Chapter 4

Discussion

The discussion section is divided into two main parts. The first part is about setting up conditions. In the second part, further results are discussed.

4.1 Setting up the Experimental Conditions

4.1.1 RNAse Assay

Everyone who has worked with RNA has might be affected by RNase contamination at some points. Admitting the experience of RNase contamination parallel with the experience of RNA work can make us more careful in order to handle the problem. However, the presence of RNases can be hardly avoided during routine laboratory research even though extra caution is shown with sterilizing the suspicious equipment or separating the working areas for different purposes. Bacterial cultures grown for plasmid preps or cloning and bacterial products, such as some restriction enzymes or microorganisms in the air or water supply can be sources for RNase. We, ourselves are also one of the main RNase sources with our skin and body fluids such as saliva, and tears. Besides of all these things, biological components that we isolate from cells for *in vitro* assays might not be RNase free.

Generally, it is often difficult and time-consuming to determine which solution or piece of equipment is the source of RNase contamination when the experiment is failed. The experiments that we followed in this thesis are mainly run off transcription, *in vitro* translation system and footprinting experiments via iodine cleavage, all should be performed in the absence of RNase contamination and degradation. Therefore, with the idea of pre-checking the experimental components, we developed an RNase assay in our laboratory. In this assay system, we used 5'-^{32}P labeled MF-mRNA, which

was mainly designed in order to use *in vitro* translation system (Triana-Alonso et al., 1995). The ΔG^0 of secondary structure stability of MF-mRNA was analyzed in order to minimize secondary structure that is an important prerequisite for efficient translation. Besides, nearly linear structure of MF-mRNA provided extra advantage to us to use it as a marker in our RNase assays since its open conformation is a good target for the RNase attack. For the assay radioactive labeling makes the system extremely sensitive and it is easy to follow the degradation spending very short time. Once mRNA is labeled with a specific activity around 5,000 dpm per pmol, it can be used eight to ten weeks until radioactivity decays to background levels.

With this method, we checked the RNase contamination for the each buffer or components that was used in the main experiments. The only severe contamination was observed in the EF-Tu. In order to understand whether this contamination was co-purified with EF-Tu or introduced afterwards, EF-Tu was purified again with the same procedure and each elution fraction of purification was analyzed (see results section 3.1.1). Our experimental results showed that EF-Tu is coming with RNase as a complex during the elution step. Interestingly, the purification conditions of EF-G and EF-Tu were only changing with the imidazole concentration of elution buffer (120 and 80 mM respectively), EF-G preparation was free of contamination (data was not shown). The only way to remove the RNase from the protein was achieved by one additional step namely gel-filtration via FPLC. The FPLC elution fractions were checked with the same RNase method for the contamination (see Figure 3.1.1.3). Fraction 51 was completely free from the contamination. This fraction was used for the phosphorothioate cleavage experiments that should be performed in RNase free environment to get correct footprinting results.

4.1.2 Poly(U): A troubleshooting against to a smear problem in the footprinting experiments

Poly(U) is commercially available and can be provided either from Pharmacia (under the name of Amersham now) or from Boehringer (under the name of

Roche Diagnostics now). In contrast to Pharmacia poly(U) that is longer and mostly of uniform length, Boehringer's poly(U) is shorter and present in a mixed population of different lengths. For this reason, each of them can be used for different purposes. For example, Pharmacia poly(U) is a good substrate for the poly(U)-poly(Phe) synthesis that is the better the more Phe can be incorporated into the polypeptide chain.

The elongating ribosome from *E. coli* covers a sequence of 39±3 nucleotides of the mRNA (Beyer et al., 1994) with respect to RNase protection experiments and about 30 nt according to a crystallographic analysis (Yusupova et al., 2001). Keeping this information in our minds, we used the Pharmacia poly(U) for our phosphorothioate experiments .The main aim of these experiments was the preparation of Pi complexes with the transcribed and 5'-labeled thioated deacyl-tRNAs and iodine cleavage as training for the further advanced cleavage experiments. From the very beginning, there were a lot of difficulties to get clear and sharp bands when ribosome complexes were analyzed; instead the bands were hidden in a smear (Figure 3.1.2.1), whereas tRNA in solution gave clear bands. The smear problem that we faced was due to the length of poly(U). There was clear differences in the sharpness of bands in the presence of either poly(U) (smear) containing 1000 nucleotides fragments or MF-mRNA (no smear) with a length of 46. The experiment where poly(U) was added just before loading to the gel to the samples which were prepared with MF-mRNA (Figure 3.1.2.2) confirmed our conclusion since the result was again smear. After poly(U) fractionation poly(U) with a length of 50 to 100 bases was ideal for our experiments.

4.1.3 Dipeptide bond formation

Although the main aim in this part of the experiments was to set up the conditions for some footprinting experiments, at the end we had two different achievements. Besides getting good conditions for the oscillating ribosome experiments, some new findings lead us to start a side project, which is still in progress.

The whole story in this side project started with the idea of checking the activity of newly charged [^{3}H]Val-tRNA^{Val} that was going to be used in the oscillating ribosome experiments. Although the binding efficiency of [^{3}H]Val-tRNA^{Val} to the A site was around 60%, the puromycin reaction, which is the best control for the translocation activity was bound to a 2-6% efficiency. After showing that EF-G is active and puromycin is working efficiently in other experiments, we needed to show if EF-Tu is active. It could have been one of the main reasons that it was forming ternary complex but not leaving the ribosome thereby causing to the poor puromycin reaction. Under conventional buffer conditions with low Mg^{2+} concentration, aminoacyl-tRNA binding to the A-site is EF-Tu dependent (P. Wurmbach and K. H. Nierhaus, unpublished). Taking advantage from this knowledge, EF-Tu was analyzed, and was shown to be completely active. Surprisingly, when aminoacyl-tRNAs were bound to the Pi site, instead of binding to the A site while the P site was occupied already with a deacyl-tRNA, they had higher puromycin reactivity, although Val-tRNA showed a low activity also in this assay. The reason of the poor puromycin reaction might be the poor translocation reaction in the complexes where deacyl-tRNA is in the P, and aminoacyl-tRNA is in the A site. In order to test this idea, we checked complexes, which were prepared with acetyl-aminoacyl tRNA in the P side instead of deacyl-tRNA. These complexes are very important for the oscillating ribosome experiments as well. Results were as expected: [^{3}H]Val-tRNA^{Val} in the A site formed a dipeptide with the AcPhe-tRNA as P site partner instead of deacyl-tRNA, and after translocation gave again about 100% puromycin.

In summary, all these results show that translocation efficiency with the deacyl-tRNA in the P site and aminoacyl-tRNA in the A-site is poor. One main reason might be that such complexes are not physiological as compared to *in vivo* conditions. Generally, ribosomes carry two amino-acylated tRNAs before peptide bond formation, or one deacyl-tRNA and one peptidyl-tRNA with at least two amino acid residues. Ribosomes are highly accurate during the protein synthesis and our artificial *in vitro* conditions with an unblocked amino group at the aminoacyl-tRNA at the A site and a deacylated tRNA at the P site might prevent translocation. Note that before translocation under physiological

conditions there is always a tRNA at the A site where the last amino acid is N-blocked (e.g. dipeptide or a longer chain). The high translocation efficiency in the complexes with the deacyl-tRNA on the P site and the acetyl-aminoacyl tRNA in the A site, which mimics a tRNA with a dipeptide, also supports this interpretation.

For corroboration, other tRNAs are going to be transcribed and charged. Binding efficiency and puromycin reaction will be analyzed.

4.2 One step further in understanding elongation cycle: How lazy are the tRNAs on the ribosome?

Not so long ago, about 15 to 20 years, an atomic resolution of the ribosomes was a dream, a dream out of reach the reality due to the high complexity of the structure. Today, the dream turned into the reality and one can easily download available crystal structures to the computer and view atomic structures of both subunits. Additionally, structure of the complete ribosome incorporating tRNA and mRNA has been solved at 5.5 to 7 Å resolution (Yusupov et al., 2001). The recent arrival of all these crystal structures revolutionized the field and opened a new phase in our understanding of protein synthesis. To identify and fill the gaps in our current knowledge of the mechanisms involved in translation might now be easier possible by correlating structural and biochemical data.

The ribosome has a dynamic structure and the next step in understanding translation will definitely come from elucidating ribosomal dynamics. One of the most amazing events during the elongation phase of protein synthesis is the movement of tRNAs on the ribosome, namely translocation. The molecular bases for translocation and mRNA-reading-frame maintenance are still questions standing out.

Experimental evidence for the nature of tRNA movement during the translocation was provided by footprinting techniques. Chemical modification studies are giving important preliminary answers. Two principles have been implied. The first one is base modification with reagents like kethoxal, DMS and others. The second method is the phosphorothioate technique that modifies phosphate groups by replacing a non-bridging oxygen with a sulfur atom. Interestingly, either technique has led to a model in elongation cycle. The first proposal came in 1989 by Noller and colleagues who applied base footprinting techniques (Moazed and Noller, 1989). They were proposing a creeping like movement of tRNAs on the ribosome right after peptide bond formation but before EF-G dependent translocation (see section 1.5). An alternative α–ε model for translocation has been proposed in our laboratory (Dabrowski et al., 1998). By measuring the protection by the ribosome of phosphorothioated tRNAs against iodine-mediated cleavage, it was concluded that there are movable domains on the ribosome that transport the tRNAs through the ribosome.

Both models assign a passive role to the tRNAs in the translocation process. However the degree of the passiveness is different in either model. How lazy are they? According to the hybrid side model, only a part of tRNA jumps from one site to the other on the large subunit with a creeping-like movement and the rest on the small subunit waits for an EF-G mediated push to complete the translocation. Or does tRNA prefer to sit on the movable domain of the α–ε model and wait for being transported during the elongation cycle via the ribosome?

There are uncertainties with aspects of the hybrid site model as discussed in the next paragraph, and this is also true for the α–ε model. These uncertainties will get answers when crystal structures are available of PRE and POST states, a very active field of the leading ribosome-crystallography groups. Correlating structural and biochemical data will be an important mean to uncover the mechanism of translocation. Two main uncertainty points related to the α–ε model will be considered in detail in the rest of the

discussion part: The first one is that if there is a movable domain, what does it consist of? The second one is how this movable domain moves with a tRNA from one site to the other without breaking the contact and resets back to the PRE state. Additionally, for the reasons listed below, we do believe that we are going on the right track of the way of translocation pathway by following the features of the α–ε model.

Problems of the hybrid-site model: Firstly, the hybrid site model was derived from the protection patterns of the 23S rRNA. However, the protection of 16 out of 17 bases was dependent on the A -3' or CA-3' of the universal CCA-3' terminus of the tRNAs (Moazed and Noller, 1989). It is obvious that the location of the CCA end of a tRNA at the A or P site is different depending on whether the α-amino group of the aminoacyl residue is free and positively charged, or whether it is involved in a peptide bond. Furthermore, the flexibility of the CCA end enables it to change its position although the rest of the tRNA molecule remains unaltered at the A or P site. This suggests that CCA end does not provide a reliable reporter for the site location of the tRNA molecule. On the other hand, α–ε model was derived from the protection patterns of 65 to 70 positions of a tRNA which means 80-90% direct information with respect to the 76 nt of the whole tRNA.

Secondly, the hybrid site model was derived from experiments, where a vast range of Mg^{2+} concentrations (5-25mM) was applied. However, it is well known that the binding properties and the interdependencies of the various sites are extremely sensitive to changes of Mg^{2+} concentrations (Rheinberger and Nierhaus, 1987). This sensitivity probably reflects an increasing distortion of both the ribosome and tRNA with increasing Mg^{2+} concentrations, which would expect to compromise a fine-structure analysis such as the chemical probing of the rRNA bases. On the other hand, the experiments leading to the α–ε model were performed under near *in vivo* conditions. In this work, we once more showed the importance of the buffer conditions and shed some light on the observation of a hybrid state under conditions of a conventional buffer system (section 4.2.1).

Also a systematic analysis of tRNA binding sites during elongation (Agrawal et al., 2000) did not provide any evidence for hybrid states of a tRNA during the elongation cycle. Furthermore, the crystal structure of a programmed ribosome containing three deacylated tRNAs at 5.5 Å resolution has identified deacylated tRNA at the classical P site (Yusupov et al., 2001). According to the hybrid site model a deacylated tRNA should be never present at the P site but rather at the hybrid site P/E.

At least we can state that a ribosome with a tRNA at a hybrid site is not significantly populated in a preparation of PRE and POST states of the elongation cycle. We therefore will discuss the data of this work in the frame of the α–ε model.

4.2.1 Polyamines contribute to the stability of the tRNA tertiary structure

There are two main different buffer systems that are in use in ribosomology (see Table 4.2.1). One of them, namely a system that we term "conventional buffer" system contains high concentration of Mg^{2+} (10 to 20 mM), does not contain polyamines. It deviates significantly from the physiological values. The other one, a polyamine buffer, is near to the *in vivo* conditions with a low concentration of Mg^{2+} of 3 to 6 mM and with polyamines.

We demonstrated *via* iodine cleavage of phosphorothioated tRNAs that, in solution, tRNAs adopt different conformations under polyamine and conventional buffer conditions. Under conventional buffer conditions a Mg^{2+} shift from 10 to 20 mM had no significant influence on tRNA conformation. In contrast to conventional buffers, strong protections were seen with polyamine buffer, for example, at the phosphate groups of the anticodon stem (24-26 and 39-43) and around phosphate 10 (residues 7, 8, 11,12 and 13; see Table 3.2.1.1). The results indicate that, despite the low Mg^{2+} concentration, the tRNA tertiary structure in the polyamine buffer is more compact and probably more stable than in the conventional buffer system with 10 to 20 mM Mg^{2+}. This is surprising, as the general wisdom is that RNA structure becomes more

stable at higher Mg^{2+} concentrations. Obviously polyamines not only compensate for the lower magnesium, but rather induce a more compact structure – a feature that might be related to the near *in vivo* performance of protein synthesis under polyamine conditions, in contrast to the conventional buffer systems (for review see (Nierhaus et al., 2000)).

	Concentrations (mM)				
System			Polyamines		
	Mg^{2+}	K^+, $NH4^+$	Spermidine	Spermine	Putrescine
Conventional Buffer	7-20	100	None	None	None
Polyamine Buffer	3-6	150	2	0.05	None
In vivo	~4	~150	1-4	~0.03	20

Table 4.2.1: Concentrations of ions and polyamines important for ribosomal functions (Adapted from (Nierhaus et al., 2000)).

Interestingly, the regions of increased protection in the polyamine buffer were located in the vicinity of two polyamine binding sites: In the crystal structure of yeast $tRNA^{Phe}$, besides four distinct Mg^{2+} ions, two spermine molecules could be located. The first is in the major groove at one end of the anticodon stem (phosphates 25, 26 and 41 to 43) and the second is near the variable loop and curls around phosphate 10 in a region where the polynucleotide chain takes a sharp turn (Figure 4.2.1.1) (Quigley et al., 1978). Since the differences in protection between the polyamine system and the conventional systems accumulate at the polyamine binding sites, we conclude that many of these additional protection sites are attributed to the bound polyamines, thus underlining both the impact of polyamines on RNA stabilization and the sensitivity of the phosphorothioate technique.

However, the accessibility pattern of a deacylated thioated tRNA in the P site of programmed ribosomes is almost identical under polyamine and conventional buffer conditions (Figure 4.2.1.2). Yet it is known from cryo-EM that the locations of the tRNA are strikingly different. A deacylated tRNA is found at the classical P site under polyamine conditions and at a P/E hybrid site under conventional conditions (Figure 4.2.1.1). Combination of our data with the cryo-EM finding (Figure 4.2.1.2) suggests that the ribosomal components that hold the 50S portion of the tRNA are located in a classical P site under polyamine conditions but slip into the E site position under conventional buffer conditions. This means that the result of hybrid site model experiments are most likely due to buffer conditions that are far from those present *in vivo*, but in spite of the non-physiological nature this observation might nevertheless provide some insight into the mechanism of translocation.

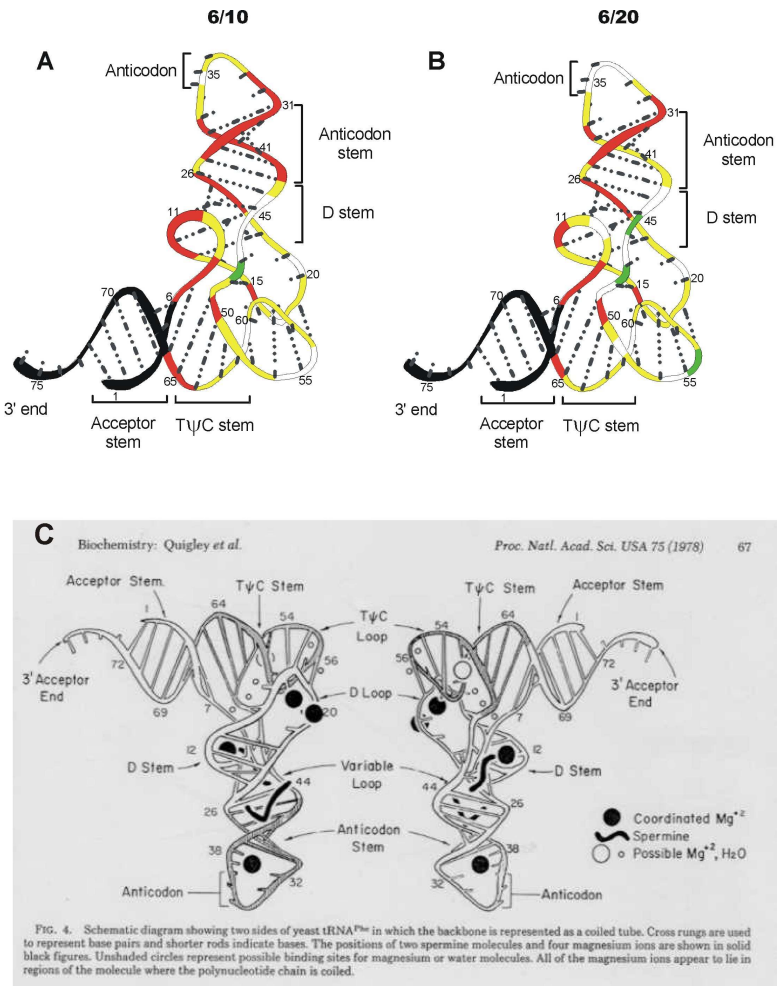

Figure 4.2.1.1: Comparison of the tRNA structure with the x-ray structure result. 6 means polyamine buffer with 6 mM Mg^{2+} concentration. 10 and 20 means conventional buffer with 10 or 20 mM Mg^{2+} concentration

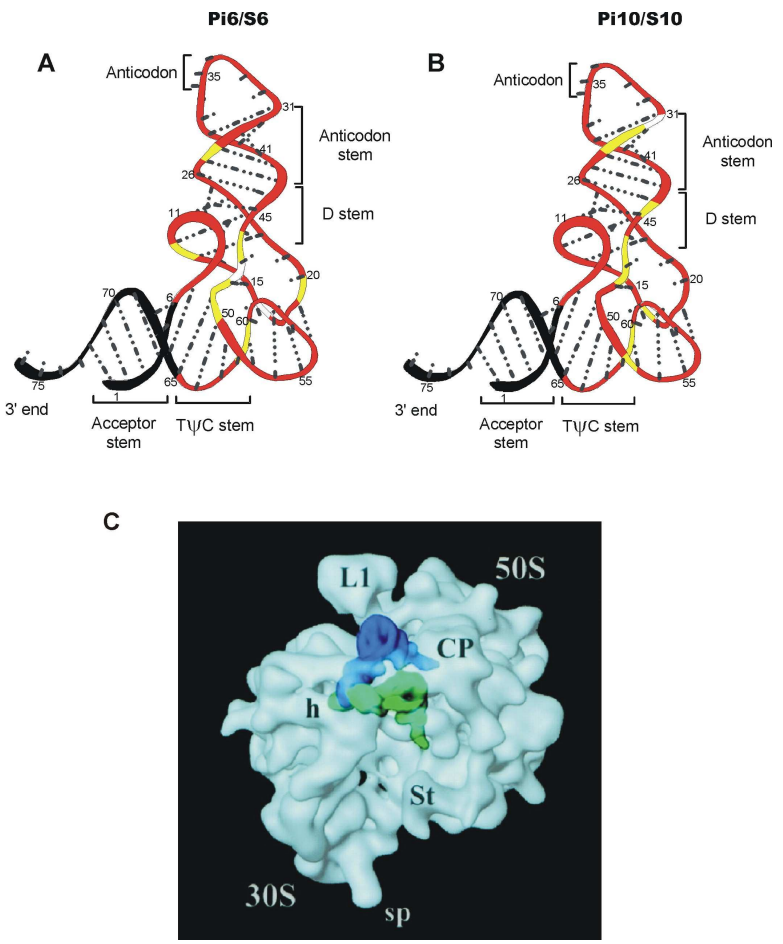

Figure 4.2.1.2: A and B) $tRNA^{Phe}$ in the Pi state under polyamine buffer or conventional buffer (10 mM Mg^{2+}) respectively. C) Cryo-EM of deacylated tRNA in different buffer conditions. Green, the classical P site seen with physiological polyamine conditions; blue, tRNA position seen under conventional buffer conditions (Agrawal et al., 1999).

4.2.2 Refining the ribosomal components responsible for fixation of a tRNA at the P site

It was shown that deacyl-tRNAs bound to the individual ribosomal subunits complement each other in a manner that the combination of both patterns as equivalent to that of a tRNA at the P site of a 70S ribosome (Schäfer, 1997; Schäfer et al., 2002). This observation allows a sharp delineation of the tRNA contact regions within the 70S ribosome that are contributed by each of the

component subunits. The small subunit contacts include the anticodon loop and the first two base pairs of the adjacent anticodon stem (positions 30±1 to 43±1), whereas the remaining 85% of the tRNA is in contact with the large subunit (Figure 3.3.2.1). In this thesis, the contribution of each subunit to the overall distribution of contacts made with the P site tRNA was analyzed in the available 5.5 Å map of the tRNA·70S complex. Schäfer's data(Yusupov et al., 2001) are in remarkable concordance with the 5.5 Å map (see Table 3 in (Yusupov et al., 2001)) and also with the mimic of an anticodon-stem loop structure in the crystal of 30S subunit (Carter et al., 2000). Figure 3.3.2.1 illustrates the excellent agreement of Schäfer's data with the 5.5 Å map of a 70S complex. The 30S protections of the anticodon stem-loop structure (positions 30±1 to 41±1 in yellow) are located exclusively in the neighborhood of 30S components (blue and green) of the 70S map, whereas the remaining portion of the tRNA (red) lies within the domain of the 50S subunits (yellow and orange).

Furthermore, we have analyzed a number of strong protections that are common between two different species of elongator tRNA, *viz.* $tRNA^{Phe}$ and $tRNA^{Met}$ as mentioned in Schäfer's thesis (Schäfer, 1997). We tested whether these phosphates may represent strategic fixation points for a deacyl- tRNA at the P site. If so one could expect conservation of the tRNA bases adjacent to these phosphates and conservation of the neighboring ribosomal components. Indeed this is the case: Eight out of ten strong protections are adjacent to conserved bases of the tRNA (Table 3.3.1.1). In this work a detailed inspection of the 5.5 Å map of the 70S complex revealed that the ribosomal components neighboring the 10 tRNA bases are remarkably conserved (Table 3.3.1.2). The rRNA bases are conserved in >95% bacteria and 80-100% across all three phylogenetic domains. Furthermore, a number of conserved positions of ribosomal proteins that neighbor these phosphates were identified. For example, position 120 (*E. coli* numbering) of S13 is always a Lys or an Arg residue and lies next to tRNA base G30, a highly conserved Arg128 of S9 protein neighbors tRNA base Y32 and positions Arg/Lys56 and Arg/Lys64 of L5 are in close proximity to C56. This further

corroborates the suggestion that the identified tRNA nucleotides are of strategic importance for tRNA fixation at the P site.

4.2.3 Are uncertainties of the α-ε model really so uncertain?

At the beginning of the discussion part, some possible uncertainties about the α–ε model were mentioned. The questions were: if there is a movable domain, what does it consist of and how this movable domain moves with a tRNA from one site to the other without breaking the contact and reset back to the PRE state. Now, it is time to return to these doubts and try to make clearer as much as possible under the light of data given in this thesis and also some other data present in the literature.

Although the protection experiments present a ´static´ picture of the ribosome, which is exemplified by the identification of fixation of the P site tRNA as described in the previous section, a comparison of protection patterns under different conditions and between different states enables an interpretation of some dynamic features of the ribosome.

Firstly, as it was shown in Schäfer's thesis (Schäfer, 1997) and analyzed in more detail with the help of available crystal data in this thesis, the protection patterns seen with isolated 30S and the 50S subunits could be combined to reconstruct the P-site pattern of 70S ribosomes. Isolated 30S subunits have a single binding site, the prospective P site after association with the large subunit, as demonstrated with binding experiments (Gnirke and Nierhaus, 1986) and *via* the toeprinting method (Hartz et al., 1989). In contrast, isolated *E. coli* 50S subunits bind exclusively deacylated tRNA to the E site and have no available P or A site (Gnirke and Nierhaus, 1986; Kirillov et al., 1983). The fact that the 50S E site pattern is practically the same as the 50S part of the 70S P site pattern seems to be reminiscent of a P/E hybrid site of the hybrid-site model for elongation, where the tRNAs are thought to creep through intermediary hybrid sites (A/P and P/E) before arriving, after translocation, at the classical P and E sites (Moazed and Noller, 1989; Nierhaus, 1996).

However, the similarity with a hybrid site does not hold here, since the 70S P site pattern was obtained under polyamine-buffer conditions, where the tRNA is found at the canonical P site (Agrawal et al., 1999).

We note that a similar conservation in protection patterns was observed between pre- and post-translocation complexes (PRE and POST respectively), with a deacylated tRNA at the P site in the PRE state and at the E site in the POST state. This led to the α-ε model for the ribosomal elongation cycle (reviewed in (Nierhaus et al., 2000)), which proposes the existence of a movable domain that binds and guides tRNAs during translocation. The movable domain contains two binding regions α and ε, each of which bind a tRNA with a characteristic protection pattern. During translocation the α-region carries a tRNA from the A to the P site and the ε-region a tRNA from the P to the E site. The protection pattern of a tRNA bound to isolated 50S subunits (E site) is the same as that of the 50S part of the 70S P site can be interpreted in the frame of the α-ε model to suggest that the ε-part of the mobile tRNA carrier is at the E site in isolated 50S subunits, but ´swings´ into the P site upon association with the 30S subunit forming 70S ribosomes.

Secondly, it is worthy to emphasis that when the protection pattern of a P-site tRNA bound to a non-programmed 70S ribosome (i.e. no mRNA, or noncognate mRNA) was assessed, as shown in the section 3.2.3, to our surprise the 30S subunit did not contribute to the protection pattern at all (Figure 3.2.2.2). On the other hand, the contact pattern of a tRNA bound to 70S ribosome in the absence mRNA is almost identical to that seen with the isolated 50S subunit (Schäfer et al., 2002). This result suggests that codon-anticodon interaction at the ribosomal P site of 70S ribosomes is essential for 30S contacts and that the additional 30S contacts, those outside of the anticodon, are not available in the absence of codon-anticodon interaction. This finding agrees with and extends a previous observation that 30S subunits in the absence of mRNA do not bind any tRNA at Mg^{2+} concentrations that are well suited for protein synthesis (Gnirke and Nierhaus, 1986). Furthermore,

this result implies that the 30S subunit undergoes a conformation change upon codon-anticodon interaction resulting in additional contacts which further stabilize the P site tRNA.

Thirdly, the accessibility pattern of a deacylated thioated tRNA in the P site of programmed ribosomes is almost identical under polyamine and conventional buffer conditions. Yet it is known from cryo-electron microscopy that the locations of the tRNA are strikingly different, *viz.* a deacylated tRNA is found at a classical P site under polyamine conditions and at a P/E hybrid site under conventional conditions (Agrawal et al., 2000). This suggests that the ribosomal components that hold the 50S portion of the tRNA are located in a classical P site under polyamine conditions but slip into the E site position under conventional buffer conditions. The physiological relevance of the latter finding is immediately compromised by the buffer conditions themselves, i.e. their non-physiological nature (see (Nierhaus et al., 2000) for discussion), but may nevertheless provide some insight into the mechanism of translocation.

In the frame of the α-ε model, the conservation of protection patterns between the P and P/E sites suggests that under conventional buffer conditions the ε module of the movable domain has slipped into the E site on the 50S subunit. This situation induced by non-physiological buffer conditions may seem again reminiscent to a hybrid-site. However, the hybrid site model does not propose a movable domain and thus would predict alternative patterns for P/P and P/E sites.

Since the protection patterns encompass the entire tRNA, from the anticodon loop to the acceptor stem, a contiguous structure spanning the intersubunit space, from the decoding center to the peptidyl-transferase center, should exist. A potential structure has been identified, termed bridge B2a, in 70S ribosomes (Gabashvili et al., 2000). A major component of bridge B2a is the universally conserved stem-loop of H69 of 23S rRNA, which has been proposed to undergo conformational change upon subunit association, enabling it to bridge the intersubunit space and to make contacts with both A

and P site tRNAs (Harms et al., 2001). Other candidates for the movable domain include the upper region of the h44 of the 16S rRNA (VanLoock et al., 2000) and parts of the ribosomal protein L2 (Diedrich et al., 2000; Willumeit et al., 2001).

4.2.4 The facts and the elongation models: A critical assessment

Let us compile the whole translocation story. Translocation process, following peptidyl transferase step, prepares the ribosome for a new round of peptide chain elongation. The functional challenge is that the very ends of the 75 Å long tRNAs have to be moved in a precise manner: The anticodon has to be moved precisely on the 30S subunit in order to maintain the reading frame, and the CCA-end of the peptidyl-tRNA has to be moved precisely in order to position its 3'-end into the PTF center of the 50S subunit and thus allow the next peptide bond.

Starting with Bretscher's theoretical paper in 1968 (Bretscher, 1968) many suggestions were made and proposed concerning this fascinating step of the elongation cycle. In spite of a fairly complete understanding of the ribosome structure, we are still far from a solution and the molecular mechanism of translocation. Here we will try to sort out and to reconcile the facts; mainly footprinting data and structural data obtained by both cryo-EM and X-ray crystallography studies.

Two models of the translocation process which were raised by different footprinting experiments, namely the hybrid side model and the α-ε model, are getting their awards and punishments from the structural data. Dynamical structure of ribosome is causing conformational changes during the translocation. It is the declared aim to make a movie showing the continuous and complete process. Up to now we are limited with the structural results of PRE and POST complexes seen in the cryo-EM. Suffice to say that we begin to fill the empty spaces between the two ending points (i.e. PRE and POST complexes) with known data.

Agrawal et al. showed the tRNA orientations in the PRE and POST states in a cryo EM study (see Figure 4.2.4.1A) (Agrawal et al., 2000). These results brings new structural aspects to the α-ε model. The α-ε model suggests that the movement of the tRNAs occurs simultaneously on both large and small subunits in a coordinated fashion. The cryo-EM reconstructions show that the tRNAs in the PRE and POST states display a similar mutual arrangement relative to each other (the angles between the tRNAs are 39° and 35° in the PRE and POST state respectively (Agrawal et al., 2000)), but that also the relative positions of the CCA ends change dramatically (Figure 4.2.3.1A). Prior to translocation the CCA-ends of the two tRNAs present at A and P sites are directly adjacent at the peptidyl-transferase center - an obvious requirement for peptide-bond formation. Following translocation the CCA-ends are separated by over 50 Å (Agrawal et al., 2000; Yusupov et al., 2001) – after formation of the peptide bond there is no need to keep the CCA-ends together. These findings indicate that the postulated α region and ε region do not move strictly side-by-side during translocation, but separate during or after translocation (Figure 4.2.3.1B).

The cryo-EM results of Agrawal et al. provide some explanations for the hybrid side model as well. According to the their results the overall orientation of the E-site tRNA is parallel to that of an earlier identified P/E state tRNA (see Figure 4.2.3.1A; (Agrawal et al., 1999)). However, a P/E hybrid state for deacylated tRNA in the P site has been seen only under conventional buffer conditions, under polyamine buffer the tRNA was still in the P site (see Figure 4.2.3.1A). Our footprinting data showed that the protection pattern of deacyl-tRNA in the P site is the same under either buffer condition. These results clearly indicate that α and ε regions of the ribosomal tRNA carrier are not strictly coupled but can move partially independent from each other: They are parallel to each other in PRE state and move during the translocation state into the POST state where the CCA ends are separated by 50 Å whereas the anticodon region sticks together allowing simultaneously codon-anticodon interaction in

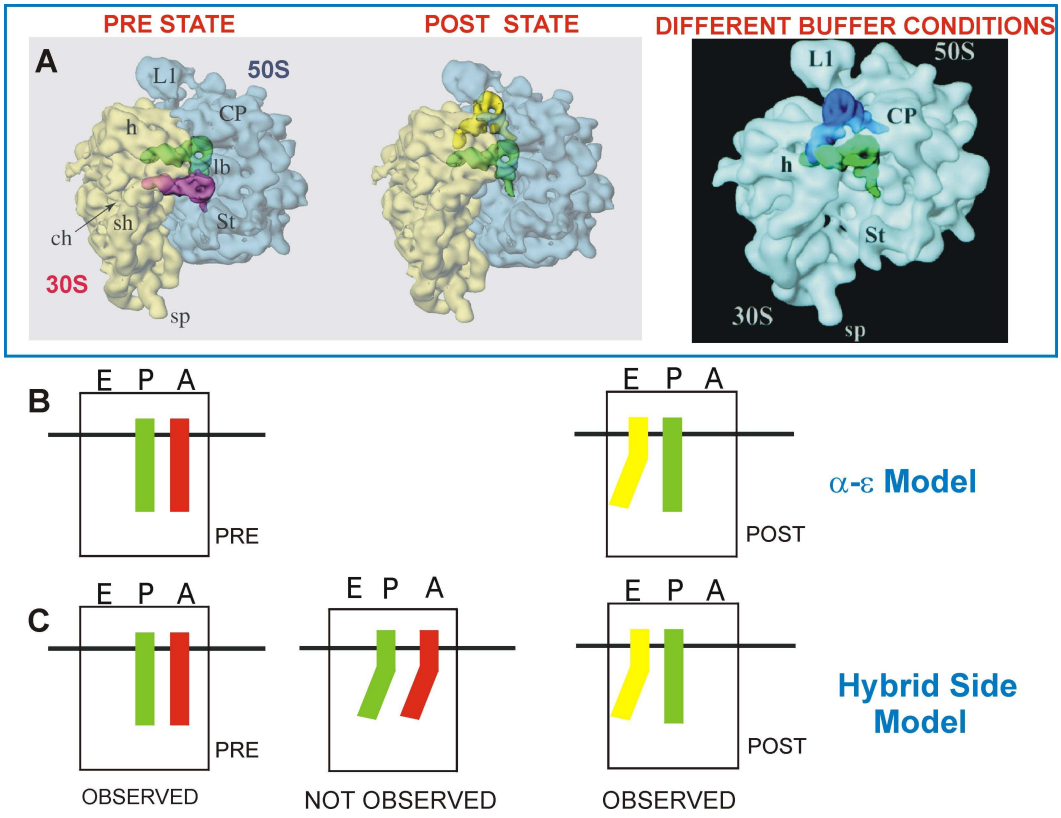

Figure 4.2.3.1: **A)** Left side: The crystal structure of $tRNA^{Phe}$ is placed into the positions corresponding to A (pink), P (green) and E (yellow) sites (Agrawal et al., 2000). Right side: The position of deacyl-tRNA in different buffer conditions. Green is in polyamine buffer and blue is in the conventional buffer (Agrawal et al., 1999). B) Movements of the tRNAs according to the α-ε model. C) Movements of the tRNAs according to the hybrid side model.

the POST state. When a deacylated tRNA is alone at the P site under polyamine buffer conditions, it is in the classical P site, but under conventional buffer conditions the ε-region of the tRNA carrier is shifted in a way as we see it in the E site after translocation. Therefore, it does not seem justified to call this buffer induced premature shifting a “hybrid site”.

The hybrid-site model is based protection patterns of the 23S rRNA. After peptide-bond formation the 16S rRNA protections due to the bound tRNAs did not change, whereas the 23S rRNA protections shifted to those of the adjacent binding site, i.e. the A site pattern shifted to that of the P site pattern

and likewise the P site pattern to that of the E site. However, the protections of 16 out of 17 bases were dependent on the A -3' or CA-3' of the universal CCA-3' terminus of the tRNAs (Moazed and Noller, 1989). In other words, the base protection data that postulate an A/P hybrid mainly come from the CCA end of tRNA. We do not think that the single-stranded ACCA-3' ends of the tRNAs are a reliable reporter structure for the location of a tRNA. But taking this opinion aside, are there other evidences for a shift of the CCA ends?

Cryo-EM structure shows that there is only a slight movement of the CCA end towards the P site from the A site immediately after the peptidyl transferase if at all. Additionally, a recent crystal structure of the 50S shows that the CCA ends do not move after peptidyl transferase, the CCA-ligands of the PTF center remain fixed to the same 23S rRNA elements (Schmeing et al., 2002). In other words, even a movement of the CCA ends is not supported by available structural data at the moment.

4.2.5 Are the elongation factors good helpers of the lazy tRNAs: One step further to clarify the uncertainties

Elongation factors EF-Tu and EF-G play a crucial role in the ribosomal elongation cycle. EF-Tu with bound GTP has a relatively high affinity for aminoacyl-tRNA^{bulk}. The binding of aminoacyl-tRNA to the A site of the ribosome is promoted by EF-Tu as a stable ternary complex of aa-tRNA·EF-Tu·GTP. On the other hand, EF-G stimulate tRNA translocation, and by shifting the tRNA from A and P sites to P and E sites, respectively, it produces a vacant A site for the next aa-tRNA·EF-Tu·GTP to bind.

The crystal structure of EF-Tu has been determined from several species. It has three domains: an N-terminal guanine nucleotide-binding domain and two smaller β-sheet domains. The protein has a relatively open conformation when GDP is its ligand (Figure 4.2.4.1a). When GTP is bound EF-Tu gets a compact conformation and can now bind a tRNA as shown in Figure 4.2.4.1.b. EF-G consists of five structural domains (Czworkowski et al., 1994).

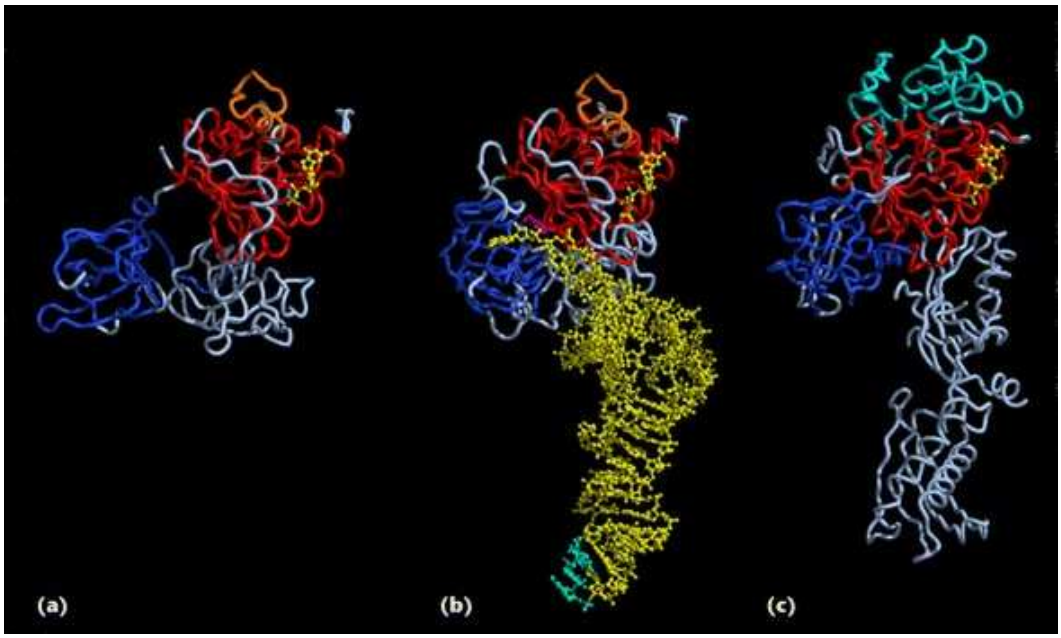

Figure 4.2.4.1: Crystal structures of EF-Tu, EF-Tu·aa-tRNA and EF-G. (a) *Thermus aquaticus* EF-Tu·GDP (Protein Data Base (PDB) entry 1TUI, Polekhina et al., 1996). (b) The complex of *T. aquaticus* EF-Tu, GMPPNP (a GTP analogue) and yeast phenylalanyl-$tRNA^{Phe}$ (PDB entry 1TTT, Nissen et al., 1995). (c) *Thermus thermophilus* EF-G·GDP (PDB entry 1DAR, al-Karadaghi et al., 1996). Proteins are portrayed as a tube representing the polypeptide backbone; effector nucleotides and aminoacyl transfer ribonucleic acid (aa-tRNA) are shown as all-atom ball-and-stick. Structurally similar residues of the G domains (on which the structures are aligned) are coloured red, structurally similar residues of domains 2 are blue, the G′ subdomain of EF-G is cyan, the G″ insertion of EF-Tu is orange and the rest of the protein is grey. The guanine nucleotides bound to the G domains are yellow, as is the aa-tRNA, except that the amino acid of the tRNA is magenta and the three anticodon nucleotides are green. (Both figures were made with the program MidasPlus from the Computer Graphics Laboratory, University of California, San Francisco; taken from http://www.els.net/els/, doi: 10.1038/npg.els.0000546).

It is interesting that the overall shape and charge distribution of EF-G is very similar to that of the aa-tRNA·EF-Tu·GTP ternary complex (Figure 4.2.4.1) (Nissen et al., 1995). Most remarkably, domains III, IV and V of EF-G mimic the tRNA structure in the EF-Tu ternary complex and domain IV can be related to the anticodon arm of tRNA.

Strikingly, EF-Tu and EF-G have significant properties in common. They bind in a mutually exclusive manner to overlapping sites on the ribosome. The position of the EF-G in the ribosome is similar to the location of the EF-Tu ternary complex observed by cryo-EM (Stark et al., 1997). Domain V of the EF-G interacts with the 50S subunit near the base of the L7/L12 stalk, whereas domain I (G domain) contacts the sarcin-ricin region. Interestingly, domain IV of EF-G reaches into the decoding region of the 30S subunit. The G domain of EF-Tu is near the sarcin-ricin region of the ribosome, and the anticodon arm of tRNA in the ternary complex is in the 30S decoding region.

These similarities of both factors concerning size, shape and orientation on the ribosome raise the question whether they use a similar mechanism to perform different tasks on the ribosome. A particular interesting problem is the question to which extent domain IV of EF-G is an important element for triggering the translocation reaction. In order to answer the latter question some groups prepared truncated variants of EF-G. One of the interesting researches is coming from Gudkov group, where they prepared two truncated EF-G proteins (Martemyanov and Gudkov, 1999). In the protein Val-481 and Pro-604, as well as Asp-492 and Pro-599, are spatially close in the EF-G tertiary structure. The residues between these neighboring amino acids were deleted and two truncated variants were prepared while keeping the stability of the protein. They demonstrated that ribosomal complex formation with EF-G and uncleavable GTP analog (GMPPNP) is decreased in the presence of truncated proteins. They also showed that truncated proteins are not active in the poly(U) directed translation systems, since they have lost the translocase function.

Their analysis led to the following conclusion: Domain IV is not involved in GTPase activity of EF-G. This domain contributes to binding to the ribosome and is strictly required for translocation. The proposed mechanism is that EF-G mimics the anticodon arm of tRNA and interacts with the A-site tRNA and the 30S subunit and, by its movement into the acceptor site, could stimulate the tRNA displacement together with the mRNA movement. The finding about the crucial role of domain IV for the translocation was also described by

Wintermeyer et al. from similar experiments (Rodnina et al., 1997). However, in contrast to Gudkov and colleagues the Wintermeyer group concluded that this domain is also involved in the GTPase activity.

Gudkov and coworkers assume that the different results of the Wintermeyer and the Gudkov groups might be related to the difficulties with respect to the solubility of the EF-G fragments, since Wintermeyer's group had constructed and analyzed *E. coli* fragments that had to be renatured *via* a transient urea dialysis in order to keep them in solution, whereas the Gudkov group used fragments derived from *Thermus thermophilus* EF-G that had no solubility problems.

All these ideas are developed in the framework of a passive movement of the tRNAs that is generated by the ribosome and is coupled to a coordinated movement of the mRNA that is linked to the tRNAs *via* two adjacent codon-anticodon interactions. We see that the idea of "lazy tRNAs" in the process of translocation is on the way to an accepted concept of of the scientific community.

Conclusions

The conformation of a tRNA in solution depends on the buffer conditions. Under *in vivo* near conditions (polyamine buffer) the conformation differs from that observed under conventional buffer systems regardless of whether the [Mg^{2+}] is 10 or 20 mM. However, the buffer systems have only little influence on the accessibility of the tRNA phosphates if the tRNA is bound to the P site. An analysis of our findings led to the following conclusions: (1) A comparison of the contact patterns of two different elongator tRNAs at the P site of programmed 70S ribosome identified ten common and highly protected sites that might be of strategic importance for the fixation of a tRNA at the P site. (2) The accessibility or contact patterns of the tRNAs with isolated subunits in the presence of mRNA can be combined to produce the pattern seen at the P site of 70S ribosomes thus allowing a sharp delineation of the regions of a tRNA in contact with the 30S and 50S subunits within the programmed 70S

ribosome. (3) The contact pattern of non-programmed 70S ribosomes is almost identical to that of isolated 50S subunits indicating that codon-anticodon interaction at the P site is required for 30S contacts with the tRNA. (4) On the basis of our results we propose the following scheme for conformational rearrangements within 70S ribosomes upon subunit association and P site tRNA binding: (i) Upon association of the ribosomal subunits forming the 70S ribosome the tRNA carrier of the 50S subunit shifts from the E site to the P site. (ii) Binding a tRNA to the P site in the presence of mRNA establishes codon-anticodon interaction. This in turn induces a conformational change of the 30S subunit that allows further stabilizing interaction with this subunit, in addition to those already existing with the 50S subunit - both of which may be important for subsequent translocation. The unchanged protection patterns of a deacylated tRNA at the P site under various buffer conditions and the different location of a corresponding tRNA seen in cryo-EM provides insight into the translocation mode, agrees well with the α-ε model but together with other findings does not support the hybrid-site model.