNA binding was affected on the ribosomes containing double mutations.

The sensitivity of mutant ribosomes to chloramphenicol and lincomycin was increased, the response to erythromycin was not affected as compared with wild-type ribosomes. Lincomycin does not inhibit poly(U) dependent poly(Phe) synthesis and erythromycin is inactive in the NacPhe-tRNA puromycin system, whereas chloramphenicol can block both steps (20). Chloramphenicol and lincomycin are known to inhibit acceptor substrate binding, erythromycin destabilizes the peptidyl-tRNA complex with ribosomes (20). In addition, erythromycin and chloramphenicol have been shown to induce oligopeptidyl-tRNA release during poly(U) translation *in vitro* (20, 15). Hypersensitivity of mutated ribosomes to chloramphenicol and lincomycin can be caused by a reduced binding affinity of tRNA 3′ end to the ribosomal peptidyltransferase centre. Several mutations in the central ring of domain V of 23S RNA are known to confer resistance to chloramphenicol, among them are 2503 in *E.coli* (10) and 2504 in mammalian mitochondria (8). G2505 is protected against chemical modification by the drug (5). In this context it is interesting to note that the G2505 to C transversion led to increased sensitivity of *in vitro* translation to chloramphenicol. Observation, that mutations at positions 2505 and 2583 conferred hypersensitivity to chloramphenicol during poly(U) directed translation but not in the puromycin reaction indicated that tRNA binding to the ribosomal A site was the likely step to be affected by these mutations. Mutant ribosomes were slightly more sensitive to lincomycin during peptidyltransferase assay, as compared to wild-type ribosomes. Obviously, chloramphenicol and lincomycin both inhibit acceptor substrate binding but each in a different manner.

It is known that mutations in both small and large ribosomal subunit proteins can affect translational accuracy (21, 22). The mutations at the position 2583 are the first examples for fidelity mutations in 23S ribosomal RNA. One possible way to explain this phenomenon is the assumption that binding of the aa-tRNA to the peptidyltransferase centre is weaker (or alternatively transfer reaction is slower) by the 2583 mutant. In this way, ribosomes would have a longer time for aa-tRNA dissociation during proofreading. Accordingly, the peptidyltransferase reaction must be limiting and an irreversible step for aa-tRNA selection, as has been suggested earlier (23). This model predicts that mutant ribosomes need more GTP's to hydrolyze to aa-tRNA binding. Moazed and Noller have demonstrated that protection against chemical modification of bases A2439, A2451 and A2602 in 23S RNA are dependent upon the acyl moiety on tRNA, protection of bases U2506, U2584 and U2585 depend on the presence of 3′ terminal A (4, see also fig.3). U2584 and U2585 were shown to cross-link with photo-reactive Phe-tRNA at the ribosomal A site. All these data are in agreement with our conclusion that the region around helix 90 in 23S RNA is involved in aa-tRNA binding to the ribosomal A site.

ACKNOWLEDGEMENTS

We gratefully acknowledge Prof. R. Villems from Estonian Biocentre and Prof. K. H. Nierhaus from MPI for Molecular Genetics for support and useful discussions, T. Margus, M. Backhof and S. Nigul for technical assistance. Our special thanks are due to Dr. C. Prescott for useful suggestions and help during preparation of the manuscript.

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Saarma, U., Lewicki, B. T. U., Margus, T., Nigul, S., and Remme, J. 1993. Analysis of mutations in the 23S rRNA. *In* The Translational Apparatus: structure, function, regulation, evolution. *Edited by* Nierhaus, K. H., Franchesci, F., Subramanian, A. R., Erdmann, V. A., and Wittmann-Liebold, B. Plenum Press, New York, pp. 445–454.

ANALYSIS OF MUTATIONS IN THE 23S rRNA

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INTRODUCTION

Several experimental approaches have been employed to study the structure and function of ribosomes (reviewed in Dahlberg, 1989, and Leclerc and Brakier-Gingras, 1990). One such approach is that of site-directed mutagenesis of the rRNA, most commonly in the rRNA of the bacterium *Escherichia coli*. This organism has seven rRNA operons and thus separation of the host encoded rRNA from that of the mutated plasmid-encoded rRNA is necessary for further biochemical studies. The problem can be overcome by using *in vitro* synthesized rRNA for reconstitution of the subunit. This has been sucessfully achieved for total reconstitution of functional 30S particles. In contrast, transcribed and unmodified 23S rRNA cannot form active particles in reconstitution assays. Therefore, 23S rRNA should be expressed *in vivo* followed by isolation of the ribosomes. It is possible to discriminate between activities of mutant and wild-type ribsomes by using a second mutation in the gene of 23S rRNA. One such mutation is the A to T transversion corresponding to the position 1067 of 23S rRNA which confers resistance to thiostrepton during cell-free translation (Thompson *et al.*, 1988). Thiostrepton has a high affinity to wild-type ribosomes. U1067 ribosomes bind the drug with a reduced affinity (Thompson and Cundliffe, 1991). Therefore, A to U substitution at position 1067 can be used for selective inactivation of wild-type ribosomes and for physical separation of mutant ribosomes. This experimental approach has been used to analyze functional properties of mutations corresponding to positions G2505 and G2583 of 23S rRNA (Saarma and Remme, 1992). The most interesting finding was that mutations at position 2583 increased the translational accuracy. These results, together with those of mutations at positions G2505, G2583, G2607, and G2608 *in vitro* and G2505 and G2583 *in vivo* will be summarized here.

Furthermore, data concerning the formation of 50S subunits are presented. The assembly of ribosomes *in vivo* is strongly coupled with the transcription of the ribosomal RNA and includes the rRNA processing and rRNA modification. All attempts to show this process *in vivo* and to mimic it *in vitro* have to date failed. As a first approach the properties

The Translational Apparatus, Edited by K.H. Nierhaus *et al.*, Plenum Press, New York, 1993

of ribosomes whose rRNA was derived either by T7 RNA polymerase or *E. coli* RNA polymerase were compared with regard to both the *in vivo* transcription of the plasmid-encoded *rrn*B operon and ribosomal assembly. To distinguish between plasmid- and chromosomal-derived rRNA the above described A to T transversion at the position 1067 in the plasmid-encoded 23S rRNA gene was used. The relative amount of the plasmid-derived ribosome fraction in the mixed population was determined by sequencing the 1067 region of the 23S rRNA by means of reverse transcriptase, whereas the activity of the mutant particles could be measured in a poly(U) dependent poly(Phe) synthesis system in the presence of thiostrepton. It was found that the 50S particles containing rRNA transcribed by the viral T7 RNA polymerase were practically inactive in contrast to ribosomes synthesized by the host transcriptase. The significance of these results is discussed.

ANALYSIS OF 50S ASSEMBLY

To study the influence of the RNA polymerase species on the *in vivo* transcription of the *rrn*B operon and the ribosomal assembly, two series of plasmids were constructed. All of them carry the A to T transversion at the 1067 position in the 23S rRNA gene. In the first set the ribosomal RNA genes are under the control of a T7 late promoter instead of the natural P1P2-tandem promoters. These constructs are designated pT7... and were transformed into *E. coli* BL21(DE3). This strain contains a chromosomal copy of the gene for the T7 RNA polymerase under control of the IPTG-inducible *lac*UV5 promoter. The *rrn*B operon on pT7-1 is complete, whereas on pT7-2 the spacer following the P2 promoter and the 16S rRNA gene was excised, except for 39 bases at the 3' end. The construct pT7-3 furthermore lacks in addition the 5' half of the spacer between the 16S and 23S rRNA genes including the 5' half of the spacer $tRNA^{Glu2}$. The second series is called ptac-1, ptac-2 and ptac-3 and contains the identical gene arrangement than the first series but here the rRNA genes are under the control of the *tac* promoter instead of the T7 promoter. The second series of constructs were transformed into *E. coli* JM109 where the transcription from the *tac* promoter by the *E. coli* RNA polymerase is induced by the addition of IPTG.

An effect on the cell growth could be observed only in the presence of IPTG with *E. coli* cells carrying the pT7 constructs. Here the cell growth stopped about 45 min after induction whereas the growth was not disturbed upon induction of the T7 RNA polymerase encoded in the chromosome in the absence of any plasmid. In contrast, the growth of the host with the ptac plasmid was hardly affected by induction (Lewicki *et al.*, 1993) . These results were not surprising since the T7 late promoter is very strong and highly specific for its own RNA polymerase resulting in a large overproduction of rRNA. It is known that a large excess of rRNA over ribosomal proteins increases the formation of inactive particles which could be one of the reasons for the premature inhibition of growth of the cells containing the pT7 constructs. This overproduction of rRNA in the presence of T7 RNA polymerase was confirmed when the cells containing pT7-3 grew with $^{32}PO_4$ either under steady state conditions (the radiolabled orthophosphate was present for at least 4 generations) or pulse labelling conditions (the label was added 15 min prior to the corresponding harvesting point). Evidently 85 % of the rRNA overproduced by the T7 RNA polymerase was degraded whereas only about 15 % of the excess 23S rRNA was stably incorporated into ribosomal particles.

To determine the functional activity of the mutant ribosomes, the distribution of ribosomal particles and their content of plasmid-born rRNA was determined by analysis of sucrose gradients (Figure 1). The particles corresponding to the 70S and 50S fraction were isolated, and the 23S rRNA sequenced around nucleotide 1067. The amounts of chromosomal and plasmid-derived rRNA were determined and the relative amounts of plasmid-born 23S rRNA calculated, as percentages of the total 23S rRNA below the 70S and 50S peaks, respectively.

As it is shown in Figure 1 (left side) all three pT7 containing cell lysates have a normal 70S peak but show overproduction of 50S subunits and the presence of precursor particles. The plasmid-coded rRNA is unequally distributed between the 70S and 50S fraction

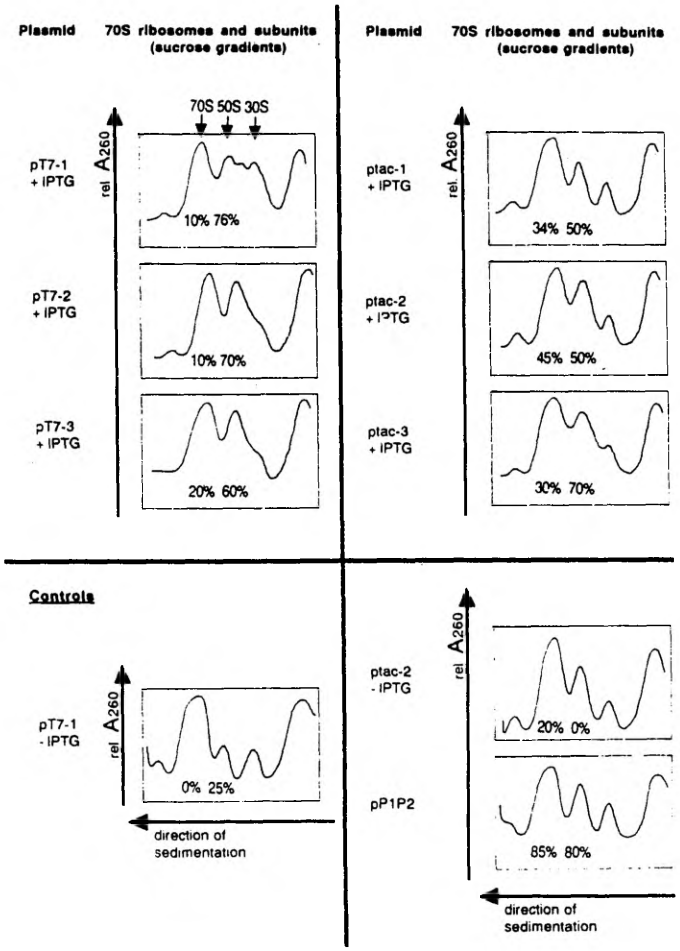

Figure 1. Sucrose gradient analysis of cell lysates with various plasmid constructs. The 23S rRNA derived from 50S and 70S fractions, respectively, were sequenced around the 1067 region and the relative amount of plasmid-born rRNA was determined as described elsewhere (Lewicki *et al.*, 1993). The percentage values below the peaks give the relative amounts of mutant (plasmid-born) 23S rRNA as compared to total 23S rRNA derived from plasmids plus chromosome.

containing 10 - 20 % and 70 ± 10 % rRNA synthesized by the viral transcriptase, respectively. Obviously, no free exchange exists between the free pool of 50S subunits carrying plasmid-encoded rRNA and the 50S subunits in the 70S ribosomes which represents the fraction active in protein synthesis. These results leads to the conclusion that

most of the particles which contain rRNA transcribed by T7 RNA polymerase seem to be inactive. A different picture was evident when the plasmid-encoded rRNA was transcribed by the *E. coli* RNA polymerase. At least the presence of pT7-1 containing the entire *rrn*B operon resulted in an indistinguishable sucrose gradient pattern in the presence and absence of the inducer and a much more equal distribution of plasmid-derived rRNA between the 50S and 70S fraction (Figure 1, right side).These findings agreed with the relative degrees of thiostrepton resistance observed with the lysates in poly(Phe) synthesizing systems. The lysates with the pT7 plasmids showed low activity in the presence of thiostrepton (Table 1), whereas about 3-fold higher activity was found with all ptac constructs.

Table 1. Poly(Phe)-synthesis activity of 70S ribosomes in the presence of thiostrepton (3 μM).

plasmid	induction	poly(Phe) synthesis - thio [cpm]	poly(Phe) synthesis + thio [cpm]	relative activity in the presence of thiostrepton [%]
no	-	4100	45	1
pT7-1	no	1800	25	1.4
	yes[1]	6700	500	7
pT7-2	no	2350	33	1
	yes	4700	300	6
pT7-3	no	2100	40	2
	yes	3200	180	5.6
ptac-1	no	5451	113	2
	yes	7450	1363	18
ptac-2	no	9617	586	6
	yes	8550	1577	18
ptac-3	no	8207	254	3
	yes	7496	1349	18

[1] In the case of induction IPTG was added 60 min before the harvesting point.

It was considered whether the inactivity of the large excess of 50S particles produced in the presence of pT7-3 was caused by functionally inactive 23S rRNA or, alternatively, by an accumulation of assembly-defective particles containing normal 23S rRNA. To test these alternatives the rRNA from the corresponding inactive 50S particles was isolated and a total reconstitution in the presence of wild-type TP50 was performed. A relative activity in the presence of thiostrepton was found which was the same as that of reconstituted particles containing ptac derived 23S rRNA. It follows that the pT7-3 derived rRNA in native particles is functional, and that the inactivity of the corresponding particles is caused by a defective assembly process. It cannot be excluded that one or more of the rRNA modifications are lacking since it was shown that modified nucleotides are important for a correct assembly process in the case of 16S rRNA (Cunningham *et al.*, 1991). A second reason could be the T7 RNA polymerase itself whose transcription elongation rate is about five times faster compared to that of the host polymerase (Chamberlin & Ring, 1973). This higher velocity of the transcriptase might cause premature folding of the nascent rRNA chain and/or premature association of ribosomal proteins.

To analyze the question whether the assembly defect is a cause of the high expression level of the rRNA or is due to the T7 RNA polymerase itself, the transcription initiation rate of the viral enzyme was decreased by using very low amounts of IPTG (0.03 mM). This condition reduces the overproduction of rRNA and should lead to an increased production

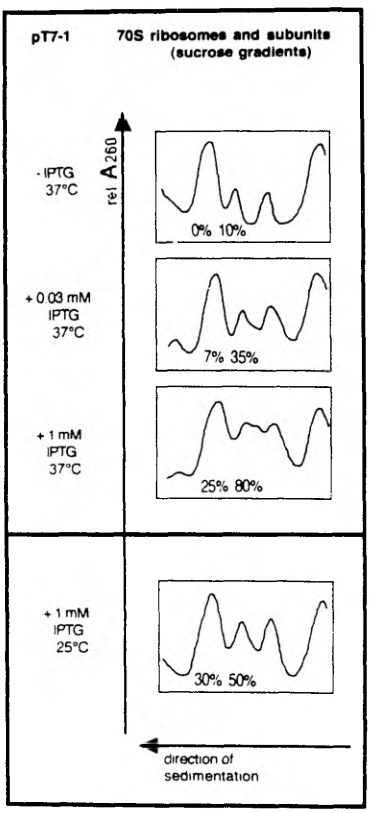

Figure 2. Sucrose gradient analysis of lysates prepared from cells grown in the presence of different IPTG concentrations and temperatures, and the relative amounts of plasmid-born 23S rRNA within the 70S and 50S fractions. For further details see legend of Fig. 1.

of active particles. Interestingly, the same unequal distribution of plasmid-encoded rRNA between the 70S and 50S fraction was observed (Figure 2, top) whereas the absolute amount of rRNA produced by T7 RNA polymerase was lowered. The exchange and therefore the activity of 50S particles containing pT7-1 derived rRNA between 50S and 70S pool is not increased indicating that the assembly defects are independent of the expression levels of the rRNA genes on the pT7-1 plasmid and therefore are caused by the T7 RNA polymerase itself. However, when the growth temperature was lowered to 25°C the rRNA analysis showed a distribution of plasmid-derived rRNA in the 50S and 70S fraction similar to that from the ptac constructs (Figure 2, bottom). Note, that both experiments lead to very similar sucrose gradient patterns with only very little amount of overproduced 50S and 30S

particles. Under conditions in which the transcription initiation rate was lowered (0.03 mM IPTG) the distribution of rRNA, synthesized by T7 RNA polymerase, between the 50S and 70S pool was unequal, whereas by lowering the transcription elongation rate of the viral enzyme (at 25°C) this distribution was much more equal indicating the increased production of active 50S particles.

EFFECT OF MUTATIONS ON THE FUNCTION OF RIBOSOMES

The mutant rRNA genes were cloned under control of an inducible promoter (lambda P_L in pNO2680, Gourse *et al.*, 1983). Conditional expression was necessary because the expression of mutant 23S rRNA had a deleterious effect on the bacterial growth. The mutations at positions G2505, G2583, G2607, and G2608 did not prevent 23S rRNA from beeing processed and assembled into 50S subunits, although the expression of 23S rRNA containing C2583 and U 2583 was accompanied by the appearence of incomplete particles (25S). Mutant rRNA formed 40 - 50 % of the total 70S ribosome population indicating that mutant 50S subunits were able to associate with 30S subunits.

The ability of mutant ribosomes to participate in cell-free translation was tested during poly(U) directed poly(Phe) synthesis. In the presence of 5-fold molar excess of thiostrepton over ribosomes, poly(U) translation on the wild-type ribosomes was inhibited 97 - 99 %. The ribosomes derived from pNO1067U (a mixed population containing 50% mutant ribosomes) were inhibited by 50 %, whereas the ribosomes encoded by the double mutant plasmid were inhibited by 54 - 71 %. This indicates that the double mutant ribosomes were able to translate poly(U). Thereby, in the presence of the drug only mutant ribosomes were active. Thiostrepton resistance of the double mutant ribosomes depended on the Mg^{2+} concentration. The effect of the magnesium ion concentration on the translational acitivity of mutant ribosomes was analyzed during poly(U) directed cell-free protein synthesis in the presence of thiostrepton. Neither thiostrepton nor the U1067 mutation had an effect on the magnesium dependence of *in vitro* translation as compared to the wild-type ribosomes. The results indicated that the nucleotide substitutions at different positions led to similar alterations of the response by the mutant ribosomes to the Mg^{2+} concentration. All mutants exhibited a lower acitivity at 10 mM $MgCl_2$ and a higher acitivity at 25 mM $MgCl_2$ as compared to the single mutant U1067 or wild-type ribosomes. G to A transitions corresponding to positions G2583 and G2608 had the smallest effect, as expected. Mutant ribosomes containing G to C transversions at positions G2505, G2583, G2607, and G2608 exhibited lower acitivity at low Mg^{2+} concentration as compared to the corresponding G to U transversions. Taking into account the known influence of magnesium concentration on the ribosome, the results suggested that mutations affected tRNA binding to the ribosome.

The sensitivity of mutant ribosomes to antibiotics, which are known to interfere ribosomal peptidyltransferase activity, was tested in two different systems. The first system was poly(U) directed translation according to Rheinberger and Nierhaus, 1990. The poly(U) translation is less sensitive to inhibitors as compared to the natural mRNA translation (Pestka, 1977). Wild-type ribosomes were inactivated by thiostrepton as previously described. By comparing the results of poly(U) translation on wild-type and U1067 ribosomes with respect to the sensitivity to chloramphenicol and erythromycin, it was evident that the U1067 mutation had no influence on the sensitivity to peptidyltransferase inhibitors. On the other hand, the sensitivity of the mixed U1067 ribosome population exhibited no difference in the presence or absence of thiostrepton. Thus, the translocation inhibitor thiostrepton does not effect the sensitivity of ribosomes to the peptidyltransferase inhibitors (chloramphenicol and erythromycin). Inhibition of poly(U) directed translation on the mutant ribosomes was tested in the presence of thiostrepton. The concentrations of the antibiotics corresponding to the 50 % inhibition level are indicated in Table 1. The results

obtained with erythromycin were similar for all the mutants within the experimental error. By contrast, inhibition of cell-free translation by chloramphenicol was effected on the U2583 and C2583 double mutant ribosomes in the reprocucible manner indicating that mutations at this position conferred hypersensitivity of poly(U) translation to chloramphenicol.

The second system to assay the sensitivity of the mutant ribosomes to antibiotics was based on the ability of thiostrepton to block translocation on the wild-type ribosomes. NacPhe-tRNA^{Phe} was bound to the ribosomal A site following preoccupation of the P site by deacylated tRNA. After EF-G promoted translocation, peptidyl-tRNA becomes competent to puromycin. When this reaction is carried out in the presence of thiostrepton, translocation occurs only on the ribosomes containing U1067. Consequently, this route allows analysis of the peptidyltransferase reaction catalyzed by mutant ribosomes using a mixed ribosome population. Chloramphenicol and lincomycin inhibited the transfer of Nac-Phe to puromycin, whereas erythromycin had no effect on the peptide bond formation, which has also been shown in earlier reports (Pestka, 1977). The concentration of drugs corresponding to 50 % inhibition of peptidyltransferase reaction on the wild-type and mutant ribosomes is indicated in Table 1. The inhibition pattern of chloramphenicol was similar indicating that mutations in 23S rRNA had no effect during this particular assay. By contrast, mutations C2505, U2583, and C2583 led to an increased sensitivity of peptide bond formation by lincomycin. It is known that both, chloramphenicol and lincomycin, inhibit the acceptor substrate binding (Pestka, 1977) but different effects of mutations on the sensitivity of these two drugs show that inhibition is caused by a different way of action. In addition, both, erythromycin and chloramphenicol, have been shown to indure the release of oligopeptidyl-tRNA during cell-free translation (Pestka, 1977; Rheinberger and Nierhaus, 1990). Hypersensitity of mutants U2583 and C2583 to chloramphenicol during poly(U) translation and to lincomycin during puromycin reaction can be caused by a reduced binding affinity of the 3' end of tRNA to the ribosomal peptidyltransferase centre.

The influence of mutations in 23S rRNA on the fidelity of translation was analyzed during poly(U) directed translation system in the presence and absence of thiostrepton. Single mutant U1067 ribosomes exhibited the same Leu misincorporation level as wild-type ribosomes both in the presence and absence of thiostrepton. Consequently, A1067 to U transversion and the presence of thiostrepton had no effect on the translational accuracy. The comparison of the missense error frequencies in the presence and absence of the drug showed that only the double mutants at position G2583 exhibited significant difference. Data on the Leu misincorporation in the presence of thiostrepton is presented in Table 2. It is interesting to note that the effect on the translational accuracy correlates with the sensitivity to chloramphenicol during cell-free translation. One possible way to explain this phenomenon is to assume that binding of the aa-tRNA is weaker (or alternatively the transfer reaction is slower) on the C or U2583 mutant. In this way, ribosomes would have more time for aa-tRNA dissociation during proofreading. Accordingly, the peptidyltransferase reaction must be limiting and an irreversible step for aa-tRNA selection. This model predicts that mutant ribosomes need more GTP's to hydrolyze for aa-tRNA binding.

The effect of mutations in 23S rRNA on the translational accuracy was tested *in vivo*. Plasmids containing mutant rRNA genes were introduced into cells with nonsense mutants in ß-galactosidase. In this system the ribosomes must translate through a stop codon in order to synthesize functional ß-galactosidase. Changes in the level of enzyme synthesis, which are due to the presence of mutant ribosomes, were detected as described in Prescott and Kornau, 1992. The strains used were suppressor-free and therefore the level of enzyme synthesis reflected the mistranslation at nonsense codons (Petrullo *et al.*, 1983). The activity of ß-galactosidase was normalized to the level of ß-lactamase activity synthesized at the same point in time. The ß-lactamase gene contains no internal in-frame stop codon. Therefore, the amount of this enzyme synthesized is a reflection of overall *in vivo* translation

activity. The ratio of ß-galactosidase activity relative to that of ß-lactamase represents the level of mistranslation to translational acitvity.

The relative level of mistranslation at UAA nonsense condon was apparently unaffected by the presence of mutation U1067a. The double mutants caused the efficiency of mistranslation to be decreased in the following order: U1067>C2505>A2583>U2583>C2583. Thus these results are in good agreement with the results obtained *in vitro*. On the other hand an increased level of translational readthrough

Table 2. Functional effects of mutations in 23S rRNA during poly(U) directed cell-free translation and peptidyltransferase reaction (PTR). Assays were performed as described in Saarma and Remme, 1992.

Ribosomes	Poly(U) translation				PTR	
	Thio[1]	Inhibition[2]		Leu/Leu+Phe)[3]	Inhibition[4]	
	resist	Cm (mM)	Ery (mM)		Cm (µM)	Lin (mM)
wild-type	0.04	>3	4	0.0122	4	0.35
U1067	0.52	>3	4	0.0123	4	0.35
U1067/C2505	0.32	2.5	5	0.0188	4	0.07
U1067/A2583	0.43	>3	4	0.0101	4	0.15
U1067/U2583	0.35	1.0	5	0.0057	4	0.10
U1067/C2583	0.29	2.0	5	0.0039	4	0.10
U1067/C2607	0.39	>3	6	0.0120	ND	ND
U1067/A2608	0.46	>3	4	0.0121	ND	ND
U1067/U2608	0.41	>3	8	0.0122	ND	ND
U1067/C2608	0.38	>3	8	0.0121	ND	ND

[1] Thiostrepton resistance is strongly dependent upon Mg^{2+} concentration. The data correspond to 12 mM $MgCl_2$ and represent the ratio of cpms ± thiostrepton (1 µM).

[2] Inhibition was detected in the presence of thiostrepton on the mutant ribosomes and in the absence of drug on the wild-type ribosomes. Poly(U) translation was inhibited by 50 % at the indicated concentrations; Cm = chloramphenicol, Ery = erythromycin.

[3] Leu misincorporation was measured in the presence of thiostrepton on the mutant ribosomes and in the absence of drug on the wild-type ribosomes.

[4] PTR was inhibited by 50 % with either both wild-type and mutant ribosomes (Cm) or with mutant ribosomes (Lin) at the indicated concentrations; Lin = lincomycin.

ND - not determined.

was observed at UAG stop codon due to the presence of U1067 ribosomes. This effect was suppressed by the second mutation at position G2583. The UAG specific readthrough on the U1067 ribosomes can be explained by affecting RF-1 and RF-2 binding in a different way. Therefore, mutations at position G2583 reduce the level of mistranslation at both UAA and UAG nonsense codons.

Taking into account that mutant ribosomes form about 40 - 60 % of the total ribosome population (Saarma and Remme, 1992), the effects of mutations in 23S rRNA on the translational accuracy should be higher than those observed in these experiments. A comparison of the results obtained *in vivo* and *in vitro* show that the effect of mutations at position 2583 of 23S rRNA on the translational accuracy are in agreement in both systems. Surprisingly, C2505 had a small effect *in vivo* but not *in vitro*.

The A1067 to U transversion is known to reduce the affinity of thiostrepton to ribosomes 100 times (Thompson and Cundliffe, 1991) allowing the physical separation of mutant ribosomes as it was proposed (Thompson *et al.*, 1988). Several solid supports were tested in respect to binding of ribosomes to the immobilized thiostrepton. It was found that the acid-hydrolyzed Sephadex can be used as a support for affinity purification of U1067 ribosomes. Evidently polysaccharide chains are long enough spacers to avoid steric hindrances in ribosome-thiostrepton interaction. Physical separation of mutant ribosomes would be important for the analysis of effects of mutations in 23S rRNA concerning the structure and function of the ribosome. Direct measurements of the binding affinities of aminoacyl-oligonucleotides to the peptidyltransferase centre of the mutant ribosomes would answer to the question whether the mutations in the 23S rRNA effect the binding of substrates to the peptidyltransferase centre.

CONCLUSION

Thiostrepton is known to inhibit both elongation factor dependent allosteric transition and nonenzymatic translocation of the wild-type ribosomes (Hausner *et al.*, 1988). The A to U transversion at position 1067 of 23S rRNA has been shown to confer resistance to thiostrepton during cell-free translation on the *E. coli* ribosomes (Thompson *et al.*, 1988). U1067 has proven to be a useful mutation to discriminate between the activities of chromosomal and plasmid encoded ribosomes.

In this study the thiostrepton resistance was used first to study the 50S assembly process. It was shown that 50S particles containing transcripts synthesized by the T7 RNA polymerase at 37°C are inactive: 1) The corresponding 50S particles accumulated in the 50S pool and could not enter the 70S pool *in vivo* indicative of their inactivity. 2) *In vitro* the plasmid-derived ribosomes were not active in poly(Phe) synthesis. 3) The pT7-1 derived transcripts accumulate in the 50S fraction even when the transcription initiation rate was lowered which caused low expression levels of the plasmid rRNA genes. 4) The 50S subunits with pT7-1 rRNA could partially enter the 70S fraction when the transcription elongation rate was decreased by incubation at 25°C. It follows that it is neither the transcription providing defective rRNA nor the overproduction of ribosomal RNA but rather the assembly of defective 50S subunits which caused the inactivity of the 50S particles *in vivo* when the rRNA is synthesized with the T7 RNA polymerase. A likely explanation is that the coupling beween rRNA transcription and ribosomal assembly ("assembly gradient", for review see Nierhaus, 1991) is a prerequisite for the formation of active particles and can be disturbed in the presence of T7 RNA polymerase. The viral enzyme is five times faster than the host transcriptase and so the finely tuned interaction between rRNA folding and protein binding might be uncoupled.

Further, the thiostrepton resistance was used as a second mutation for the functional characterization of single point mutations at the peptidyltransferase region of *E. coli* 23S rRNA. It was found that mutations corresponding to positions G2505 and G2583 affected ribosomal sensitivity following exposure to the inhibitors of peptide bond formation, whereas mutations at G2607 and G2608 had no effect. An increase in translational accuracy was attributed to the mutations at position 2583. The hyperaccurate phenotype of these mutations was confirmed *in vivo*. In addition, it was found that the U1067 mutation had an influence on the UA$\bar{A}$ directed termination event. The nucleotide 1067 is situated at a region which might be related to the factor dependent GTPase activity. Therefore, mutations at this position might affect factor directed reaction on the ribosomes and are possibly not suitable for the study of mutations which interfere with factor-dependent reactions. On the other hand, U1067 had no influence on the ribosomal peptidyltransferase centre and can indeed be used as a second mutation to study the role of 23S rRNA in the peptide bond formation.

Acknowledgements

K. H. Nierhaus from Max-Planck Institute of Molecular Genetics and R. Villems from Estonian Biocentre are acknowledged for support and discussions. We would like to thank C. Prescott for advise and help in experiments *in vivo*. We thank A. Liiv and T. Tenson for assistance and C. Prescott and Mrs. K. Ustav for correcting the manuscript.

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Saarma, U., Remme, J., Ehrenberg, M., and Bilgin, N. 1997. An A to U transversion at position 1067 of 23S rRNA from *Escherichia coli* impairs EF-Tu and EF-G function. J Mol Biol 272: 327–335.

J. Mol. Biol. (1997) 272, 327–335

JMB

An A to U Transversion at Position 1067 of 23 S rRNA from *Escherichia coli* Impairs EF-Tu and EF-G Function

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Escherichia coli ribosomes with an A to U transversion at nucleotide 1067 of their 23 S rRNA are impaired in their effective association rate constants (k_{cat}/K_M) for both EF-Tu and EF-G binding. In addition, the times that EF-G and EF-Tu spend on the ribosome during elongation are significantly increased by the A to U transversion. The U1067 mutation impairs EF-Tu function more than EF-G function. The increase in the time that EF-Tu remains bound to ribosome is caused, both by a slower rate of GTP-hydrolysis in ternary complex and by a slower EF-Tu·GDP release from the mutated ribosomes. There is, at the same time, no change in ribosomal accuracy for aminoacyl-tRNA recognition. With support from these new data we propose that nucleotide 1067 is part of the ribosomal A-site where it directly interacts with both EF-G and EF-Tu.

Keywords: translation; EF-Tu; EF-G; ribosome kinetics; rRNA

Introduction

Two soluble protein elongation factors, EF-Tu and EF-G, use GTP as a co-factor to enhance speed and accuracy of mRNA translation in *Escherichia coli* (Kaziro, 1978; Kurland *et al.*, 1996). Two well defined, local regions of *E. coli* 23 S rRNA appear to interact directly with elongation factor G (EF-G). The first is situated in domain II of 23 S rRNA. It is defined by bases 1051 to 1108 and is commonly referred to as the thiostrepton binding structure, TBS. The second is the α-sarcin stem-loop structure, SRL, in domain VI. The latter domain, but not the former, has been shown to interact directly also with elongation factor Tu (EF-Tu). These two domains are distal in sequence, but they may be proximal in the tertiary fold of 23 S rRNA (for reviews see Cundliffe, 1990; Thompson, 1996; Czworkowski & Moore, 1996).

A previous study demonstrated profound effects on the function of EF-Tu as well as EF-G by a base change at position 2661 in the SRL of *E. coli* ribosomes (Bilgin & Ehrenberg, 1994). The present work extends these investigations of functional interactions between rRNA and elongation factors to the thiostrepton binding structure.

The TBS is conserved in all three kingdoms, as indicated by computer simulations of its secondary structure (De Rijk *et al.*, 1996) and by the striking result that TBS regions in *E. coli* and yeast can be exchanged without loss of ribosome activity (Musters *et al.*, 1991; Thompson *et al.*, 1993).

Direct interaction between EF-G and TBS is indicated by early experiments (Sköld, 1983), demonstrating crosslinking between TBS and EF-G. Later it was shown that EF-G protects bases in TBS from chemical modification (Moazed *et al.*, 1988).

Further evidence for an intimate interplay between EF-G and the TBS comes from observations that, first, thiostrepton and micrococcin bind to the TBS and that, second, these drugs strongly affect the function of EF-G. The binding sites for both drugs were early ascribed to the TBS (Cundliffe & Thompson, 1981; Egebjerg *et al.*, 1989) and subsequent experiments confirmed this conclusion. It was found that binding of both thiostrepton and micrococcin change the pattern of chemical modification of N1 position of base A1067 by DMS. Thiostrepton reduces, while micrococcin enhances the accessibility of the DMS (Egebjerg *et al.*, 1989). Experiments also demonstrated that mutations A1067 to U or C give high resistance to both thiostrepton and micrococcin (Thompson *et al.*, 1988; Rosendahl & Douthwaite, 1995). The binding of both drugs is modulated

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Abbreviations used: EF-Tu, EF-G, elongation factors Tu and G; TBS, thiostrepton binding structure; SRL, stem-loop structure; w.t., wild-type.

0022–2836/97/380327–09 $25.00/0/mb971254

by ribosomal protein L11, which has been shown to stabilize the tertiary structure of the TBS, thus being an important factor for the folding of this 23 S rRNA region (Draper, 1996 and references therein). Furthermore, the thiostrepton producing organism *Streptomyces azureus* protects itself against the drug by methylating A1067 in its 23 S rRNA (*E. coli* numbering) (Thompson *et al.*, 1982).

Evidence for the existence of direct interactions between thiostrepton or micrococcin, bound in the TBS, and EF-G comes from how these drugs affect the ribosome-dependent GTPase activity of EF-G, which is stimulated by micrococcin and inhibited by thiostrepton (Cundliffe & Thompson, 1981). Earlier experiments also show that thiostrepton and micrococcin inhibit other ribosome functions involving EF-G (Vazquez, 1979).

While chemical protection studies suggest that EF-G, but not EF-Tu, interacts directly with the TBS (Moazed *et al.*, 1988), the functional experiments by Vazquez (1979) show that micrococcin and thiostrepton inhibit the action not only of EF-G, but also of EF-Tu. This suggests that the TBS may, in fact, interact directly also with EF-Tu. The reason why chemical protection experiments have failed to reveal such contacts (Moazed *et al.*, 1988), may simply be experimental conditions where the ribosome was not in the right configuration to interact with EF-Tu. If so, base substitutions in the TBS may influence not only the kinetics of EF-G, but may also affect the function of EF-Tu.

The present work is an experimental study of how the above mentioned A to U transversion at position 1067 of 23 S rRNA in the TBS affects EF-Tu as well as EF-G function in translation.

The experiments were performed with an optimized *in vitro* translation system with *in vivo* like properties (Ehrenberg *et al.*, 1990). Both steady state and fast kinetic methods (quench-flow) were used to study how this mutation influences the cycling times of EF-G and EF-Tu, the rate of GTP-hydrolysis in a cognate ternary complex in the A-site and the rate of peptide bond formation.

The experiments reveal significant functional effects of the A1067U transversion on both elongation factors, and demonstrate that the mutation inhibits the EF-Tu much more than the EF-G function. The results suggest that not only the α-sarcin stem-loop structure, but also the TBS may be a constitutive part of the ribosomal A-site.

Results

Content of U1067 23 S rRNA in the ribosome population

Ribosomes were isolated from an *E. coli* strain (POP2136), transformed by the pNO-U1067 plasmid, coding for 23 S rRNA containing a U at position 1067 of its gene for 23 S rRNA (Spahn *et al.*, 1996). Sequencing of 23 S rRNA from ribosomes purified from the transformed cells revealed that about 51% of 23 S rRNA was of U1067 and the rest of wild-type (w.t.: Materials and Methods, data not shown). The fraction of U1067 in 23 S rRNA extracted from polysomes, 70 S and 50 S particles are the same (Spahn *et al.*, 1996), indicating that ribosome activity is unchanged by the A to U transversion. We confirmed this result by measuring peptide bond formation kinetics for the w.t./U1067 mixture in the presence and absence of thiostrepton and found that 50% of the ribosome population was drug resistant (data not shown, N. Bilgin, unpublished). This result showed that the fraction of U1067 ribosomes found by sequencing (51%) also represents the fraction of U1067 in the translationally active ribosomes.

EF-Tu cycle

We first studied the influence of an A to U transversion at position 1067 in 23 S rRNA on the full EF-Tu cycle. EF-Tu was kept at a small, constant concentration and the ribosome concentration was titrated from small to high values, with all other translation components in excess. The experiment measured the effective association rate constant (k_{cat}/K_M) for the binding of ternary complex to the ribosomal A-site and the average time ($1/k_{cat}$) that EF-Tu stays either on the ribosome or on the path from free EF-Tu·GDP to ternary complex (see Ehrenberg *et al.*, 1990 for details).

The Eadie-Hofstee plot in Figure 1 shows that k_{cat}/K_M is $28 \times 10^6\ M^{-1}\ s^{-1}$ for wild-type ribosomes and $17 \times 10^6\ M^{-1}\ s^{-1}$ for the w.t./U1067 mixture. The same plot gives $k_{cat} = 6.8\ s^{-1}$ for wild-type ribosomes and $k_{cat} = 5.4\ s^{-1}$ for the w.t./U1067 mixture. Knowledge of the fraction of U1067 in 23 S rRNA allows estimation of k_{cat}/K_M and k_{cat} also for pure U1067 ribosomes (Bilgin & Ehrenberg, 1994, details in Materials and Methods) and estimates of $6.9 \times 10^6\ M^{-1}\ s^{-1}$, respectively $3.1\ s^{-1}$ were obtained. These data show that the A to U transversion at position 1067 of 23 S rRNA significantly affects both the rate of binding of ternary complex to the A-site (k_{cat}/K_M reduced about threefold) and the time EF-Tu stays on the ribosome ($1/k_{cat}$ increased about twofold; Table 1).

The rate of GTP hydrolysis on EF-Tu and peptide bond formation

The effects of the A to U change at position 1067 on the rate of hydrolysis of GTP, when a cognate ternary complex is in the A-site, as well as on the rate of peptide bond formation were studied next. The experiments were done with the quench flow technique (Bilgin *et al.*, 1992), which allows rapid mixing and subsequent quenching of the reaction components for short incubation times. One quench-flow syringe was loaded with poly(U)-programmed ribosomes and the other with (cognate) ternary complex containing EF-Tu, GTP and Phe-

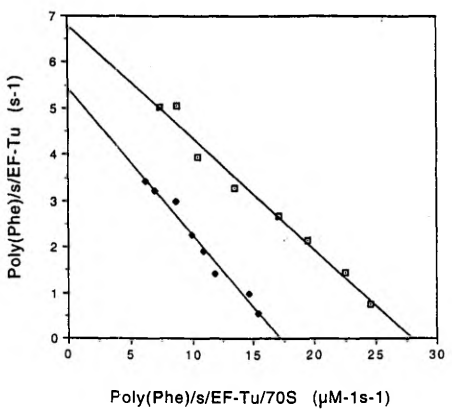

Figure 1. EF-Tu cycle. Eadie-Hofstee plots from titrations with wild-type (□) or with w.t./U1067 ribosome mixture (◆) at a fixed and rate limiting concentration of EF-Tu. The rate of poly(Phe) synthesis normalized to the amount of EF-Tu (poly(Phe)/s/EF-Tu) is plotted as a function of the rate of poly(Phe) synthesis normalized to the amount of EF-Tu and to the concentration of active ribosomes (poly(Phe)/s/EF-Tu/70 S). The intercept at the ordinate is the k_{cat} for the EF-Tu cycle and the intercept at the abscissa is the k_{cat}/K_M for the EF-Tu-ribosome association.

$tRNA^{Phe}$ in large excess so that the time to fill the ribosomal A-site could be neglected (Materials and Methods). Figure 2 shows the result of two such experiments, where the extent of GTP hydrolysis and dipeptide formation is measured as a function of time for wild-type ribosomes as well as for the w.t./U1067 mixture. GTP hydrolysis appears as a first order reaction for wild-type ribosomes and we estimated the rate of GTP hydrolysis (k_{GTP}) to be 122 s^{-1} in this case (Materials and Methods). For the w.t./U1067 mixture, the experimental data reflects the sum of two exponentials corresponding to the extent of GTP hydrolysis for wild-type and for U1067 ribosomes. By using the known fraction of U1067 in the mixture and that $k_{GTP} = 122\ s^{-1}$ for wild-type ribosomes, we estimated that $k_{GTP} = 71\ s^{-1}$ for pure U1067 ribosomes (Materials and Methods). These two rate constants correspond to average times (time $= 1/k_{GTP}$) of GTP-hydrolysis of 8.2 ms for wild-type and 14.1 ms for mutant ribosomes.

The plots for dipeptide formation show a clear lag phase at short times (Figure 2(a) and (b)) which means that the reactions have several consecutive steps. The average time for dipeptide formation (τ_{dip}) is the time it takes to hydrolyze GTP on EF-Tu plus the time for EF-Tu·GDP release from the ribosome plus the peptidyl transfer time. τ_{dip} can be estimated by integrating the probability, $P(t)$, that no dipeptides have formed from zero to infinite time (Materials and Methods, Bilgin *et al.*, 1992). We obtained a τ_{dip} of 22.5 ms for peptidyl transfer on wild-type ribosomes.

In order to calculate τ_{dip} for pure 1067 ribosomes, the contribution from wild-type ribosomes to the dipeptide formation was subtracted. For this, data for wild-type ribosomes (Figure 2(a)) and the measured fraction (0.49) of wild-type in the w.t./U1067 mixture were used (Materials and Methods). We estimated then, as in Figure 2(a) for wild-type, that τ_{dip} is 43 ms for pure U1067 ribosomes.

For these altered ribosomes the average time to hydrolyze GTP is 5.9 ms longer than for wild-type ribosomes (14.1 ms − 8.2 ms = 5.9 ms). Since the average time for peptide bond formation

Table 1. Kinetic parameters for wild-type and U1067 ribosomes

	EF-Tu cycle			
Ribosome	k_{cat} (s^{-1})	Fraction of w.t.[a] (%)	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	Fraction of w.t.[a] (%)
W.t.	7.3 ± 0.7	100	26 ± 3	100
W.t./U1067	5.4 ± 0.1	74 ± 8	17 ± 0.1	65 ± 6
U1067(calculated)	3.0 ± 0.02	42 ± 4	8.4 ± 2.2	32 ± 11
	EF-G cycle			
	k_{cat} (s^{-1})	Fraction of w.t.[a] (%)	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	Fraction of w.t.[a] (%)
W.t.	91 ± 3	100	66 ± 5	100
W.t./U1067	76 ± 1	83 ± 5	59 ± 7	90 ± 4
U1067(calculated)	66 ± 4	72 ± 7	55 ± 9	83 ± 8
	GTP hydrolysis on EF-Tu and peptidyl transfer			
	k_{GTP} (s^{-1})	τ_{GTP} ($1/k_{GTP}$) (ms)	τ_{dip} (ms)	
W.t.	122 ± 11	8.2 ± 0.7	22.5 ± 2.1	
U1067 (calculated)	71 ± 7	14.1 ± 1.4	43.0 ± 1.4	

[a] The ratios between wild-type and mutant values, obtained in parallel experiments, were averaged over separate experiments (two experiments for EF-Tu, and three experiments for EF-G) and σ-values calculated.

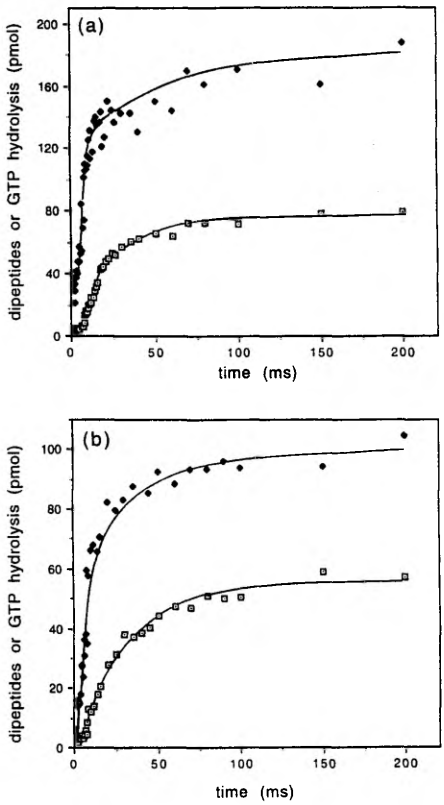

Figure 2. The extent of GTP hydrolysis and dipeptide formation for wild-type ribosomes (a) and for w.t./U1067 mixture (b). The amount of GTP hydrolyzed (◆) or the amount of dipeptides formed (□) is plotted as a function of time.

includes the time to hydrolyze GTP, one would expect an at least 5.9 ms longer τ_{dip} for the 1067U mutant than for the wild-type. Interestingly, the increase in τ_{dip} is $43 - 22.5 = 20.5$ ms, indicating that other steps, in addition to GTP hydrolysis, are slowed down by the U1067 mutation. One possibility is that the peptidyl-transfer reaction itself is slowed down. Another is that the release of EF-Tu.GDP from the ribosome takes longer time in the U1067 case.

Accuracy

If the binding of ternary complexes to the ribosome gets weaker, or if the rate of GTP hydrolysis on EF-Tu gets slower, a high accuracy phenotype may arise (Kurland & Ehrenberg, 1984). In order to see if the observed impaired binding of ternary complex (k_{cat}/K_M) to the U1067 mutant and the observed slower rate of GTP hydrolysis on EF-Tu are associated with such an accuracy phenotype we compared the wild-type ribosomes with the w.t./U1067 mixture in a missense error assay (Ehrenberg *et al.*, 1990). Leucine misincorporation into poly(Phe) was studied and it was found that the normalized missense error frequency is 0.7×10^{-3} for both ribosome variants when Leu-tRNA^{Leu4} competes with Phe-tRNA^{Phe} in poly(U) reading (data not shown, Materials and Methods).

Proofreading of cognate aminoacyl-tRNA

The number of GTPs hydrolyzed on EF-Tu per cognate peptide bond (f_c) in poly(U) translation is near two for both wild-type and for U1067 ribosomes in dipeptide formation (Figure 2). We also measured f_c for wild-type and U1067 ribosomes in steady-state assays by two different methods. In the first, the extent of poly(Phe) synthesis was measured as a function of EF-Tu·GDP in the absence of EF-Ts (Materials and Methods). f_c was calculated by using the known exchange rate of GTP on EF-Tu ($k_d = 0.011\ s^{-1}$) and well determined EF-Tu concentration. We obtained f_c-values of 1.96 and 2.08, respectively, for wild-type and for mutated ribosomes (data not shown). In the second method, the extent of GTP hydrolysis on EF-Tu per peptide bond formation was directly measured after separating the action of EF-Tu from that of EF-G (Ehrenberg *et al.*, 1990). Here, we took advantage of the slow exchange rate of GTP in the ternary complex and used [^{3}H]GTP to measure the extent of GTP hydrolysis on EF-Tu, whereas a high concentration of unlabelled GTP was used to drive the cycle of EF-G (Ehrenberg *et al.*, 1990, Materials and Methods). We obtained with this method similar f_c values for wild-type and for U1067 ribosomes (1.9 and 2.3, respectively). We conclude from these results that U1067 ribosomes do not affect the proofreading of the cognate aminoacyl-tRNA's.

Dissociation rate of peptidyl-tRNA from the A-site

In a translation system that lacks EF-G, dipeptidyl-tRNA remains in the A-site after peptidyl-transfer, waiting to be translocated. Addition of EF-G after various times leads to completion of long poly(Phe) chains, provided that peptidyl-tRNA has not yet dissociated from the A-site during these waiting times before EF-G addition (Karimi & Ehrenberg, 1994). We compared, using this technique, the dissociation rates of peptidyl-tRNA from wild-type and from w.t./U1067 ribosomes and found dissociation rates of *N*-acetyl-Phe-Phe-tRNA^{Phe} corresponding to $2.3 \times 10^{-3}\ s^{-1}$ for both ribosome types (data not shown).

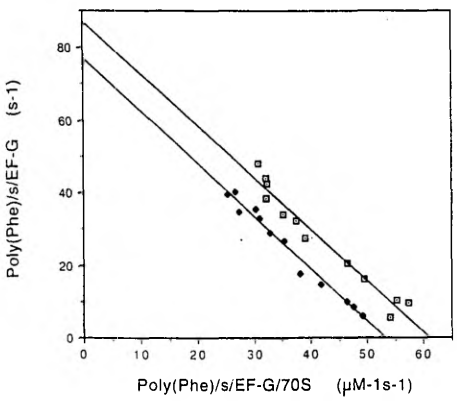

Figure 3. EF-G cycle. Eadie-Hofstee plots from titrations with wild-type (□) or with w.t./U1067 ribosome mixture (◆) at a fixed and rate limiting concentration of EF-G. The rate of poly(Phe) synthesis normalized to the amount of EF-G (poly(Phe)/s/EF-G) is plotted as a function of the rate of poly(Phe) synthesis normalized to the amount of EF-G and to the concentration of active ribosomes (poly(Phe)/s/EF-G/70 S). The intercept at the ordinate is the k_{cat} for the EF-G cycle and the intercept at the abscissa is the k_{cat}/K_M for the EF-G-ribosome association.

EF-G cycle

The effects of the A to U change at position 1067 on the full cycle of EF-G were also studied as described by Ehrenberg *et al.* (1990). A ribosome titration experiment, that is principally similar to the one in Figure 1, was done but with EF-G, instead of EF-Tu, kept at a small rate limiting concentration with all other components in excess. One such experiment where wild-type ribosomes are compared with w.t./U1067 mixture is shown in Figure 3. From these Eadie-Hofstee plots k_{cat}/K_M for the effective binding of EF-G to the ribosome was estimated to be 61×10^6 $M^{-1}s^{-1}$for wild-type and 53×10^6 $M^{-1}s^{-1}$ for the w.t./U1067 mixture. For the pure U1067 ribosome we estimated k_{cat}/K_M to be 47×10^6 $M^{-1}s^{-1}$ (Materials and Methods). The mutation also decreased the maximal turnover rate for EF-G ($k_{cat} = 87\ s^{-1}$ for wild-type and $k_{cat} = 77\ s^{-1}$ for w.t./U1067 mixture). For the pure U1067 mutant k_{cat} was estimated to be $70\ s^{-1}$.

Discussion

We have found that a base change from A to U at position 1067 of 23 S rRNA from *E. coli* has significant effects on both EF-Tu and EF-G function. The strongest effects are seen for EF-Tu. First, the effective association rate constant (k_{cat}/K_M) for cognate ternary complex binding to the ribosomal A-site is reduced more than a factor of two by the base change and, second, the average time for GTP-hydrolysis in a cognate ternary complex in the A-site is increased almost twofold (Table 1). Since the time for dipeptide formation increases more by the rRNA mutation than the corresponding increase in GTP-hydrolysis time, the A to U change probably has multiple effects on EF-Tu function, and we suggest that the release of EF-Tu·GDP from the ribosome is slowed down. This gets support from the corresponding increase in the average time for a full EF-Tu cycle (Figure 1, Table 1). It is, however, still possible that the U1067 mutation perturbs also the peptidyl-transfer reaction itself.

The A to U transversion has also a small but a significant effect on the effective rate of association (k_{cat}/K_M) of EF-G to the ribosome as well as on the translocation rate, leaving the K_M unchanged (Figure 3, Table 1).

Our functional data confirm that the TBS region is important for the interaction between EF-G and the ribosome, but indicates that this part of 23 S rRNA may be even more crucial for the interaction between the ribosome and EF-Tu. Beside the TBS, both elongation factors have previously been shown to interact with the SRL region (Moazed *et al.*, 1988), indicating that domains II and VI are quite close in the tertiary fold of 23 S rRNA within the *E. coli* ribosome.

The impairment of EF-Tu function, that we have observed *in vitro* , is consistent with *in vivo* experiments by Thompson *et al.* (1988), demonstrating a growth rate deficiency of mutants containing U1067. This decreased growth rate is readily explained by the reduction in k_{cat}/K_M for the interaction between cognate ternary complex and the A-site in combination with the prolonged time for peptidyl transfer (τ_{dip}; Table 1). Each of these defects could by themselves account for why U1067 bacteria grow slower than wild-type (Kurland *et al.*, 1996; Pedersen, 1984), while the reduction in k_{cat}/K_M for EF-G is expected to have a much smaller effect on growth rate.

Given the significant effect on EF-Tu function that is caused by the A to U transversion at 1067, the lack of experimental data that directly point to an interaction between EF-Tu and the TBS-sequence is surprising. There exists, however, a number of indirect observations that implicate intimate structural and functional relations between TBS and EF-Tu.

Studies with thiostrepton and micrococcin (see Introduction) indicate that the TBS is a constitutive part of the ribosomal A-site (Gale *et al.*, 1981). These antibiotics inhibit such ribosomal functions that normally are associated with the A-site. This may indicate that the drugs, and therefore the nucleotides to which they bind, may be present in the A-site itself. Among A-site related functions, the inhibition of (p)ppGpp formation, peptide chain termination and the binding of ribosomal factors IF-2, EF-Tu and EF-G to the ribosome are influenced by thiostrepton (Gale *et al.*, 1981). Furthermore, thiostrepton may, depending on

experimental conditions, stimulate ribosomal peptidyltransferase (Kutay *et al.*, 1990) and genetic evidence for a functional interaction between domains II and V of 23 S rRNA has been provided (Mankin *et al.*, 1994)

A different type of experimental support to the idea, that the TBS might be part of the ribosomal A-site, comes from studies showing that enzymatic binding of aminoacyl-tRNA to the A-site moderately protects bases G1068 and G1071 from chemical modification (Moazed & Noller, 1989).

Interestingly, the U1067 mutant does not have an accuracy phenotype, different from that of wild-type ribosomes, despite its reduced rate of GTP hydrolysis on EF-Tu and impaired k_{cat}/K_M for ribosome-ternary complex interaction. This means that k_{cat}/K_M for cognate and near-cognate ternary complex interaction with the ribosome must be reduced to the same extent by the base change. This behavior is unusual, since a reduced k_{cat}/K_M value for the cognate ternary complex normally is associated with reduced missense errors (Kurland *et al.*, 1996). A similar case was found by Tapio *et al.* (1990) for an EF-Tu variant (Aa) with dramatically reduced k_{cat}/K_M for cognate ternary complex-ribosome interaction but with the same missense error level as wild-type EF-Tu. The most likely interpretation of these results is that the amino acid substitution in EF-Tu (Tapio *et al.*, 1990) and the base change at 1067 both affect interactions between EF-Tu and rRNA in a step before codon-anticodon contact. We have also found that the number of GTPs hydrolyzed per peptide bond for cognate ternary complex (f_c) and that the drop-off rate of cognate peptidyl-tRNA from the A-site, both are unchanged by the U1067 mutation. Since the f_c-value is inversely proportional to the probability that a cognate aminoacyl-tRNA survives ribosomal proofreading (Kurland *et al.*, 1996), these experiments also suggest that the change of base at position 1067 may affect a direct interaction between this base and EF-Tu rather than between rRNA and aminoacyl-tRNA.

Recent crystallographic data reveal that the the structure of EF-G·GDP (Czworkowski *et al.*, 1994; Aevarsson *et al.*, 1994) and of the ternary complex between aminoacyl-tRNA, GTP and EF-Tu (Nissen *et al.*, 1995) are remarkably similar. This may mean that not only EF-Tu, but also EF-G, directly interacts with the ribosomal A-site. It is thus conceivable that EF-G ends up in the A-site after translocation of peptidyl-tRNA from A- to P-site (Nyborg *et al.*, 1996; R. Karimi & M. Ehrenberg, unpublished data). Assuming that not only EF-Tu but also EF-G have direct interactions with the A-site, we may explain our kinetic data on the effects of the A to U transversion at position 1067 of 23 S rRNA in a unified way. If TBS in general and base 1067 in particular are part of the ribosomal A-site, then it follows naturally that a base change at position 1067 may directly affect the action of both elongation factors by changing their binding interactions with the ribosome.

With support from the present finding that the rate of GTP hydrolysis on EF-Tu is slowed down by the A to U transversion (Table 1), we also suggest that the TBS region is crucial for the activity of a common GTPase center, that stimulates both EF-Tu and EF-G-dependent GTPase activity. Further, indirect, evidence in this direction is the more recent observation that GTP hydrolysis on EF-Tu is inhibited by thiostrepton (U. S. & N. B., unpublished results).

Materials and Methods

Strains and plasmids

E. coli strain pop2136, comprising a chromosomally located gene encoding for the temperature-sensitive lambda repressor cIts857, was used as a host to tranform the plasmids. Plasmid pNO2680 which carries the *E. coli* rrnB operon under the control of lambda leftward promoter was used to derive pNO-U1067T. Both pNO2680 and pNO-U1067T were transformed into pop2136 strain and selected on ampicillin plates (100 μg/ml).

Preparation of ribosomes

Transformants pNO2680 and pNO-A1067T of *E. coli* strain pop2136 were grown in TY2 medium supplemented with 0.4% (w/v) glucose and ampicillin (100 μg/ml) at 30 C to $A_{480} = 0.14$. Thereafter, expression of RNA from plasmids was induced by temperature shift to 42°C and the bacteria were harvested after two hours. Ribosomes were purified by gel filtration chromatography on Sephacryl-S300 (Pharmacia) (for details, see Jelenc 1980), dialyzed and stored in polymix buffer (Jelenc & Kurland, 1979, see below).

Preparation of enzymes and tRNAs

EF-Tu, EF-Ts, EF-G, Phe-tRNA synthetase and Leu-tRNA synthetase were purified from *E. coli* MRE600 by following the protocols of Ehrenberg *et al.* 1990). $tRNA_{bulk}$ was purified from *E. coli* MRE600 as described by Kelmers *et al.* (1971). $tRNA^{Phe}$ and $tRNA^{Leu4}$ were purified from $tRNA_{bulk}$ according to Gillam *et al.* (1967). *N*-acetyl-Phe-$tRNA^{Phe}$ was prepared from purified $tRNA^{Phe}$ as described by Wagner *et al.* (1982).

tRNAs and enzymes were all dialyzed and stored in polymix buffer.

Analysis of rRNA content in ribosomes

rRNA was extracted from the purified ribosomes used in the assays and sequenced. Purified rRNA obtained from wild-type and from w.t./U1067 mixture was sequenced by primer extension using reverse transcriptase as described by Bilgin *et al.* (1990). Fractions of mutant RNA in purified ribosomes were estimated from the autoradiograms of the sequencing gels according to Bilgin & Ehrenberg (1994).

Assays

All experiments were performed at 37°C in polymix buffer (5 mM magnesium acetate, 5 mM potassium phos-

phate (pH 7.3), 95 mM potassium chloride, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine and 1 mM 1,4-dithioerythritol). Steady-state experiments were described by Ehrenberg *et al.* (1990) and pre-steady-state assay with time resolved quench-flow is described by Bilgin *et al.* (1992) in detail.

Factor cycles

EF-Tu and EF-G cycle experiments were carried out as described by Ehrenberg *et al.* (1990) and references therein. The elongation factor was kept at a rate limiting concentration and the ribosome concentration was varied with all other components in excess. Cycling rates of elongation factors obey Michaelis-Menten kinetics. k_{cat} and K_M for EF-Tu and EF-G were directly determined from Eadie-Hoffstee plots (Ehrenberg *et al.*, 1990) and parameters for pure mutant were calculated as described by Bilgin & Ehrenberg (1994) using the equations:

$$k_{cat2}/K_{M2} = [(k_{cat}/K_M) - (1 - \alpha)k_{cat1}/K_{M1}]/\alpha$$

and

$$K_{M2} = \alpha/[(1/K_M) - (1 - \alpha)/K_{M1})]$$

where α is the fraction of the mutated ribosomes in the wild-type-mutant mixture, k_{cat1}, k_{cat2} and k_{cat} are the maximum elongation factor cycling rates for wild-type, mutant and wild-type-mutant mixture, respectively. Similarly K_{M1}, K_{M2} and K_M are the corresponding K_M values.

GTP hydrolysis on EF-Tu and dipeptide formation

The experiments were performed on quench-flow apparatus (Kin-Tec USA). EF-Tu·[^{3}H]GTP·Phe-tRNA^{Phe} ternary complex was rapidly mixed with ribosomes, initiated with *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} and poly(U) in the absence of EF-G and EF-Ts (see Bilgin *et al.*, 1992 for details).The GTP hydrolysis on EF-Tu and the appearance of dipeptides were followed as a function of time. The extent of GTP hydrolysis was determined by the GDP/(GDP + GTP) ratio, calculated after thin-layer chromatography of the quenched samples, multiplied by the total amount of [^{3}H]GTP initially present in the ternary complex before mixing with ribosomes. The extent of dipeptide formation was calculated by using the ratio of the *N*-acetyl-Phe-Phe dipeptides to the total *N*-acetyl-Phe plus *N*-acetyl-Phe-Phe dipeptides after separating the *N*-acetyl-Phe-Phe dipeptides from *N*-acetyl-Phe by HPLC. This ratio was then multiplied by the total amount of *N*-acetyl-Phe initially present in the ribosome mix (Bilgin *et al.*, 1992). The time curves were evaluated as described by Bilgin *et al.* (1992).

In order to calculate the rate of GTP hydrolysis and the average time for peptide bond formation for pure mutants, contributions of the wild-type ribosomes were substracted from the corresponding data for the w.t./U1067 mixture by taking into account the percentage of the wild-type ribosomes in the mixture (49%).

The apparent GTP hydrolysis for w.t./U1067 mixture was the sum of two exponentials corresponding to the rates of GTP hydrolysis (k_{GTP}) for wild-type and for 1067U mutant. The known k_{GTP} for wild-type ribosomes and the fraction of wild-type RNA in the w.t./U1067 mixture was used in a MatLab program (courtesy of M. Pavlov) to estimate the k_{GTP} for pure U1067 ribosomes.

For peptide bond formation, the reaction cannot be characterized by a single rate constant. Dipeptides appear after a lag phase indicating several steps before peptidyltransferase. For wild-type ribosomes, the average time for dipeptide formation was estimated by integrating the probability that no peptide has occured from zero to infinite time as described by Bilgin *et al.* (1992). In order to calculate the average time for dipeptide formation for pure mutant, we first substracted the contribution of the wild-type ribosomes to dipeptides at each time point from the data set for w.t./U1067 mixture. We used, for this purpose, the data set for wild-type ribosomes and the percentage of the wild-type ribosomes in the w.t./U1067 mixture. This new data set was then treated as described by Bilgin *et al.* (1992), to calculate the average time for dipeptide formation for pure U1067 ribosomes.

Missense errors

Leu misincorporation into poly(Phe) chains was measured by varying Leu-tRNA^{Leu} concentration at a fixed Phe-tRNA^{Phe} concentration where EF-Tu was in excess over the total tRNA (Ehrenberg *et al.*, 1990). Leu misincorporation increases linearly with Leu-/Phe-tRNA ratio. We plotted Leu/(Leu + Phe) incorporation against Leu-/Phe-tRNA ratio. The normalized missense error frequency (P_E) was obtained from the slope of this line (Ehrenberg *et al.*, 1990).

Number of GTP molecules hydrolyzed per correctly formed peptide bond (f_n) in steady-state

In the absence of EF-Ts, the rate of GTP exchange on EF-Tu is very slow ($k_d = 0.011\ s^{-1}$). In an elongation burst assay, with an exception that EF-Tu·GDP is added with the ribosomes and EF-Ts is excluded, the extent of poly(Phe) elongation depends on the concentration of EF-Tu which is exclusively in the EF-Tu·GDP form and the rate of exchange of GDP to GTP on EF-Tu. Poly(Phe) synthesis is then given by the equation:

$$poly(Phe)/s = [EF - Tu] \cdot k_d/f_n$$

Poly(Phe) synthesis was measured at varying EF-Tu concentrations and f_n was calculated by taking $k_d = 0.011\ s^{-1}$ (Ehrenberg *et al.*, 1990).

f_n was also determined by directly measuring the [^{3}H]GTP hydrolysis on EF-Tu in the absence of EF-Ts while EF-G was driven by high concentration of unlabelled GTP. The amount of GTP hydrolyzed was obtained by isotope dilution. This f_n assay was described by detail by Ehrenberg *et al.* (1990).

Peptidyl-tRNA stability at the A-site

Stability of the *N*-acetyl-Phe-Phe-tRNA in the A-site was measured as described by Karimi & Ehrenberg (1990).

Acknowledgments

This work was supported by The Swedish Natural Science Research Council.

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Edited by D. E. Draper

(Received 25 March 1997; received in revised form 27 June 1997; accepted 2 July 1997)

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Saarma, U., Spahn, C. M. T., Nierhaus, K., and Remme, J. 1997. Mutational analysis of the donor substrate binding site of the ribosomal peptidyltransferase centre. (Manuscript submitted to RNA).

Mutational analysis of the donor substrate binding site of the ribosomal peptidyltransferase centre

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Abstract

Previous experiments have shown that the top of helix 90 of 23S rRNA is highly important for the ribosomal peptidyltransferase activity and might be part of the donor (P) site. Developing on these studies, mutations in the 23S rRNA at the highly conserved positions G2505, G2582 and G2583 were investigated. None of the mutations affected assembly, subunit association or the capacity of tRNA binding to A and P sites. A "selective transpeptidation assay" revealed that the mutations specifically impaired peptide bond formation. Results with a modified "fragment" assay using the minimal donor substrate pA-fMet are consistent with a model where the nucleotides ΨGG2582 form a binding pocket for C75 of the tRNA.

Introduction

Peptide-bond formation is the central enzymatic activity of the ribosome. The underlying biochemical reaction is the transfer of the peptidyl residue from a peptidyl-tRNA in the P site to the α-amino group of an aminoacyl-tRNA at the A site. The peptidyltransferase (PTF) is an integral activity of the large ribosomal subunit (Monro, 1967) and is localized at the base of the central protuberance of the 50S ribosomal subunit (Stöffler & Stöffler-Meilicke, 1986; Oakes *et al.,* 1986). It has been demonstrated with ribosomes from *Escherichia coli* that the 23S rRNA and the large subunit proteins L2, L3 and L4 are possibly involved in this catalytic activity (Hampl *et al.,* 1981; Franceschi & Nierhaus, 1990). The importance of the 23S rRNA in the PTF activity has been shown by several independent methods. A complex derived from the large subunit of *Thermus aquaticus* ribosomes consisting of 23S rRNA and only up to 8 proteins has significant PTF activity (Noller *et al.,* 1992; Noller, 1993). Using footprinting and cross-linking techniques, substrates specific for both the A and the P site have been demonstrated to interact with domain V (reviewed in Garrett & Rodriguez-Fonseca, 1995). Antibiotics, known to inhibit PTF, have been

extensively used to map the ribosomal PTF centre and the majority of inhibitors have also been shown to interact with domain V (Cundliffe, 1990; Rodriguez-Fonseca *et al.,* 1995; Spahn & Prescott, 1996 and references therein).

Site directed mutagenesis is a powerful method for the identification of nucleotides and amino acid residues that are important for the PTF activity. Using this method, two regions of the 23S rRNA have been shown to be particularly important for peptide bond formation. Mutations at G2252 and G2253 interfere with PTF activity (Lieberman & Dahlberg, 1994; Samaha *et al.,* 1995; Porse *et al.,* 1996). Moreover, the partial restoration of activity by a combination of a 23S rRNA mutation and a compensating tRNA mutation established a Watson-Crick base-pair in the ribosomal donor site between 23S rRNA nucleotide G2252 and tRNA nucleotide C74 within the 3' CCA end (Samaha *et al.,* 1995; Porse *et al.,* 1996). However, the neighboring nucleotides G2251 and G2253 do not interact with the adjacent C75 of the 3' CCA end (Samaha *et al.,* 1995; Green *et al.,* 1997). Therefore, a complete set of interactions between the 3' CCA end of tRNA and ribosomal the PTF centre, as well as the nucleotides or aminoacyl residues involved in the enzymatic activity, are yet to be identified.

The second most sensitive region with respect to peptide bond formation has been identified around the top of helix 90 of 23S rRNA shown in Fig. 1 (Saarma & Remme, 1992; Porse & Garrett, 1995; Porse *et al.,* 1996; Spahn *et al.,* 1996a; Spahn *et al.,* 1996b; Green *et al.,* 1997). We have previously analysed the involvement of two highly conserved UGG sequences in binding the 3' CCA end of tRNA (Spahn *et al.,* 1996a; Spahn *et al.,* 1996b). Although no evidence for a canonical base pair interaction was found, the results suggested that G2581 and Ψ2580 might participate in the fixation of C75 of the CCA 3' end of peptidyl-tRNA in the ribosomal P site.

In this study we extend our analysis to mutations of the three universally conserved nucleotides G2505, G2582 and G2583 in the putative PTF centre of *E. coli* 23S rRNA. Cross-linking and footprinting experiments mapped the binding sites of the substrates of the PTF centre close to these nucleotides (Garrett & Rodriguez-Fonseca, 1995). In order to distinguish between the activities of chromosome-encoded wild-type ribosomes and the plasmid-borne mutant ribosomes, a 'selective transpeptidation assay' has been used with thiostrepton as the selective agent. We show that mutations at all three positions severely inhibit peptide bond formation. Furthermore, experiments with pA-fMet as minimal donor of the PTF centre suggest that G2582 in addition to Ψ2580 and G2581 might interact with C75 of a tRNA located at the P site.

Results

Mutations of *E. coli* 23S rRNA in the peptidyltransferase centre have a high probability to give a lethal phenotype. Indeed, the G2505C and G2583A/C mutations caused a lethal phenotype in combination with A1067U (Saarma *et al.,* 1992) like many other mutants in the centre of domain V of 23S rRNA (Lieberman & Dahlberg, 1994; Porse & Garrett, 1995; Porse *et al.,* 1996; Spahn *et al.,* 1996a). Furthermore, G2582A and G2582U linked to A1067U have a dominant lethal phenotype (not shown). In order to allow expression and isolation of mutant ribosomes, a conditional expression system has been used.

In addition to the plasmid borne mutant rRNA, transcription occurs also from seven chromosomal 23S rRNA genes. Following expression of the mutant RNA and isolation of the ribosomes, we determined the fraction of plasmid-derived 23S rRNA with the primer extension method (Sigmund *et al.,* 1988, see materials and methods) using the A1067U mutation as a marker. All mutant 23S rRNA species characterized in this paper (G2505C, G2582A, G2582U, G2583A, and G2583C) were readily incorporated into 70S ribosomes and polysomes, indicating that the base substitutions did not cause any assembly defect. The expression system produces a mixed ribosome population, approximately half of which was of plasmid origin (data not shown).

It is difficult to distinguish the kinetics of peptidyl transfer of wild-type and mutant ribosomes in a mixed ribosome population. Therefore, a 'selective transpeptidation assay' has been employed which depresses the activities of the wild-type (chromosome-encoded) ribosomes almost completely (Spahn *et al.,* 1996b). The method exploits a second mutation in the plasmid-borne 23S rRNA, i.e. A1067U that renders the ribosomes resistant towards the antibiotic thiostrepton, whereas chromosome-borne ribosomes will be blocked by the antibiotic. The combination of a 'peptidyl transfer' mutations with the thiostrepton resistance mutation on one and the same 23S rRNA gene enables the measurement of the peptidyl transfer kinetics exclusively of the mutant ribosomes.

In the presence of the heteropolymeric MF-mRNA (coding for Met and Phe) the ribosomal P site was filled with $tRNA_f^{Met}$, followed by the binding of AcPhe-$tRNA^{Phe}$ to the ribosomal A site. Thiostrepton trapped the chromosome-derived ribosomes in the pre-translocational state. Translocation of AcPhe-$tRNA^{Phe}$ to the ribosomal P site on ribosomes containing plasmid borne 23S rRNA was triggered by EF-G, thus leading to a puromycin reaction specific for mutant ribosomes.

The amounts of AcPhe-tRNA bound to the A and P sites were determined before and after translocation, respectively (for details, see Spahn, *et al.,* 1996b). The overall tRNA binding to both the ribosomal A and P sites was about the same for all mutants (not shown).

Fig. 2A demonstrates that A1067U ribosomes are fully active in the puromycin reaction. In the presence of thiostrepton, half of the ribosomes can carry out peptidyl transfer as compared with the minus-drug control. The result

correlates well with the sequencing data that revealed a fraction of about 50% of ribosomes containing plasmid-borne 23S rRNA. The curve minus-EF-G represents the background of the reaction. In contrast to the resistant ribosomes, the activity of wild type ribosomes is blocked in the presence of thiostrepton (see Table).

Figs. 2B, C, and D demonstrate the consequences of the nucleotide substitutions at position 2505, 2582 and 2583. In all cases the level of peptidytransferase activity is severely reduced. In the presence of thiostrepton, only little activity is seen with the mutants G2505C (Fig. 2B), G2582A (Fig. 2C), G2582U (Table 1), and G2583A (Fig. 2D). Ribosomes containing the G2583C mutation in 23S rRNA have practically no peptidyltransferase activity under conditions used (Table 1).

The Table 1 summarizes the peptidyl transferase activity of ribosomes containing mutations around helix 90 determined in this study and in Spahn, *et al.,* 1996b according to the test system shown in Fig. 2. The results indicate that a cluster of nucleotides at the top of helix 90 is of pivotal importance for the PTF reaction (Fig. 1).

pA-fMet is the minimal donor for the ribosomal peptidyltransferase reaction, but it's donor activity is significantly lower than that of pCA-fMet (Cerna, *et al.,* 1973). Intriguingly, the donor activity of pA-fMet becomes strongly stimulated by CMP (Cerna, 1973), whereas for pCA-fMet activity, CMP even shows a small inhibitory effect (Cerna, 1975). The interpretation of this findings is that binding of nucleotide C75 of the tRNA 3' end in the ribosomal donor site is important for the PTF activity and that CMP stimulates the donor activity of pA-fMet by taking the place of C75 (Cerna *et al.,* 1973; Cerna, 1975). Previously, we have detected that CMP stimulation is lost with mutations Ψ2580C and G2581A (Spahn *et al.,* 1996b). A similar assay was performed with ribosomes containing mutations at the positions G2505, G2582, and G2583.

We have tested the transpeptidation reaction from pA-fMet to Phe-tRNA^{Phe} in the presence and absence of CMP. Mutant ribosomes G2505C and G2583A exhibited similar stimulation by CMP as the wild-type (A1067U). In contrast, CMP stimulation was reduced by a factor of 2 in the presence of ribosomes containing the mutations G2582A or G2582U (stimulation factors in Table 2). In this assay, both wild-type and mutant ribosomes can participate in peptide bond formation. If the mutant ribosomes are inactive, *i. e.* only the wild-type ribosomes participate in the catalysis, the stimulation factor should be the same as that observed with wild-type ribosomes. A twofold decrease in the stimulation factor observed with G2582 mutants indicates that the mutant ribosomes were active in this assay. Consequently, transfer of the fMet residue from pA-fMet to Phe-tRNA was not stimulated by CMP on the mutant ribosomes containing the mutations G2582A/U.

The relative CMP stimulation of the mutations at G2505, G2582, and G2583 compared with our previous results are shown in Fig. 3. The mutations at G2582 cause a loss of the CMP stimulation as reported earlier for mutations at positions Ψ2580 and G2581 (Spahn *et al.,* 1996b), whereas the mutations at the adjacent G2583 or G2505 showed a full stimulation effect of CMP.

Discussion

Transpeptidation reaction takes place in close proximity to the location of the 3' CCA ends of the tRNAs at the donor and acceptor sites. The region of 23S rRNA around the top of helix 90 has been implicated in binding of the donor substrate. In footprinting experiments, peptidyl-tRNA analogues protect the bases U2506, U2584 and U2585 (Moazed & Noller, 1989; Moazed & Noller, 1991). These protections disappeared after the removal of 3' terminal A of P site bound tRNA. G2505 is protected by a tRNA that lacks the 3' terminal CA. G2583 and the nucleotides in the helix 90 were not accessible for chemical modificators (Moazed & Noller, 1989). In a recent cross-linking study with $[2N_3A76]tRNA^{Phe}$ derivatives, the 23S rRNA segments 2500-2520 and 2570-2590 were labeled from A and P sites, respectively, showing direct proximity of helix 90 of 23S rRNA to the PTF centre (Wower *et al.,* 1995). Further support comes from a mutation (lack of methylation) at position U2584 of 23S rRNA from *Halobacterium salinarium* that renders the ribosomes resistant to sparsomycin, an antibiotic that stabilizes the binding of the 3'-terminal fragments of peptidyl-tRNA analogues to the P site (Lazaro *et al.,* 1996).

In this paper we are extending our study of the effects of mutations at the peptidyltransferase region of 23S rRNA (Saarma & Remme, 1992; Spahn *et al.,* 1996a; Spahn *et al.,* 1996b). Mutations at the highly conserved positions G2505 (to C), G2582 (to A, U) and G2583 (to A, C) show a dominant lethal phenotype. An equal distribution of the mutated 23S rRNA in the polysomal fraction the 70S ribosomes and the 50S subunits shows that the mutations did not cause an assembly defect and that the mutant 50S subunits are not impaired in subunit association. Since the efficiencies of tRNA binding to the A and P sites are not affected, the mutations do not seem to induce a gross-conformational change.

A 'selective transpeptidation assay' (Spahn *et al.,* 1996b) was used to study the effects of the mutations on the formation of AcPhe-puromycin. All studied mutants were drastically repressed with respect to peptidyltransferase activity (Table 1). The fact that the impairments are restricted to the PTF activity suggests the involvement of the nucleotides G2505 G2582 and G2583 in the ribosomal peptidyltranferase. The severely reduced PTF activity might explain the dominant lethal phenotype of these mutations (Saarma & Remme, 1992).

While our experiments were in progress a similar analysis concerning the effect of mutations around helix 90 on PTF activity was presented (Porse & Garrett, 1995). A different method for assaying PTF activity was used as well

as a different combination of antibiotic and resistance mutation in order to measure exclusively the peptide bond formation with mutant ribosomes. Both studies are in quite good agreement and imply an important role of this rRNA region for the PTF activity. Porse et al. 1996 propose a role in the donor substrate binding for the region around helix 90 in agreement with our previous analysis (Spahn *et al.,* 1996a). Based on the fact that significant activity of the U2585G mutation was found in contrast to the A and C mutations at the same position, the possibility of a Hoogsteen pair between the 3' terminal A of the donor substrate and U2585 of 23S rRNA was suggested (Porse *et al.,* 1996). However, using a chimeric reconstitution approach Green et al. found similar activity for U2585G and U2585C, rendering a Hoogsteen pair at this position unlikely (Green *et al.,* 1997).

Previously, we have shown that the pA-fMet reaction of ribosomes carrying the mutations Ψ2580C and G2581A cannot be stimulated by CMP (Spahn *et al.,* 1996b). The CMP stimulation was interpreted as an indication for the importance of C75 fixation of the tRNA at the donor (P) site of the PTF center (Cerna, 1975; Quiggle & Chladek, 1980). Here we extend this effect to mutations at G2582 (to A, C). The loss of CMP stimulation seems to be restricted to mutations within the ΨGG2582 sequence. Mutations at the directly adjacent G2583 show a full response to CMP, as well as mutations at G2505 and C2507 which are neighboured in the secondary structure (Fig. 1). Interestingly, the mutation Ψ2580C does not abolish ribosomal peptidyltransferase activity in puromycin reaction (Spahn, *et al.,* 1996b). In contrast, mutations at G2505 and G2583 are inactive in puromycin reaction but are stimulated by CMP.

There is an apparent contradiction between the results obtained in two different assay systems. In the 'puromycin reaction' G2582 mutants were found to be inactive. In contrast, the loss of CMP stimulation can be explained only if these mutant ribosomes were active in the pA-fMet reaction. The problem is solved, if we assume that G2582 is invovled in an interaction with C75 of a tRNA. This interaction can take place only in the 'puromycin reaction', since pA-fMet is a 'minimal donor' and does not have the corresponding nucleotide C75. Therefore, inactivity of the G2582 mutants in the puromycin reaction can be due to a disturbance of the G2582-C75 interaction. Likewise, the lack of the CMP stimulation is also explained by such a disturbance. The loss of CMP stimulation and the restriction to mutants of the triplet ΨGG2582 has a clue for a direct involvement of this sequence in binding C75 of the P site located peptidyl-tRNA. The fact that CMP stimulation cannot be compensated with other nucleoside phosphates indicates that the sequence ΨGG2582 forms a binding pocket for C75 of tRNA instead of Watson-Crick base-pairing. However, indirect effects cannot be ruled out.

C74 of the 3' terminal end of peptidyl-tRNA is base-paired with G2252 within the helix 80 region of 23S rRNA (Samaha *et al.,* 1995; Porse *et al.,*

1996). If the remaining 3' CCA end would be bound by the helix 90 region, both 23S rRNA regions have to be in close proximity. 3' CCA dependent footprints can be found in both regions (Moazed & Noller., 1989; Moazed & Noller, 1991). Moreover, a functional coupling between both regions has been shown: mutations in the helix 80 region change the reactivity of bases in the helix 90 region (Samaha *et al.*, 1995; Green *et al.*, 1997).

Materials and Methods

Preparation of ribosomes

23S rRNA mutants were constructed and cloned into the expression plasmid ptac-2, that contains the thiostrepton resistance mutation A1067U in its 23S rRNA gene (Saarma & Remme, 1992). *E. coli* strain XL-1 was used to express the mutant genes.

The isolation of ribosomes was performed in two steps: (i) crude ribosomes were pelleted from a S30 lysate through a 20% sucrose cushion. (ii) 70S ribosomes were fractionated and collected from a 10%-30% (w/v) sucrose gradient (for details see Spahn *et al.*, 1996a).

The relative amount of mutant 23S rRNA was determined by the primer extension method (Sigmund *et al.*, 1988) using 5'-[^{32}P]-labeled primer complementary to bases 1069-1087 of 23S rRNA.

Selective transpeptidation assay

The assay was performed under the following buffer conditions: 20 mM Hepes, pH 7.6; 6 mM $MgCl_2$; 150 mM NH_4Cl; 4 mM β-mercaptoethanol; 2 mM spermidine and 0.05 mM spermine. 70S ribosomes were programmed with MF-mRNA (Triana-Alonso *et al.*, 1995) that contains the codons AUG (Met) and UUC (Phe) in the middle. The ribosomal P site was occupied with $tRNA_f^{Met}$ (1.5 fold molar excess over 70S ribosomes, incubation at 37°C for 20 min), followed by filling the A site with Ac[^{14}C]Phe-$tRNA^{Phe}$ (1030 dpm/pmol, 1.1 tRNA/70S). After incubation for 30 min at 37°C, two aliquots were taken to determine the levels of binding and the remaining reaction mixture was divided into three aliquots. Thiostrepton (1 μM f. c.) and EF-G were added to one aliquot, and the incubation was continued for 10 min at 37°C. The puromycin reaction was performed at 0°C. EF-G only was added to the second aliquot. The third aliquot served as a mock control and neither thiostrepton nor EF-G were added to check whether the pre-translocational state was established.

Fragment reaction with pA-fMet

The conditions used were essentially as described previously (Spahn *et al.*, 1996b). 25 μl of the reaction mixture contained 50 mM Tris·HCl, pH 7.4, 400 mM KCl, 20 mM $Mg(COO)_2$, 1 pmol 70S, 1.5–2 pmol [^{3}H]Phe-$tRNA^{Phe}$

(~80 000 dpm/pmol) 1 mM pA-fMet and when indicated 1 mM CMP. The reaction was initiated by the addition of 25 µl methanol; incubation was for 60 min at 0°C. The reaction was terminated by addition of 25 µl 3 M NaOH followed by an incubation for 30 min at 37 °C to hydrolyse the ester bonds. 100 µl 10 M HCl was added and the fMet-Phe was extracted with ethyl acetate. The ethyl acetate phase was washed successively with 100 µl 1 M HCl and 100 µl water before counting in order to decrease the background. Blank values were obtained by omitting pA-fMet, ribosomes or methanol.

The data were processed in the following way: the activity stimulated by CMP (pA-fMet formed in the presence of CMP minus pA-fMet formed without CMP) was divided by the reactivity without CMP. This relative stimulated activity was compared with the corresponding value of the A1067U control ribosomes. A 50% relative stimulated activity caused by CMP stimulation reflects the presence of the 50% ribosomes containing chromosomal-borne 23S rRNA. This case, where the fraction of ribosomes with plasmid-borne 23S rRNA cannot be stimulated, was scaled to one. These values are given in Fig. 3.

Acknowledgments

We thank Dr. Susan Joyce and Dr. Tanel Tenson for help and discussions, Ülo Maiväli for excellent technical assistance, H-V Tolk for help and M. Walsh for correcting the English. This work was supported by DFG Grant Ni174/7-1 and EC Grant ERBCIPA CT930136.

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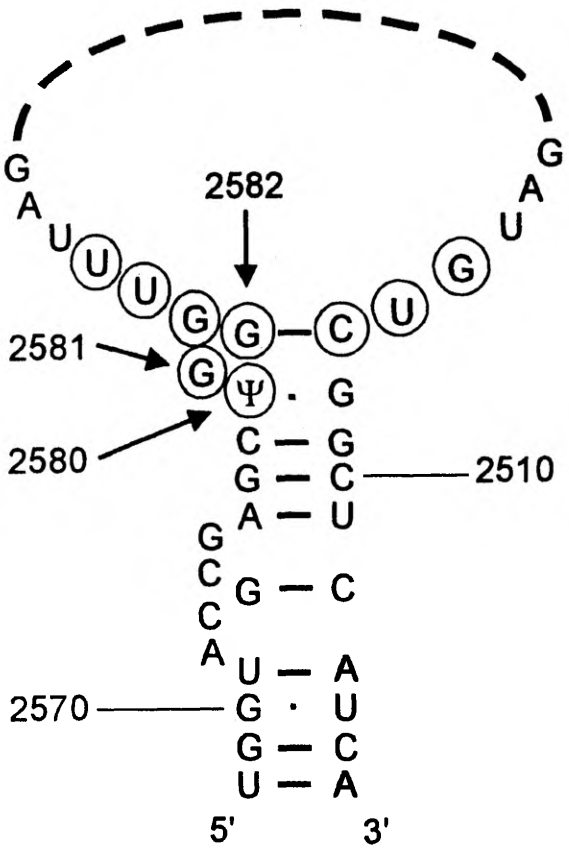

Figure 1. Secondary structure of helix 90 in domain V of *E.coli* 23S rRNA according to Gutell *et al.*, 1993. Positions where mutations impair peptide bond formation are encircled (data from refs. Porse *et al.*, 1996, Spahn *et al.*, 1996b, and this paper). Arrows indicate positions where a mutation interferes with the CMP stimulation of the pA-fMet (Spahn *et al.*, 1996b and this paper). These nucleotides might interact (directly or indirectly) with C75 of the P site bound tRNA.

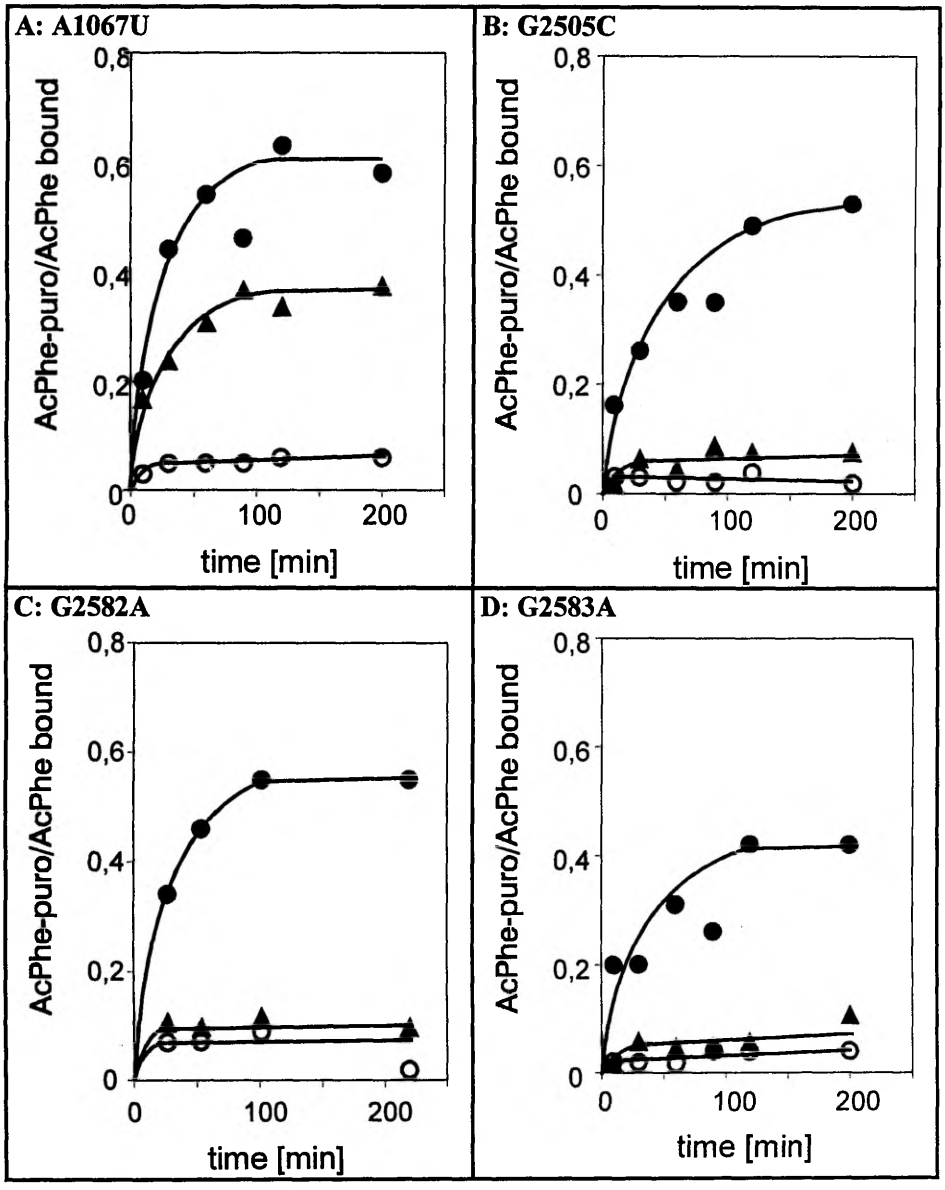

Figure 2. Kinetics of the puromycin reaction. The puromycin reaction of the pre-translocational ribosomes (minus EF-G, open circles) represents the background, whereas the puromycin reaction of the mixed population is shown after an EF-G dependent translocation (filled circles). The curve after an EF-G dependent translocation in the presence of thiostrepton (filled triangles) indicates the puromycin reaction of ribosomes exclusively with plasmid-derived 23S rRNA. For details see text.

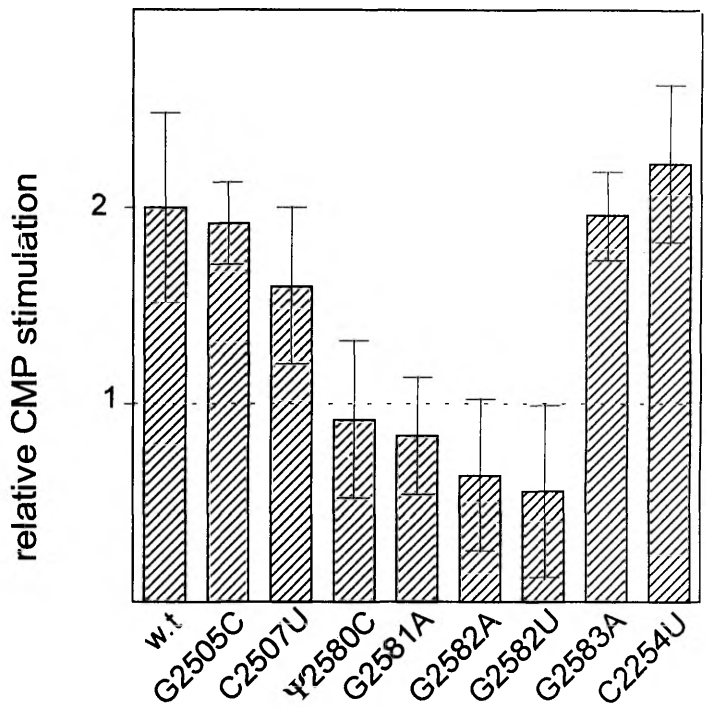

Figure 3. Relative stimulation of the pA-fMet reaction by CMP. A relative stimulation of one indicates that the fraction of ribosomes with plasmid-derived 23S rRNA within the mixed population cannot be stimulated (for details see material and methods). The data for the C2507U, Ψ2580C, G2581A and C2254U mutants are taken from Spahn *et al.,* 1996b.

Table 1. Relative puromycin reaction of ribosomes with plasmid-borne 23S rRNA. After establishing a pre-translocational state, an EF-G dependent translocation was performed in the presence and absence of thiostrepton and a puromycin reaction was performed. To calculate the relative activity the values in the saturation phase of the puromycin reaction (incubation time 3h-24h) were taken. The background values (minus EF-G) were subtracted and the values in the presence of thiostrepton (plus EF-G, plus thiostrepton) were divided by the values minus thiostrepton (plus EF-G). The ratio for the A1067U ribosomes, 0.47, correlates well with approximately 50% of ribosomes with plasmid-derived 23S rRNA and was set to 100%.

Ribosomes	Relative puromycin reaction [%] A1067U = 100%
wild type (background)	4
A1067U	100
G2505C	9
C2507U	56[a]
Ψ2580C	38[a]
G2581A	5[a]
G2582A	8
G2582U	8
G2583A	12
G2583C	1
C2507U/G2581A	6[a]
C2507Δ/G2581A	0[a]

[a] Data taken from Spahn *et al.*, 1996b.

Table 2. Stimulation of dipeptide bond formation with CMP on mutant ribosomes. The fMet-Phe synthesis is shown in the presence and absence of CMP using [^{3}H]Phe-tRNA^{Phe} as the acceptor substrate and pA-fMet as the donor. Background values (35-62 DPM in different experiments) are substracted.

Ribosomes	DPM (-CMP)	DPM (+CMP)	Stimulation factor
A1067U (wt)	758	3713	4.9
G2505C	406	1929	4.75
G2582A	435	898	2.07
G2582U	828	1515	1.83
G2583A	501	2415	4.82

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1992–1997	Ph. D. student in Dr. J. Remme's lab at University of Tartu, Institute of Molecular and Cell Biology, Department of Molecular Biology. Research subject: ribosomal peptidyl transferase centre and the importance of 23S rRNA in transpeptidation reaction

EMPLOYMENT HISTORY

1990–1995	in Estonian Biocentre as a junior scientist
Since 1995	in Estonian Biocentre as a scientist

COLLABORATIVE RESEARCH

1992–1994	visiting scientist in Dr. M. Ehrenberg's lab at Dept. of Molecular Biology, University of Uppsala. Research subject: fast kinetic analysis of ribosomal mutants
1994	visiting scientist in Dr. K. Nierhaus lab at Max Planck Institute, Berlin. Research subject: kinetic analysis of ribosomal peptidyltransferase mutants

RESEARCH COURSES

1990	Participant in the Cold Spring Harbor Laboratory 'Undergraduate Research Program' in Dr. A. Stenlund lab. Research subject: Bovine papillomavirus replication — the role of BPV protein E1 in initiation of replication
1993	Participant in NOMBA course 'Protein Engineering'

RESEARCH

Since 1987 I have been working in Dr. Jaanus Remme's lab at Tartu University. Major interest is to evaluate the role of ribosomal 23S RNA in protein synthesis and for ribosomal peptidyl transferase, in particular.

CURRICULUM VITAE

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1974–1982	Pärnu 6. Kaheksaklassiline kool
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1985–1990	Tartu Riikliku Ülikool, bioloogia-geograafiateaduskond, bioloogia eriala. Tartu Ülikooli lõpetasin bioloog-biokeemikuna
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1992–1997	Tartu Ülikooli Molekulaar- ja rakubioloogia instituudi molekulaarbioloogia kateedri doktorant

TÄIENDÕPE

1990	*Cold Spring Harbor Laboratory, Undergraduate Research Program.* E1 valgu osalus veise papilloomiviiruse replikatsiooni initsiatsioonil
1993	NOMBA kursus *Protein Engineering*

TEADUSLIK KOOSTÖÖ

1992–1994	külalisteadlane Uppsala Ülikooli molekulaarbioloogia kateedris dr. M. Ehrenbergi laboratooriumis. Uurimisteema: mutantsete ribosoomide kineetiline analüüs
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TEADUS

Alates 1987. aastast töötan dr. Jaanus Remme juhendamisel. Peamiseks huviks on olnud ribosomaalse 23S RNA osaluse selgitamine valgusünteesil, rõhuasetusega ribosoomi peptidüüli transferaasile.

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ISSN 1024–6479
ISBN 9985–56–274–7