CHAPTER 4: Discussion

4.1 RNase method

For many years, protein translation *in vitro* has been used to study the expression of proteins and the factors involved, i.e., ribosome, initiation factors, elongation factors, etc. One of the most important agents that can reduce the efficiency of translation systems are RNases. The primary targets for contaminating RNases are mRNAs. This is particularly true for our designed mRNAs, since one criterion for our constructs is the absence of significant secondary structure. This feature increases the efficiency of mRNA translation *in vitro* (Jermutus *et al*., 1998), but on the other hand it makes the mRNA extremely sensitive against the attack of RNases.

Although we used RNases deficient strains, e.g. CAN20-12E with a number of knocked out RNases (RNase BN^-, II^-, D^-, I^-), some fraction derived from this strain such as a high-salt-wash protein fraction (HSWP) still contained significant amounts of RNases.

One way to overcome the problem is modification of mRNAs to make them more insensitive against RNases, e.g. modifications at the 5' and 3' of the mRNA have been demonstrated to improve the stability of the template (Lamla and Erdmann, 2001) similar to the capping of mRNAs in eukaryotes. However, we followed a different strategy. We tried to prepare all components for an *in vitro* system in a way that they did not contain appreciable amounts of RNases. To this end, an RNase test had to be developed. We took one of our mRNAs (MF-mRNA) and labeled it at the 5'-end with [^{32}P]. Traces of RNases will degrade this labeled mRNA easily indicated by a reduction of the labeled mRNA in an RNA gel.

Since the RF2 mRNAs designed in this study were free of a convoluted secondary structure (Figure 3.2, pages: 73-75), they were target for an RNases attack. In all translational assays re-associated 70S were programmed with both $(UUC)_{12}$ containing mRNAs and primed with labeled N-AcPhe-$tRNA^{Phe}$. Translational reactions were performed, and the incorporation into the polypeptide chain of different radioactive amino acids per primed ribosome was evaluated by hot TCA precipitation. The *in vitro* translation system developed in this work used only components that were free of RNases. We had to replace the classical *E. coli* energy

regeneration system pyruvate-kinase/phosphoenolpyruvate (PK-PEP) by acethyl phosphate (Ryabova *et al*., 1995), since some preparations of commercially available pyruvate kinase was contaminated with RNases. The free energy of hydrolysis of acethyl phosphate ($\Delta G°' = -10.3$) is lower than that of PEP ($\Delta G°' = -14.8$ kcal/mol), but still very high (Stryer, 1999).

4.2 In vitro translation system

It is already known that in leaderless mRNAs 70S ribosomes initiate translation at the 5' end of the template quite efficiently (Balakin *et al*., 1992; Moll and Bläsi, 2002). Furthermore, tightly coupled ribosomes translating homopolymeric poly(U) mRNA have been shown to start the translation at the 5' end (Bartezko and Nierhaus, unpublished results). Taking advantage of this fact and, considering that a canonical 30S type initiation could not be performed because crude initiation factor preparation were contaminated with RNases, the idea that ribosomes start protein synthesis efficiently in an elongation mode attracted our attention.

We found in this study that a $(UUC)_{12}$ sequence that does not contain secondary structure (Figure 4.1) can substituted, to some extent, the ribosome binding site. Messengers designed with a $(UUC)_{12}$ sequence in front of a heteropolymeric region were effectively translated by the ribosomes. The ribosomal active fraction was considerable higher than normal messengers including the classical ribosomal binding site (SD sequence) and initiator codon. Additionally, the $(UUC)_{12}$ sequence allowed us to prime ribosomes with a peptidyl-tRNA analogue (N-AcPhe-$tRNA^{Phe}$).

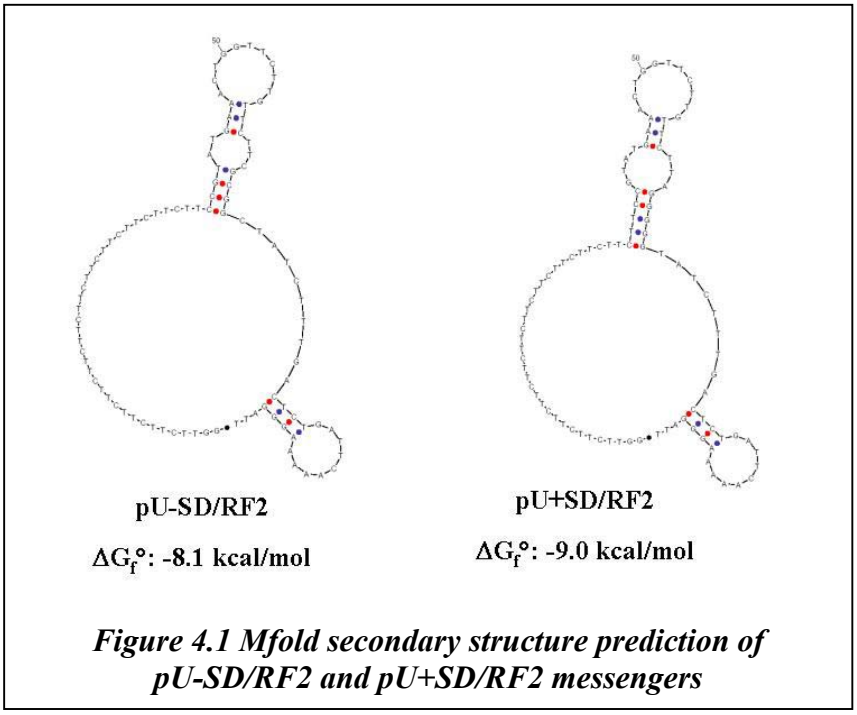

Figure 4.1 Mfold secondary structure prediction of pU-SD/RF2 and pU+SD/RF2 messengers

Our results showed that once 70S ribosomes started translation in the homopolymeric region, they translated downstream into the heteropolymeric region all along the mRNA until the stop codon.

Total translation of the heteropolymeric region of RF2 mRNAs model was strictly dependent on N-AcPhe-tRNA^{Phe}. These results are in agreement with those found by Moll et. al. (Moll *et al*., 2002) and Grill et. al. (Grill *et al*., 2000), where translation initiation of leaderless mRNAs required a 70S-ribosome-tRNA initiator complex in order to recognize the 5' terminal start codon. But on the other hand, the finding was unexpected that some translation was found when the ribosomes where only primed with AcPhe-tRNA but lacking Phe. According to the poly(U) dependent poly(Phe) synthesis on long poly(U), the polypeptide synthesis can occur without priming ribosomes with AcPhe (Bartetzko and Nierhaus, 1988).

We envisage three possible explanations: (I) it could possible that ribosomes programmed with AcPhe might "slide" on the homopolymeric region of the RF2 mRNAs model until they found the heteropolymeric region of these messengers. (II) Another possibility could be that in the absence of RF2 recycling of ribosomes could have occurred without binding of AcPhe-tRNA, whereas a correct RF2 function might improve the accuracy of translation thus preventing a re-initiation without priming the ribosomes with AcPhe-tRNA. Sliding events in the presence of AcPhe-tRNA would be a new feature of the 70S-re-associated ribosomes not studied or not demonstrated until now. In the same way that re-associated 70S ribosomes bind more efficiently AcPhe-tRNA than 70S tight couples (Triana-Alonso *et al*., 2000), this sliding possibility cannot be excluded. (III) Another possibility to explain these results could be that ''trickle'' charging could occur on tRNA^{Phe} present in the translational reactions (from tRNA^{bulk}), because some proteins in the S-100 might be degraded during translational incubation providing the reaction mixture with amino acids.

We did not follow up these observations since the central topic of our project, namely the analysis for the frameshift mechanism in the translation of the RF2 mRNA, had to be followed with priority. However, we suggest here an experiment that can test whether or not re-associated 70S can scan along the mRNA primed with AcPhe-tRNA. For example repeat the same experiments like the described translational reactions, but rather using S-100 pre-charged tRNAs and purified EF-Tu and EF-G should be used. The pre-charged tRNAs should correspond to the heteropolymeric part ± Phe-tRNA. If aa-tRNAs in the absence of Phe-tRNA could be

incorporated into a chain primed with AcPhe, a sliding would have been demonstrated.

The low secondary structure of the 5' terminal $(UUC)_{12}$ template guarantees a high proportion of ribosomes engaged in the protein synthesis, since 100 % of them were primed with N-AcPhe-$tRNA^{Phe}$ in the binding or pre-incubation reaction. However, more than 50% of the initial binding was lost after mixing the binding reaction with the charging mix. It is possible that although we are working at near *in vivo* conditions with respect to the final ionic concentration, a loss of about 50% of the initial active fraction may be an intrinsic consequence of an *in vitro* translation system that is still not properly reflecting *in vivo* conditions. It is already known that during translation of poly(U) mRNA a mass abortion of peptidyl-tRNAs from ribosomes occurred at the level of $\leq$3 incorporated Phe residues. A straightforward explanation is that at least a minimal length of a tri-peptide is necessary to be inserted into the ribosomal tunnel (Rheinberger and Nierhaus, 1990), and that AcPhe primed poly(Phe) chains do not represent a standard nascent peptide chain.

An U-rich region located at the 5' end of the start codon has been identified in several *E. coli* genes (Boni *et al*., 1991). One of them is the *rnd* gene, which encodes the tRNA processing enzyme RNase D. The sequence and proposed structure of *rnd* gene comprises a stem-loop followed by a U_8 sequence, the SD region and the start codon UUG (Zhang and Deutscher, 1992). Deletion of the stem-loop and uridine residues eliminated almost completely the RNase D expression (Zhang and Deutscher, 1989). Although a SD sequence is present in this gene a few nucleotides downstream, another set of experiments revealed that the sequence of uridine was essential for expression of the *rnd* gene *in vivo* and for binding of its mRNA to ribosomes *in vitro* (Zhang and Deutscher, 1992). It is possible that in this case an U-rich region may only become necessary for mRNAs that contain a weaker initiation codons (e.g. GUG instead of AUG) or a weaker SD sequence. Nevertheless, our results showed that an U-rich region could replace the SD sequence promoting a 70S type initiation instead of the classical 30S type.

In agreement with this general observation, the mRNA "catcher" S1 is known to favor interactions with pyrimidine-rich sequences (Subramanian, 1983; Subramanian, 1984). Taking together these finding and our results, we can suggest that the affinity of S1 for the U-rich region plays an important role in the interaction of $(UUC)_{12}$ containing mRNAs with the 70S-tRNA ''initiator'' complex.

In eukaryotes, U-rich elements are also found, but only at the 3' untranslated regions as part of AU rich segments (ARE). Class III ARE contains a U rich region differing from the others two (I and II) in which the consensus sequence AUUUA is observed (Mignone *et al*., 2002). It is not astonishing that not only the location of the U-rich region is different in prokaryotes and eukaryotes, but the function as well. In eukaryotes, the U-rich region is a signal for the mRNA degradation.

On the other hand, perhaps the scanning mechanism found in the initiation of protein synthesis in eukaryotes, could be an evolutionary relict from prokaryotes, where an U-rich region at the 5' end of mRNA could play a similar role for scanning as proposed by our experiments with 70S ribosomes *in vitro* in the presence of $(UUC)_{12}$ containing mRNAs.

Another feature of our *in vitro* translation system developed in this work is its simplicity. Several approaches based on purified translational factors and partially purified mixture of aminoacyl-tRNA synthetases (Ganoza *et al*., 1985; Kung *et al*., 1977; Pavlov and Ehrenberg, 1996; Pavlov *et al*., 1997) have been performed. More recently a cell-free translation system (PURE for protein synthesis using recombinant elements) was reconstituted with highly purified translational components (Shimizu *et al*., 2001). Although the latter system was able to produce protein at a rate of about 160 µg/ml/h in a batch mode, the fact that it depends on all 31 components makes it prohibitively difficult to find a mistake, if the system fails. In this respect, our system is easier to optimize because the content of elongation factors and aminoacyl-tRNA synthetases in the S-100 fraction freed of tRNA is more or less constant from preparation to preparation and reflects the optimal relative amounts of the various components in the cell. Additionally, our system is very ''flexible'' in the sense that it allows the involvement of other factors added exogenously, e.g., release factor 2.

An essential feature of the developed system is the fact that termination of protein synthesis occurs efficiently and thus could be easily studied. We found that the exogenous addition of RF2 (T246A) to our *in vitro* translation system resulted in a very efficient hydrolysis of the nascent peptide chain from the peptidyl-tRNA. Sucrose gradients of ribosomal complexes with the oligopeptide bound showed that after the addition of RF2 almost 70% of the peptidyl-tRNA was hydrolyzed and released from the ribosomes when pU-SD/RF2 mRNA was used. Less hydrolysis (30%) was found in the case of pU+SD/RF2 (Figure 3.15; page 94). This result is a consequence of

the fact that in the latter mRNA the SD sequence fosters ribosomes to move +1 nucleotide downstream even in absence of aspartic acid in the amino acid mixture.

The RF2 protein used was derived from an *E .coli* strain K12. In this strain a threonine residue is located at position 246 of RF2, whereas it is a conserved alanine or serine in other bacterial sequences including other strains of *E. coli* (Dincbas-Renqvist *et al*., 2000). Over-expression of wild type RF2 from *E. coli* strain K12 is toxic, but cell viability is restored, if a threonine 246 is mutated for alanine (Uno *et al*., 1996) or serine (Wilson *et al*., 2000).

Some decades ago a termination systems that is far away from the physiological conditions has been reported. It is based on the RF-mediate release of f[^{3}H]]Met-tRNA$_f^{Met}$ from the ribosomal P site (Tate and Caskey, 1990). The substrate for this assay, f[^{3}H]Met-tRNA$_f^{Met}$-AUG-ribosome complex, is formed by incubating the ribosomes with an extremely high specific activity of f[^{3}H]Met-tRNA$_f^{Met}$ (100,000 cpm/pmol) and about 50-times excess of AUG triplet codons per ribosome. f[^{3}H]Met release is determined after the addition, in a second incubation, of a stop codon triplet and release factors (codon-directed) or ethanol (codon-independent). The hydrolyzed fMet is extracted by ethyl-acetate (Brown *et al*., 1990). Some criticisms of this system: (1) the high specific activity of f[^{3}H]Met-tRNA$_f^{Met}$ can provoke a high radioactive background; (2) the conditions of this system are far from those found *in vivo*, since ribosomes are never programmed with two separated codons. (3) Conventional buffers are used that are far from the *in vivo* ionic conditions. (4) Experimental artifacts could be generated upon the addition of ethanol (30% final concentration) in order to stabilize the complex, to improve the interaction of the factor with the ribosome's functional center and to promote peptidyl-tRNA hydrolysis (Tate and Caskey, 1990).

The second *in vitro* translation system is that developed by the Ehrenberg group (Zavialov *et al*., 2001), who used highly purified translational components. This system is very efficient no only in the termination of protein synthesis but also in post-termination studies.

We demonstrate here for the first time an authentic termination complex with a polypeptidyl-tRNA containing 24 amino acids and carrying a deacylated tRNA at the E site (Figure 3.15, page: 94). After the action of RF2 (release of the peptidyl residue; Figure 3.15, page: 94) the ribosome complex contains deacylated tRNAs at the P

and E sites. Further studies are planned in the group to demonstrate when the tRNAs at the P and E sites are released.

4.3 Di-peptide formation

We demonstrate here that empty ribosomes do not synthesizes di-peptides (Figure 3.20, page: 103) in contrast to results from another group where a di-peptide formation was found to be template independent (Belitsina *et al*., 1981; Belitsina *et al*., 1982). Our results are challenging the Belitsina reports; we conclude that a prerequisite for peptide bond formation are programmed ribosome, without template ribosomes cannot catalyze a peptide bond.

The P_i and POST complexes constructed here both displayed a stop codon UGA at the A site. Asp-tRNA was added that could make a dipeptide only if the ribosome was flexible enough to bridge the +1 nucleotide gap to the GAC codon. Accordingly, small amounts of dipeptides were formed, in fact the same for both Pi and POST ribosomal complexes between (13-17% of the bound AcPhe-tRNA in the absence of RF2). Interestingly, none of the dipeptides could be anchored on the ribosome, but all of them were quantitatively released explaining the corresponding decrease of AcPhe-tRNA bound (Table 8 on page 102). In other words, the decrease of N-Ac[^{3}H]Phe binding values found after the addition of ternary complex corresponded to the fraction of ribosomes that participated in the di-peptide formation. Two aspects require a closer inspection: (1) Why there is no significant difference between the P_i and the POST states for dipeptide formation, although we might expect that the presence of a second tRNA at the E site (POST state) should prevent a frameshift? (2) Why the efficiency of dipeptide formation is marginal in contrast to the highly efficient frameshifts with the RF2 mRNA models?

Point 1: One possibility is that the frameshifted ternary complex releases the E-site bound tRNA as does the in-frame bound ternary complex under standard conditions according to the negative cooperative between A and E sites. This seems to be in fact the case, since under POST conditions the reduction of E-site bound tRNA is at least as large as the amount of dipeptide formation and therefore ternary complexes interacting with the A site obviously could trigger tRNA release form the E site, even if this interaction was not in all cases productive in dipeptide formation (see for example 1 in Table 9, page 106). It follows, that in a POST complex at least for

each ternary complex, that triggers the formation of a dipeptide, one tRNA is released from the E site thus generating a situation equivalent of that of a P_i state.

Point 2: Biochemical evidence (Rheinberger and Nierhaus, 1990) and analysis of the entrance of the peptidyl-harboring tunnel (Hansen *et al*., 2002) have indicated that a dipeptidyl residue is too short to be anchored into the tunnel. In our RF2 mRNA model a 24 oligopeptide was synthesized probably firmly fixed in the tunnel. Therefore, the product of the frameshift could be found exclusively on the ribosome. The high efficiency of frameshifting was probably due to both the "pressure" arising from the short spacer between peptidyl-tRNA at the P site and the SD sequence, and from the fact that the sequence in the RF2 mRNA allowed slippage of the peptidyl-tRNA in contrast to the AcPhe-tRNA present at the P site during the dipeptide assays.

4.4 RF2 and the release of deacylated tRNA from the E site

Two models have been proposed in order to explain how post-termination complexes are disassembled. One was derived from Ehrenberg's group, which suggest that RRF in concert with EF-G dissociates the 70S ribosomes after release factors have been removed by RF3. In this model RRF·EF-G do not release the mRNA from the ribosome or deacylated tRNA from the P site, since both were still found on the 30S subunit, IF3 removed the terminating tRNA (Karimi *et al*., 1999). This model proposes that EF-G is involved in a dissociation function rather than a translocation reaction (reviewed by Wilson *et al*., 2002).

The second model, now questioned by his own author (Lancaster *et al*., 2002), comes from Kaji and coworkers. Based on the similarity between crystal structures of RRF and that of a tRNA, the model proposes that RRF binds to the A site and is translocated in an EF-G dependent manner. Previous results from Hirokawa and colleagues (Hirokawa *et al*., 2002) suggested that RRF behaves like a tRNA in function as well. However more recently the orientation of RRF on the ribosomes has been derived from cleavage experiments employing OH-radicals: The authors concluded that RRF is located crosswise over A and P sites between the ribosomal subunits, the protein with the most convincing tRNA mimicry does not make use of the mimicry according to these authors (Lancaster *et al*., 2002).

None of the models makes explicit statements about the release of the E-site tRNA. In this work we demonstrate that the E-site tRNA is strongly bound in a termination complex after the RF2 dependent hydrolysis of the peptidyl-tRNA, where

the ribosome carries tRNAs at P and E site (Fig. 3.19, page: 98). The RRF location is of decisive importance, and the question whether or not this factor can undergo an EF-G dependent translocation can and probably will be answered by a cryo-EM analysis.

4.5 Frameshifting mechanism in translation of the RF2 mRNA

The task of maintaining the reading frame during translation is a prerequisite for translation of the genetic information, and maintenance of the reading frame an astonishing achievement of the ribosome. A frameshifting event is extremely rare and its frequency has been estimated to be below 1:30,000 translocational events, i.e. one frameshift in more than 30,000 elongation cycles (Jorgensen and Kurland, 1990). However, for the expression of the RF2 gene a frameshift has to occur, and it does so with an extremely efficiency of 50 to 100 % (Craigen *et al*., 1985). There must be a mechanism that maintains the reading frame, and this mechanism is obviously switched off at the frameshifting site or the RF2 mRNA. The aim of this thesis was to study the translational regulation of RF2 expression and to learn about the mechanism that helps the ribosome to maintain the reading frame.

We designed the following experimental strategy:

Ribosomes were programmed with either $(UUC)_{12}$ containing mRNAs and primed with N-Ac[^{14}C]Phe-tRNA^{Phe}. In assays where the E site occupation was studied, the binding of [^{32}P]-tRNA^{Tyr} was determined. The frameshifting event was measured by the incorporation of [^{14}C]Asp into the precipitable polypeptide chain (Figure 3.4D, page 78). Binding of [^{32}P]tRNA^{Tyr} and +1 frameshifting (FS) was evaluated by nitrocellulose filtration and hot TCA precipitation, respectively.

Our results showed that in absence of RF2, ribosomes programmed with either $(UUC)_{12}$ containing mRNAs were prone to slide +1 nucleotide downstream, thus incorporating aspartic acid into the polypeptide chain. The fact that in the absence of RF2 both messengers induced a +1 frameshifting (Figure 3.16A, page: 95; and Table 5 on page: 90) was not expected, because according to our hypothesis, only ribosomal complexes programmed with a messenger that contained the Shine Dalgarno sequence characteristic for the RF2 mRNA should change their reading frame. It follows that *E. coli* ribosomes pausing for long periods at a stop codon are able to change the frameshift and to continue protein synthesis. These results are

consistent with previous observations, where a frameshift window led the more to a frameshift the less efficient decoding took place at the in-frame A site codon (Craigen and Caskey, 1986; Curran and Yarus, 1988).

However, in the presence of RF2 only the SD containing mRNA provoked a frameshifting (Figure 3.16B, page 95) underlining the importance to maintain a minimal RF2 concentration in the cell that prevents undesirable or even dangerous frameshifting events.

The frameshifting window in the messenger pU+SD/RF2 defined by the SD sequence and the codons for Tyr, Leu and Stop (UAU-CUU-UGA respectively) creates an environment that actively supports a +1 frameshifting. (1) The SD sequence with the extremely short spacer of two nucleotides pushes the mRNA downstream. This effect was directly shown in binding experiments, where in the presence of an SD arrangement similar to that in the RF2 mRNA an AcPhe-tRNA could be hardly bound to a UUC codon (Table 10 on page: 108) at a position corresponding to the Leu codon in the RF2 mRNA. Further, frameshift-events were manifested faster with the SD sequence rather than without one in the absence of RF2 (Figure 3.16A, page: 95). (2) The $tRNA^{Tyr}$ at the E site is a tRNA with one the lowest intrinsic affinities of 20 tRNAs tested (Gnirke and Nierhaus, 1986) and therefore can be easily removed from the E site, if codon-anticodon interaction is disturbed. However, with codon-anticodon interaction the $tRNA^{Tyr}$ is bound in a stable fashion as shown here for the first time *in vitro* with an oligopeptidyl-tRNA with 24 aminoacyl residues at the P site. This result confirms ample evidence for codon-anticodon interaction at the E site from *in vitro* studies (Gnirke *et al*., 1989; Rheinberger and Nierhaus, 1983; Rheinberger and Nierhaus, 1986b) and from analysis of native polysomes (Remme *et al*., 1989). The presence of a “weak” tRNA at the E site of the frameshift window (mostly $tRNA^{Tyr}$, sometimes $tRNA^{Lys}$, see Table 11) is conserved in this type of frameshift. (3) The peptidyl-$tRNA^{Leu}$ can slide one nucleotide downstream maintaining codon-anticodon interaction at the P site after the frameshift. Therefore, sliding of this tRNA has not to overcome high-energy barriers. (4) UGA is the least efficient of the termination codons in particular if followed by C as in the RF2 mRNA (Poole *et al*., 1995).

Ribosomes carrying only a peptidyl-tRNA at the P site could have the same probability to move leftward (-1 frameshifting) or rightward (+1 frameshifting). In the case of the RF2 mRNA the SD:antiSD interaction not only provokes the release of

the deacylated $tRNA^{Tyr}$ from the E site, but its extremely short spacer to the Leu codon at the P site also prevents a movement backward of the ribosome.

Table 11. RF2 frameshifting sites

Alignment of RF2 frameshifting sites

Microorganism	*Ribosomal Sites* Anti SD SD E P A
Bacillus firmus	5'-ACTTT 3'-UCCUCC A-5' AGGGGG TCT CTT TGA CTTA-3'
Borrelia burgdorferi	5'-ACATTT CCUCC GGAGG AAG CTT TGA CAAA-3'
Chlamydia trachomatis	5'-TAACTC CCUCC GGAGG TCT CTT TGA CCCG-3'
Deinococcus radiodurans	5'-CGCTCC C CU(C)C A GGGAG TAC CTT TGA CATT-3'
Escherichia coli	5'-TTCTT UCCUCC A AGGGGG TAT CTT TGA CTAC-3'
Haemophilus influenza	5'-TGCTTC C CUCC A GGGGG TAT CTT TGA CTTC-3'
Pseudomonas aeroginosa	5'-CCATTC CCUCC A GGGGG TAT CTT TGA CTAC-3'
Salmonella typhimurium	5'-TTCTT UCCUCC A AGGGGG TAT CTT TGA CTAC-3'
Streptococcus pyogenes	5'-GCTTC UCCUCC A AGGAGG TCT CTT TGA CTTA-3'
Synochocystis PCC6803	5'-AAACCC UCCU AGG AC TAT CTT TGA CCTG-3'
Treponema pallidum	5'-AGGTA T CCUCC GGGGG AGT CTT TGA CGTT-3'

Alignment of differents mRNAs sequences from different eubacterial species that require a +1 frameshift to the complete the synthesis of RF2.

A molar ratio RF2 per ribosome of 0.3 was enough to abolish the spontaneous frameshifting occurred in ribosomes programmed with an mRNA without SD, whereas in the presence of SD the same concentration of RF2 did not prevent an efficient frameshifting. The frameshift frequency on ribosomes programmed with pU+SD/RF2 was reduced to background values, when the concentration of RF2 was increased to molar ratios up to 2 (Figure 3.13, page: 91). This result demonstrates that not only a critical lower concentration of RF2 exist, but rather the RF2 concentration must be regulated within a window, where the lower limit has to be observed in order to prevent unwanted frameshifts, and within the window efficient frameshifting on the RF2 mRNA occurs, that is a prerequisite for RF2 expression, and above the window no frameshifting occurs on the RF2 mRNA.

Two observations indicate that the frameshift occurs at the ribosomal P site: (i) $tRNA^{Tyr}$ at the E site is lost also in the absence of Asp that fixes the frameshifting event leaving only the peptidyl-tRNA at the P site to undergo a frameshift (Figure 3.17, page: 96). (ii) As mentioned above the SD impairs tRNA binding to a codon corresponding to the Leu due to a downstream movement of the mRNA. Thus, it is not the Asp-tRNA that promotes the frameshift, rather the incorporation of Asp fixes the new +1 frame.

A characteristic signal of our analysis is the reciprocal relationship between the tRNA loss from the E site and the incorporation of the Asp residue as a signal for a frameshift event. The seemingly contradictory result with an SD sequence shifted two nucleotides upstream as compared for the "wild type" RF2 mRNA could be resolved. We found a release of the E site $tRNA^{Tyr}$ and a lack of frameshift (Figure 3.19, page: 99). However, an analysis at lower temperature (30 °C instead of 37 °C) clarified that the tRNA was present at the E site when RF2 terminated the in-frame oligopeptide thus preventing a frameshift. The short spacer between the Leu codon and SD weakened the tRNA interaction at the E site and triggered a release of the tRNA during the time span between frameshifting and filtration (about 15 min).

Formally the following sequence of events around the frameshift window can be distinguished: (i) post-translocational state formation with the stop codon UGA at the A site, (ii) SD:antiSD interaction promoting the release of deacylated tRNATyr from the E site, (iii) peptidyl-Leu-tRNA slips from the P site +1 nucleotide downstream (frameshifting), and (iv) finally decoding of the Asp-tRNA at the A site that fixes the frameshift.

Step (i) should be a fast step, the steps (ii) and (iii) probably will take a little more time, since UGA decoding is an inefficient termination signal, in particular with the forth nucleotide C (Poole *et al*., 1995). Also the shift of the peptidyl-tRNA at the P site one nucleotide is probably a slow reaction, since a frameshift occurs more often the longer the ribosome pauses at a stop codon (Mottagui, 1998) (Craigen and Caskey, 1986) (Adamski *et al*., 1993). Once step (iii) has occurred, number (iv), decoding of Asp-tRNA, is expected to be fast. This sequence of events explains why a time lag of about 20 and 40 seconds was necessary in order to manifest the frameshift in kinetic experiments without and with RF2 (Figure 3.16, page 95). This time seems to be required for the ribosomes to be prepared for the frameshifting event, i.e., to release the deacylated $tRNA^{Tyr}$ from the E site and to decode the Asp

codon at the ''A'' site for the frameshifted Asp-tRNA^{Asp}. Furthermore, the prolonged lag phase observed in the presence of RF2 indicates that this factor interferes with the frameshifting event, even if a frameshift occurs instead of a hydrolysis of the nascent peptide chain.

We conclude the following scenario for the mechanism of the frameshift event at the RF2 mRNA: **Step 1** (Figure 4.2, b and c): The SD-antiSD interaction in the coding region provokes a steric clash with codon-anticodon interaction of the tRNA at the E site. The latter is essential for a stable binding of a deacylated tRNA at the E site (Gnirke *et al*., 1989; Rheinberger and Nierhaus, 1986a; Rheinberger *et al*., 1986). Consequently, the tRNA is lost from the E site; the SD-antiSD literally squeezes out the tRNA from the E site. **Step 2** (Figure 4.2 c): A sliding of the peptidyl-tRNA^{Leu} at the P site occurs one nucleotide downstream. This event has two aspects: (i) The "pressure" of the SD due to the extremely short spacer of two nucleotides between of the peptidyl-tRNA at the P site and the SD sequence shifts the mRNA one nucleotide downstream, whereas the peptidyl-tRNA is kept at the P site due to fact that the peptidyl residue is fixed at the tunnel. (ii) Energetically it is of importance that codon-anticodon interaction is maintained after the shift thus avoiding significant energy barriers for this event due to non-compensated losses of hydrogen bonds (Lim and Curran, 2001). **Step 3** (Figure 4.2 d): The GAC codon in the +1 frame is exposed at the A site, and its decoding and the subsequent incorporation of Asp fixes the frameshift.

The fixation of the GAC codon at the A site and the frameshift event is supported by an effect linked to the loss of E-site tRNA: An elongating ribosome never envisages a situation with only one tRNA on the ribosome. There are always two tRNAs, either at the A and P sites before translocation or at the P and E sites after translocation. An occupied E site induces a low affinity A site that might be related to the ternary complex interaction with the ribosome, where most of the aminoacyl-tRNA is far from the A site except the codon-anticodon interaction (Valle *et al*., 2002). In contrast, a free E site induces a high affinity A site as demonstrated first with E. coli ribosomes and then, even more striking, with yeast ribosomes (Triana-Alonso *et al*., 1995a). The high A-site affinity facilitates the binding of the Asp-tRNA and therefore the fixation of the frameshift. An alternative explanation can be derived from an analysis of the influence of the SD:antiSD interaction, where it has been shown that this interaction can functionally replace the codon-anticodon interaction at

the E site for a low affinity of the A site and thus for high accuracy (V. DiGiaco, V. Marquez, F. Triana-Alono and K. H Nierhaus, unpublished results; see also Nierhaus, 1990). Also in the RF2 case the SD:antiSD interaction could stabilize the ribosomal complex and compensate the missing deacylated tRNA at the E site and the transient detachment of oligopeptidyl-$tRNA^{Leu}$ from the P site during the peptidyl-slippage.

The results have more far reaching consequences than to explain the mechanism of translational regulation of RF2 expression. Coming back to the point outlined a the beginning of this section saying that a mechanism must exist for maintaining the reading frame that is switched off at the frameshift site of the RF2 mRNA: The analysis of the frameshift event documented that a critical point essential for the frameshift is the removal of the tRNA from the E site. It follows that the occupation of the E site and probably codon-anticodon interaction at this site play an important role to maintain the reading frame during translation and thus being critical for the translation of the genetic information.

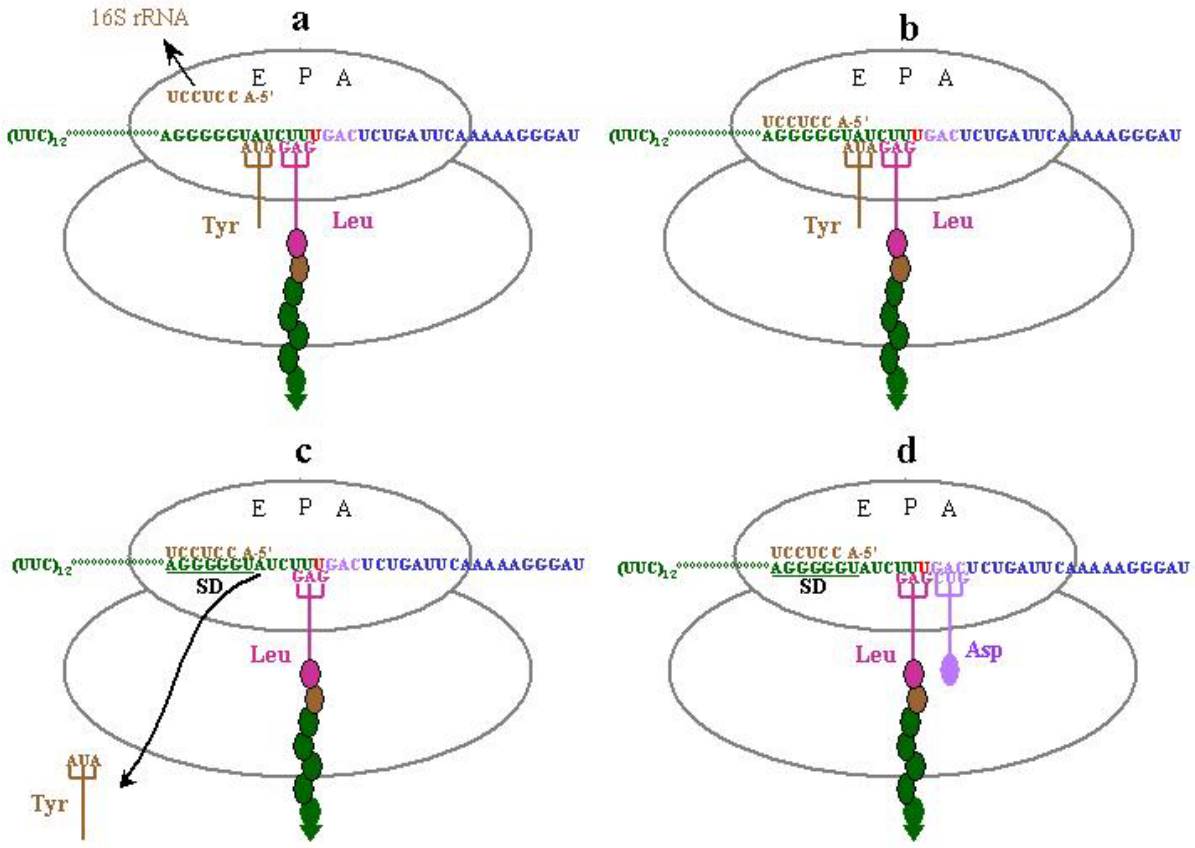

Figure 4.2 Model proposed to explain the highly frameshifting efficiency on the expression of the termination factor RF2. *(a) Ribosomes translate the RF2 mRNA until the codon number 25 positioning in the frameshifting window. Deacylated tRNA^{Tyr} is bound at the E site and peptidyl-tRNA^{Leu} at the P site. The codon number corresponds to the UGA stop codon. (b) Under shortage of RF2, ribosomes stall at the stop codon. The SD:antiSD interaction promote (c) the release of the deacylated tRNA^{Tyr} from the E site. Now the ribosome carries only one tRNA that moves one nucleotide downstream pushed by the adjacent SD:antiSD interaction. (d) +1 frameshifting is fixed by binding Asp-tRNA^{Asp} and incorporating Asp into the nascent peptide chain.*