Chapter 3: Results

3.1 Pre-requisites for the analysis of RF2 frameshifting mechanism

3.1.1 Development of a novel method for the detection of mRNA degradation: RNase Assay

RNase contaminations are one of the predominant factors that impair the efficiency of the expression of proteins using *in vitro* systems, whereas in coupled transcription/translation systems the presence of proteases seems to be stronger compromising the efficiency of protein synthesis. A method that detects the presence of RNases in components of an *in vitro* translation system would be very useful.

Taking advantage of the messengers designed in this study and, considering that the best substrates for RNases are RNA molecule free of intrinsic secondary structure; a very sensitive method was developed that allowed us to detect traces of RNase contamination. The MF-mRNA was 5' labeled with [^{32}P] and incubated for 3 min at 37 °C with the component to be tested for RNase contamination. Even traces of RNases degraded the MF-mRNA, and scanning the remaining MF-mRNA band allowed a relative quantification of the activity of RNases present.

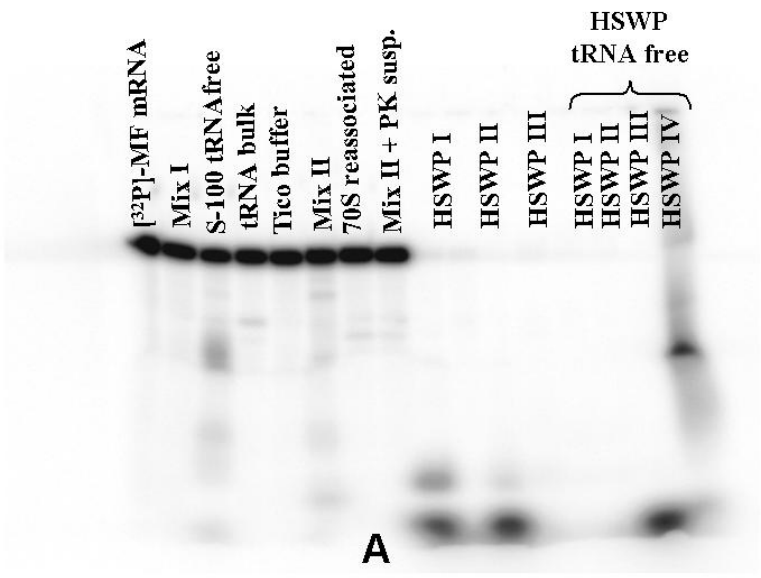


Figure 3.1 [^{32}P]-MF mRNA degradation

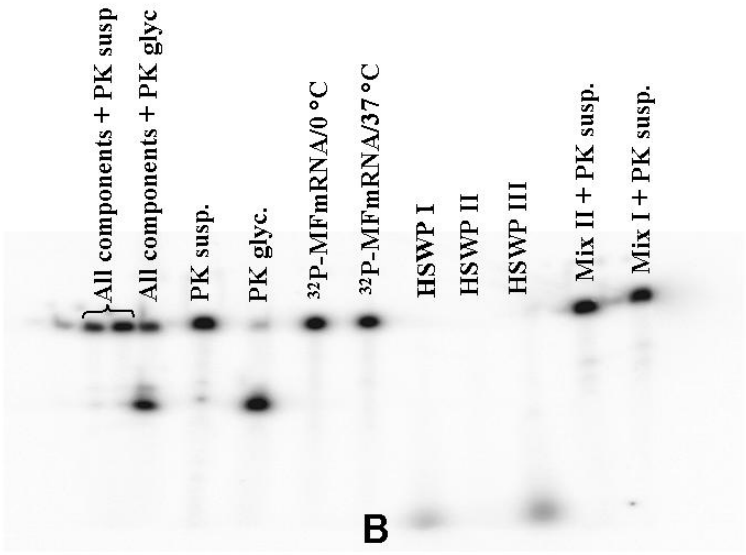


Figure 3.1 [32P]-MF mRNA degradation

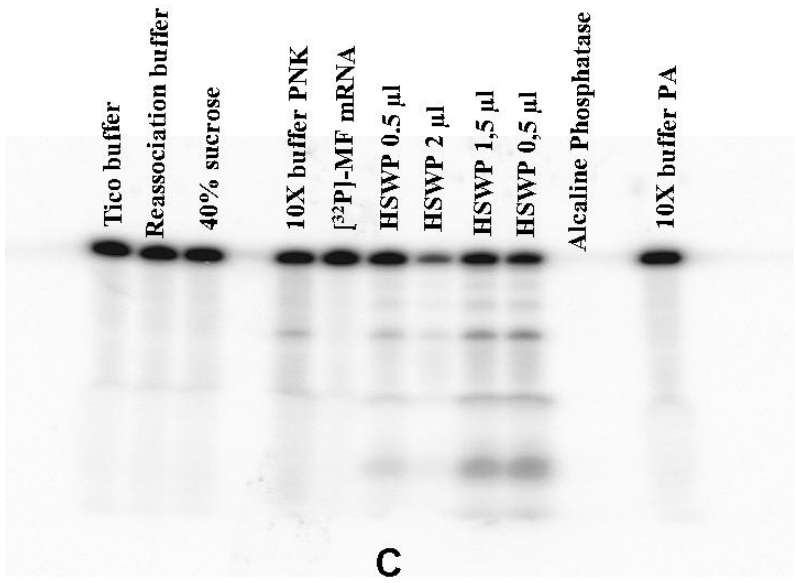


Figure 3.1 [32P]-MF mRNA degradation. *[32P]-MF mRNA was incubated with different components and mRNA degradation was detected. (A) HSWP: high salt wash protein (crude initiation factors), PK susp.: pyruvate kinase suspension preparation. (B) PK glyc.: pyruvate kinase glycerol preparation. (C) PNK: polynucleotide kinase, PA: phosphatase,alcaline*

The major RNase contamination was found in the preparation of crude initiation factors in the form of High Salt Wash Proteins (HSWP). Different HSWP preparations prepared by myself or by colleagues of the lab degraded the [^{32}P]-MF mRNA until the nucleotide level (Figure 3.1A). Interestingly, the S-100 preparation freed of tRNA did show only traces of Rnase contamination. Obviously, the RNases were removed in

complex with tRNA, since DEAE binds anions, i.e. negatively charged molecules, whereas RNases like ribosomal proteins are positively charged proteins to counteract the negative charge of their ligands. However, a similar treatment of the fraction of high-salt washed proteins (HSWP) did not remove RNases. Possibly, the HSWP did not contain sufficient amounts of tRNA to function as carrier for RNases. One should repeat the procedure but then adding before DEAE treatment some tRNAs to give the RNase a chance to bind and then to become removed.

Neither re-associated ribosomes (Figure 3.1A) nor all mixes used in the *in vitro* translation system (Figure 3.1A and B) showed RNase activity. The RNase activity found in the HSWP preparation was concentration dependent (Figure 3.1C): Degradation of [^{32}P]-MFmRNA was larger the more HSWP fraction was added.

A control assay was the addition of alcaline phosphatase that cleaves off the 5'-labeled ^{32}P residue. One expects a loss of the mRNA band what in fact is observed

Another interesting result was the fact that the batch of pyruvate kinase (PK) in glycerol contained RNases that partially degraded the [^{32}P]-MFmRNA . However, the preparation in the salt suspension containing 1 M $(NH_4)_2SO_4$ was almost free of Rnase contamination (Figure 3.1B).

3.1.2 In vitro translation system for RF2 mRNA expression

Poly(U) dependent poly(Phe) synthesis works with excellent performance under near *in vivo* buffer conditions. Furthermore, poly(Phe) synthesis can be started with $tRNA^{Phe}$ bypassing the need of initiator fMet-tRNA and initiation factors. In order to employ this simple and effective design, two mRNAs containing the frameshift window seen in the RF2 mRNA were designed, one plus the internal SD sequence and the other without, after the transcription initiation site GGU an oligo$(UUC)_{12}$ sequence followed just in front of the heteropolymeric region corresponding to the RF2 mRNA. These messengers coded for the same sequence of amino acids as observed in the RF2 mRNA before and after the frameshifting window.

Below the sequences of the main messengers used are shown in order to make clear the strategy for the experiments discussed below:

pU+SD/RF2

UUC Sequence SD Tyr Leu Stop

GG UUC $(UUC)_{11}$CGU AUG AAACUG GUU CUU GUU CUU AGG GGG UAU CUU U*GAC*

UC UGA UUC AAA Asp

pU-SD/RF2

GG UUC$(UUC)_{11}$CGU AUG AAACUG GUU CUU GUU CUU CGG GGC UAU CUU U*GAC*

UC UGA UUC AAAOriginally, the designed messengers started with the codon corresponding to Gly (GGU). In this way transcription initiation is improved and moreover ribosomes could be primed with $tRNA^{Gly}$ to start protein synthesis. However, we took advantage of the fact that 70S ribosomes start translation at the 5' end of the messenger (A. Bartetzko and K. H. Nierhaus, unpublished). Furthermore, ribosomes can be primed with AcPhe-tRNA easily. Therefore, the 5' end GGU codon was reduced to a GG followed immediately by the oligo$(UUC)_{12}$ sequence. UUC rather than UUU were chosen to guarantee a correct phasing of the reading frame with respect to the heteropolymeric region of the messengers.

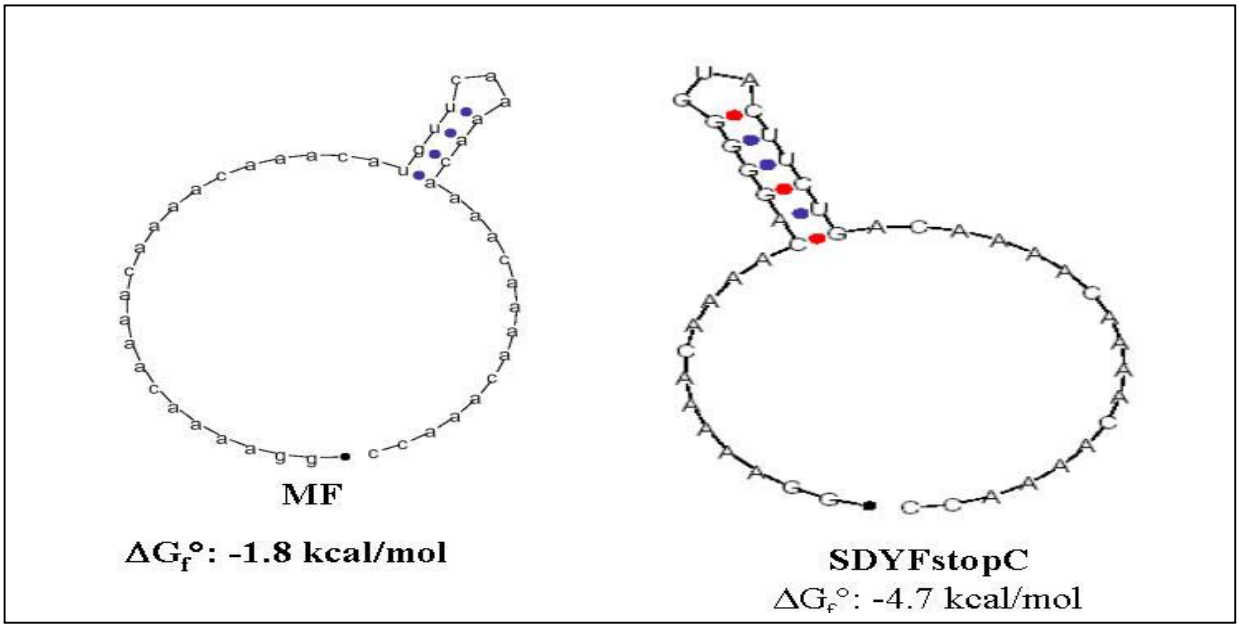
3.1.2.1. MFold secondary structure prediction of the designed mRNAs

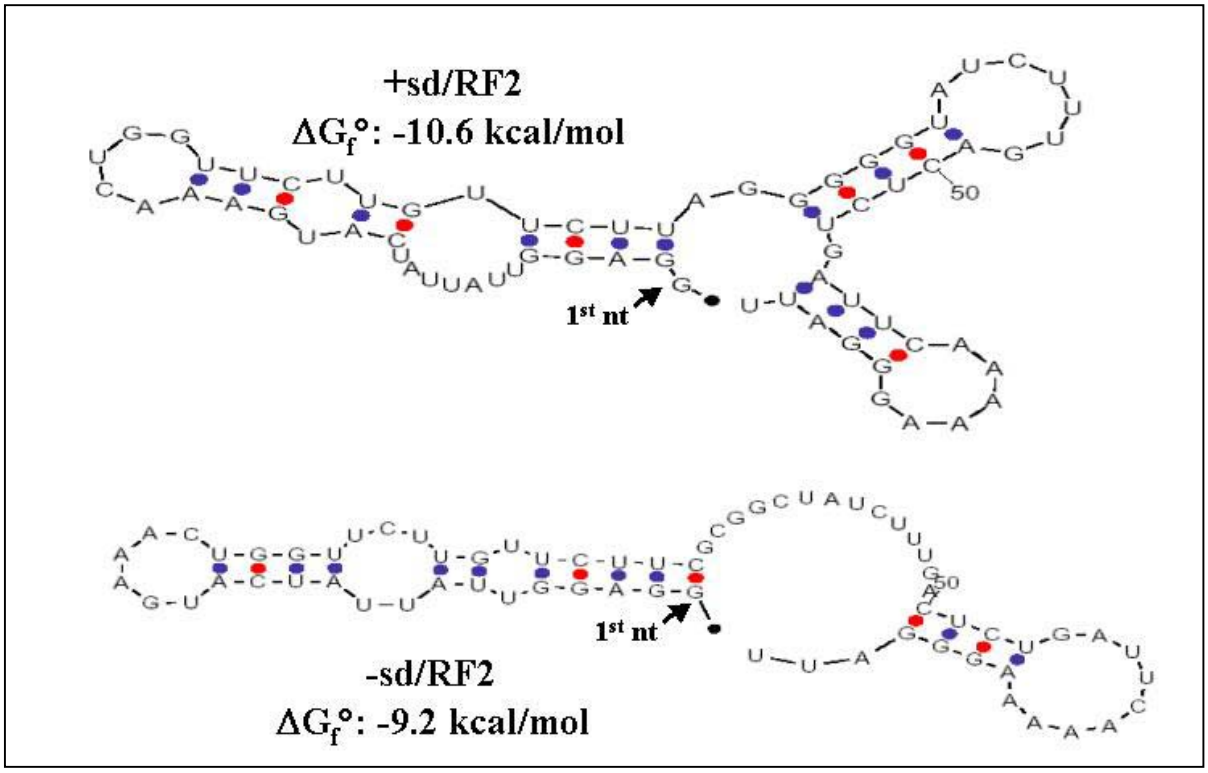
The secondary structure prediction of all mRNAs used in this study was performed with the MFold software. Normally, families of structures are obtained and those whose $\Delta G_f°$ is ≥ -10 kcal/mol (≥ means more positive and thus less stable) are structures were chosen as candidates for "run-off" transcription. The more positive the $\Delta G_f°$ of the mRNA sequences the less stable is the secondary structure of the mRNA.

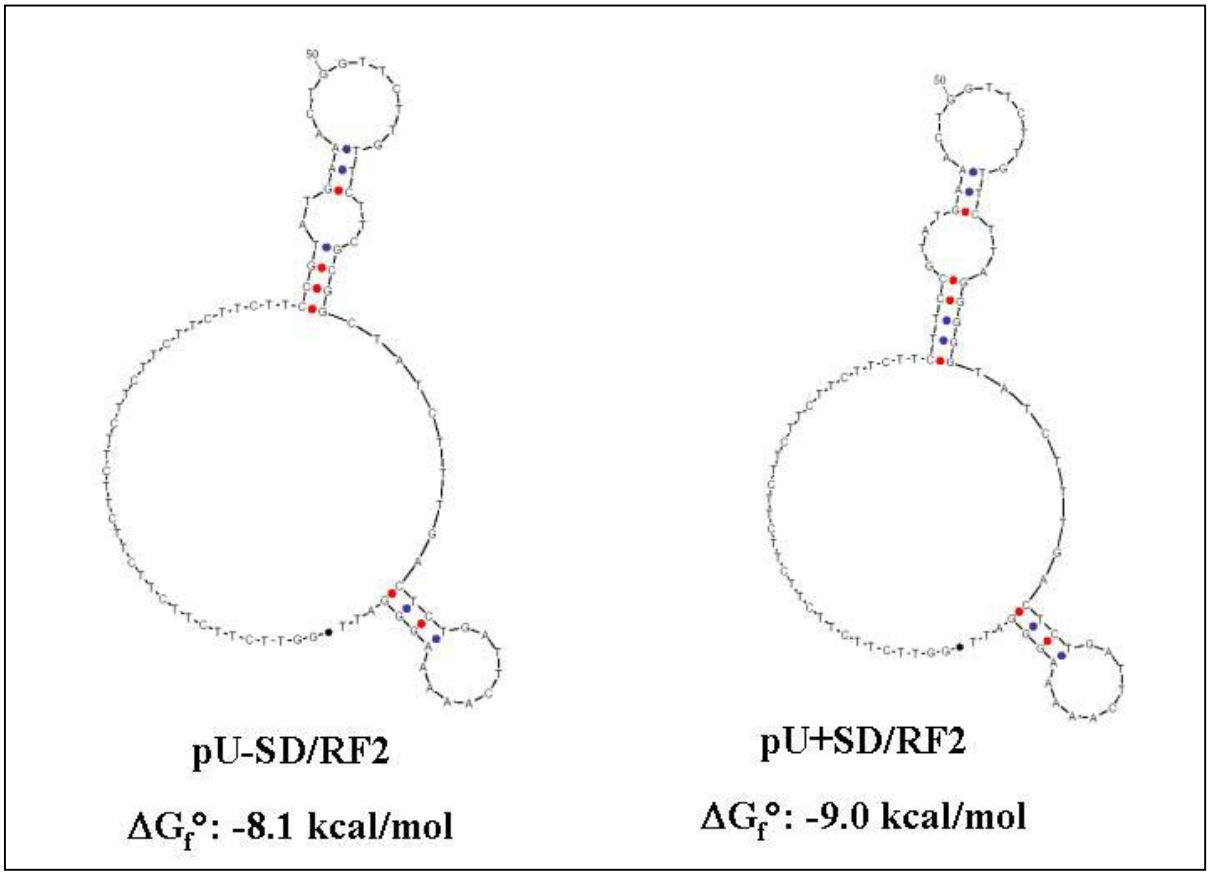
Figure 3.2 (this page and next ones) shows that in almost all mRNAs designed in this work, little stable secondary structures were found. Messengers designed for the di-peptide experiments showed values of $\Delta G_f°$ between -4.7 to -1.7 kcal/mol. These secondary structure can be easily melted by the translating ribosome, since $\Delta G_f°$ on these messengers is far from the "stability threshold" for initiating ribosomes of ≥ -6 kcal/mol (de Smit and van Duin, 1990). In contrast, values below $\Delta G_f°$ = –6 kcal/mol were found in the messengers containing the $(UUC)_{12}$ sequence. However, one has to consider that the secondary structures found in one mRNAs are not linked, e.g., via stacking interaction. If one measure the stability of the individual secondary structures of one mRNA, one obtaines values above the energetic

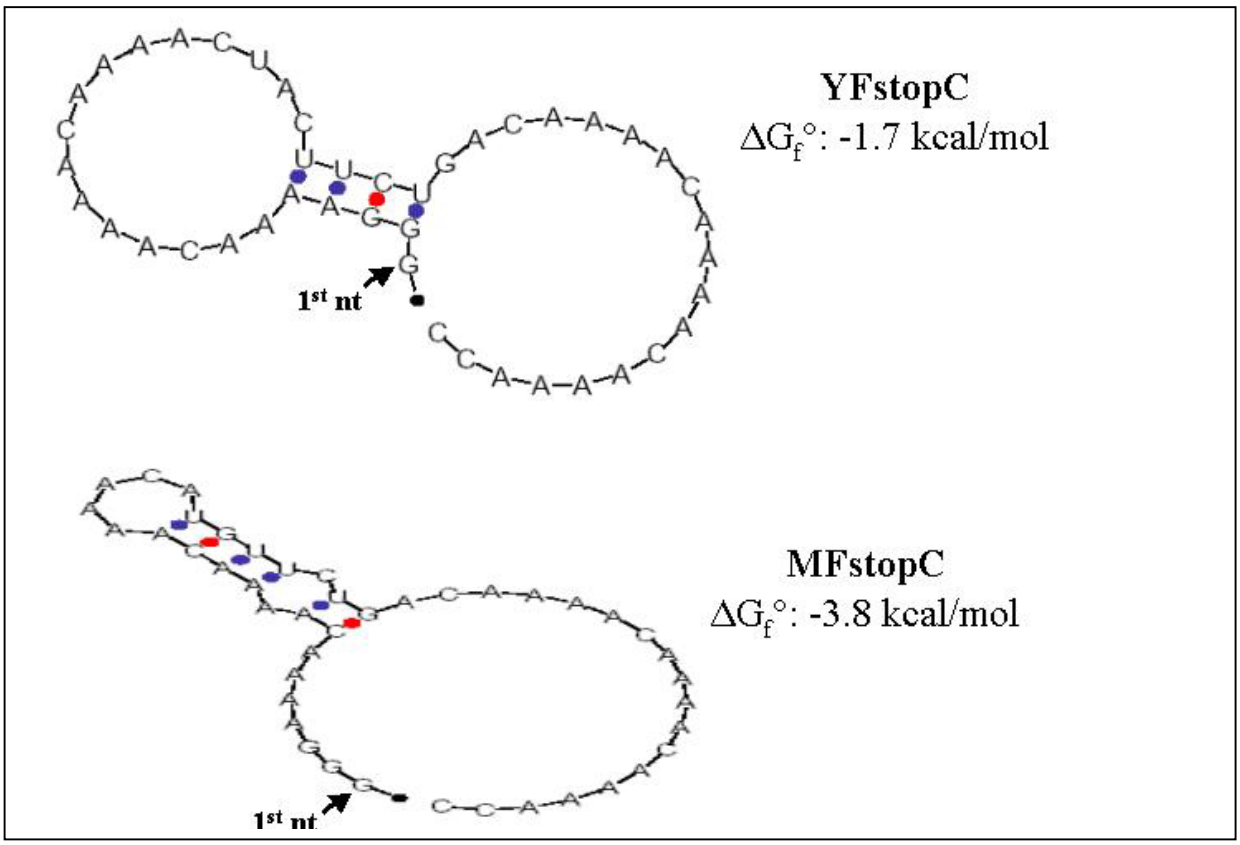
threshold of -6 kcal/mol. E.g., the mRNA pU+SD/RF2, the indiviual structures have a $\Delta G_f°$ of 1.7 and –0.6 kcal/mol, respectively. Furthermore, an open structure is observed at the 5' end of messengers containing the $(UUC)_{12}$ sequence facilitating the start of translation.

In general, acceptable secondary structures were found in all mRNAs.






pU-SD/RF2
ΔG$_f$°: -8.1 kcal/mol
pU+SD/RF2
ΔG$_f$°: -9.0 kcal/mol


1st nt
YFstopC
ΔG$_f$°: -1.7 kcal/mol
1st nt
MFstopC
ΔG$_f$°: -3.8 kcal/mol

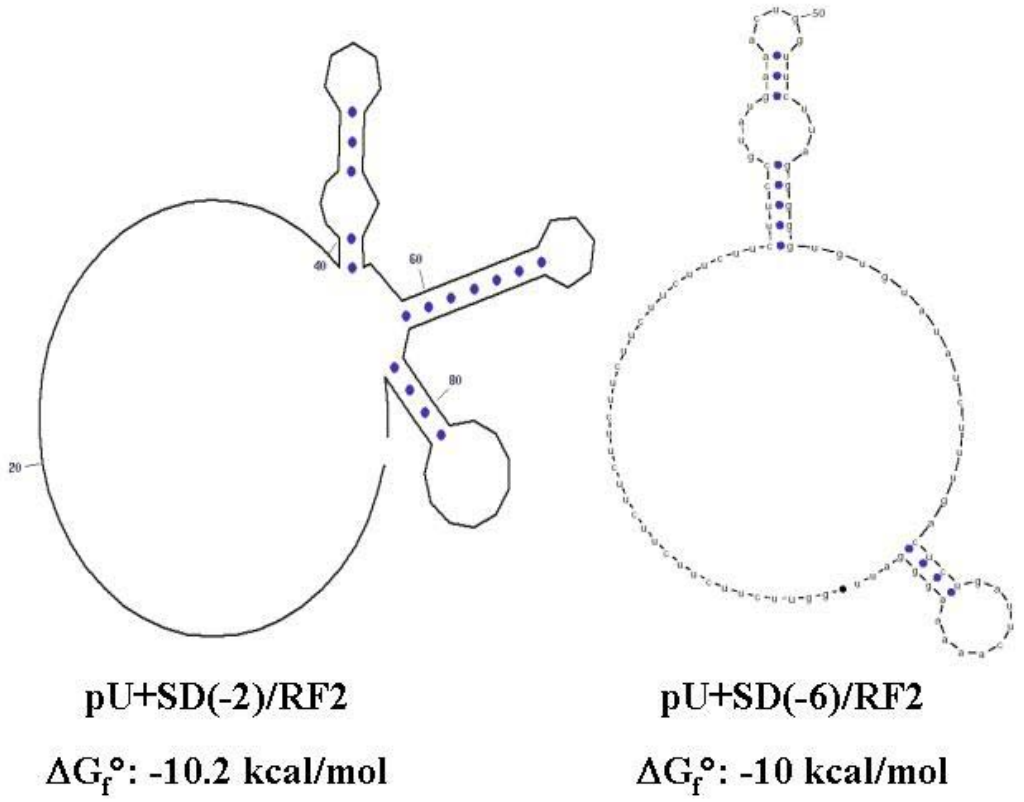


Figure 3.2 MFold Secondary structure predictions

3.1.2.2. Purity of mRNAs

All mRNAs were transcribed *in vitro* using the protocol of "run-off" transcription with T7 polymerase described in materials and methods (section 2.5.1, page 36). The figure 3.3 shows a polyacrylamide gel of both $(UUC)_{12}$ containing mRNAs after a preparative transcription and purification by gel, as described in the section 2.5.1.2, page 37. In both messengers a second band migrated over the main product. This result could be due to the known property of the T7 polymerase to prolong transcripts over the coded region, when the concentration of UTP is high (3.75 mM final concentration per 100 μl of reaction) (Triana-Alonso *et al*., 1995b). Nevertheless, all transcribed mRNAs can be used, since Triana-Alonso has demonstrated that the coded region in both the short and the long form of mRNAs are identical. The yield of the transcription reaction is given as "transcription factor" that indicates the copy number of the RNA products per DNA template. In the case of mRNAs containing the (UUC)12 sequences the transcription factor oscillated between 1000 to 1500 (Figure 3.3).

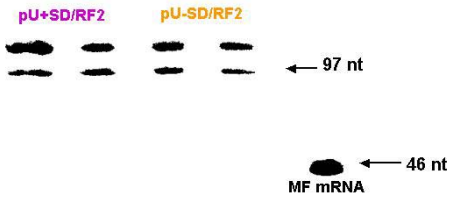


Figure 3.3 Purity of mRNAs. *Messengers transcribed in vitro were purified by PAGE as described in Material and methods (section 2.5.1.2, page 37). RNA polyacrilamide gel (12%) was used to evaluate the purity of mRNAs used in this work. MF mRNA (ggg(aaaag)$_3$aaa**AUGUUC**(aaaag)$_3$aaau) was used as a molecular marker.*

3.1.2.3. Translational control experiments with the newly designed mRNAs

In the following experiments re-associated ribosomes were programmed with both messengers and primed with labeled N-Acetyl-Phe-tRNA^{Phe}. In this way 70S type initiation complexes were obtained. The SD containing mRNA model contained the frameshifting windows plus the internal SD sequence in front of the stop codon (UGA) identical to the RF2 mRNA (pU+SD/RF2). For the second mRNA we exploited the degeneration of the genetic code in order to maintain the sequence of coded amino acids but to remove the SD sequence at the same time (pU-SD/RF2; see list of messengers: 2.5.1.5, page 40). The AcPhe binding allowed us to determine the number of peptide chains synthesized in an assay.

Translational reactions were performed in the presence of another isotope in one of the amino acid as a marker into the amino acids in order to monitor the progress of the translational process. For example, in order to test the translation of the oligo(UUC)$_{12}$ sequence the ribosomes were primed with with N-Ac[^{14}C]Phe-tRNA^{Phe} (1073 dpm/pmol), the amino acid mixture was composed of Arg, Met, Lys, Leu, Val, Tyr, Gly and [3H]Phe (100 dpm/pmol; Figure 3.4A). In the same way incorporation of Gly (Figure 3.4B) and Leu (Figure 3.4C) into the polypeptide chain was determined.

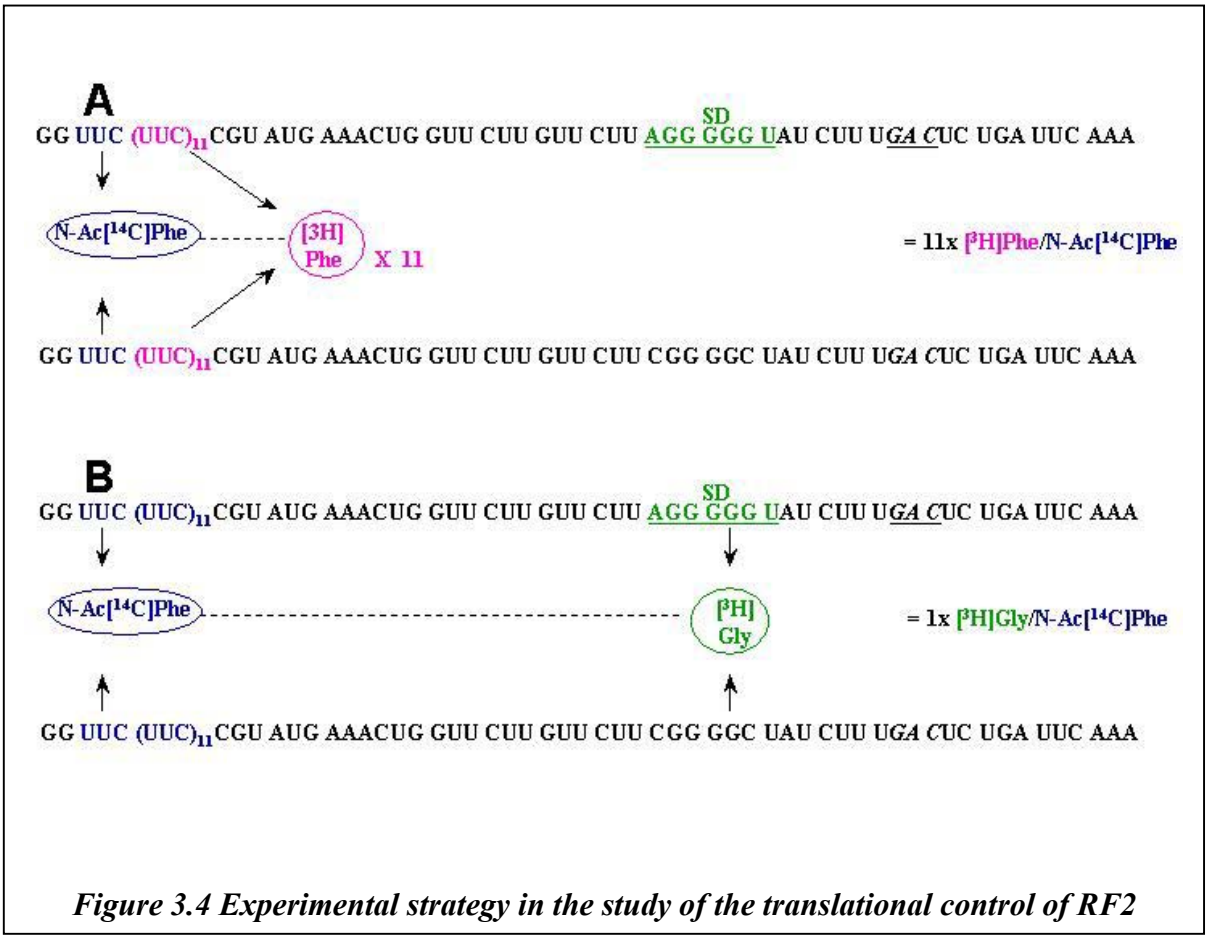


Figure 3.4 Experimental strategy in the study of the translational control of RF2

In those assays where the E site occupation was studied, the binding of [32P]-tRNATyr was assessed. The frameshifting event was measured by the incorporation of [14C]Asp into the precipitable polypeptide chain (Figure 3.4D). Binding of [32P]tRNATyr and +1 frameshifting (FS) was evaluated by nitrocellulose filtration and hot TCA precipitation, respectively. The active fraction of ribosomes were satisfying (up to 60%) that quantitatively synthesized an oligopeptide of 24 amino acids.

3.1.3 Does S-100 tRNA free fraction contains RF2?

As was mentioned before, the genetic expression of the RF2 gene is feedback regulated, i.e., the total expression of the protein depends on the concentration of RF2 in the medium. Then, for us was very important to check if the S-100 tRNA free fraction was rich or not in termination factors. Western blot analysis

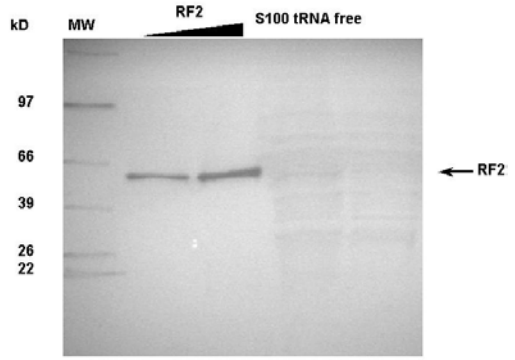


***Figure 3.5 Western blot analysis of S-100 tRNA* free fraction** *Concentration of RF2 used: 50 and 100 pmol. S-100 tRNA free: 100 µl of fraction II.*

showed that the S-100 freed of tRNA was practically free of RF2 (Figure 3.5). It follows that this extract could be used in all translational reactions without risking a premature termination. Moreover, the RF2 effects were exclusively caused by the added RF2 amounts.

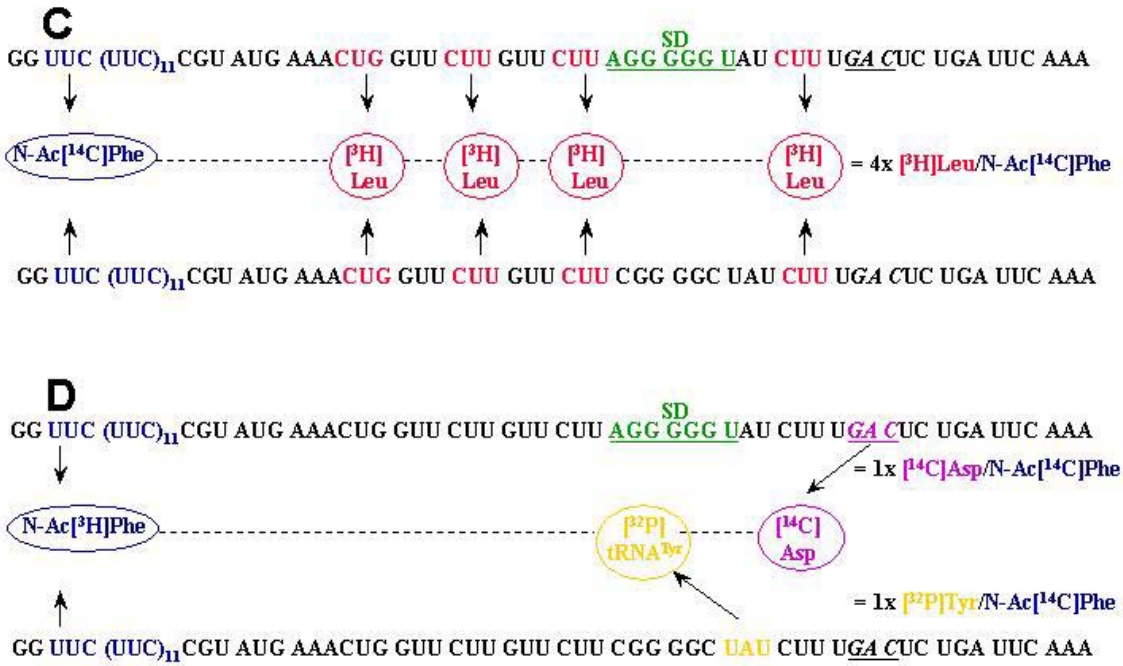


Figure 3.4 Experimental strategy in the study of the translational control of RF2. *Re-associated 70S were programmed with both mRNAs containing the (UUC)12 sequence and primed with labelled N-Ac[^{3}H]Phe-tRNA^{Phe}(1073 dpm/pmol). Amino acid mixtures in (**A**): Arg, Met, Lys, Leu, Val, Tyr, Gly, [^{3}H]Phe (100 dpm/pmol); (**B**): Phe, Arg, Met, Lys, Leu, Val, Tyr and [^{3}H]Gly (1073 dpm/pmol); (**C**) Phe, Arg, Met, Lys, Val, Tyr, Gly, [^{3}H]Leu, N-Ac[^{14}C]Phe-tRNA^{Phe} (792 dpm/pmol). (**D**) Binding of [^{32}P]tRNA^{Tyr} and +1 frameshifting (FS) was measured by nitrocellulose filtration and hot TCA precipitation, respectively. Amino acid mixture consisted of Phe, Arg, Met, Lys, Val, Gly, Tyr and [^{14}C]Asp. [^{32}P]tRNA^{Tyr} was present in the charging mix (40 pmol/aliquot; 500-3000 dpm/pmol).*

3.1.3.1. Purity of Release Factors

The release factors (RF1 and RF2) used in this study were a kind gift of Dr. Daniel Wilson. The purity of these proteins is shown in the figure 3.6. *E. coli* strain, JM109 were transformed with pET vectors (**p**lasmid for **e**xpression by **T**7 RNA polymerase) containing genes specific for RF1 and RF2 and anion exchange

chromatography (QAE Sepharose) was used to purify the decoding factors.

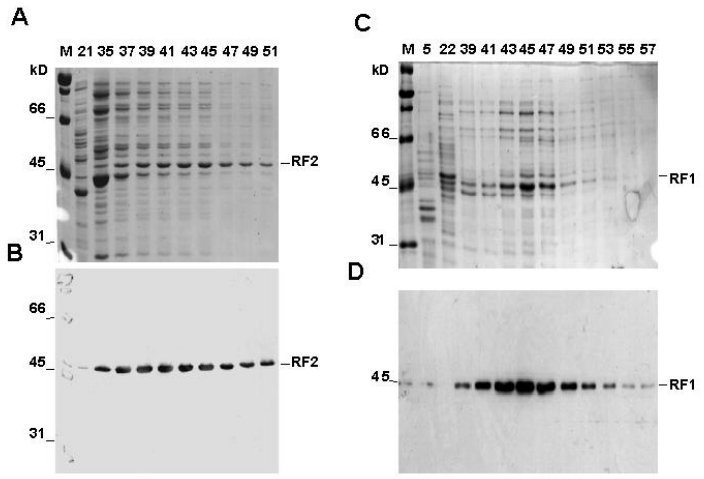


Figure 3.6 SDS-PAGE geles of Release Factors after QAE-Sepharose column purification of RF2 (T246A). *(A,C) Fractions were loaded onto SDS-PAGE gels and visualised by Coomassie staining and (B,D) immunoblotting with anti-RF2. Fraction numbers for each lane are shown and correspond to the elution profile. Molecular weight marker sizes are indicated (left). The position of the RFs band is labeled (right).*

3.1.4. tRNA bulk minus tRNA Tyrosine ($tRNA^{bulk - Tyr}$)

The tRNA bulk from *E coli* was the source of tRNAs used in this work. It contains the total tRNAs, although 5S rRNA and tmRNA are also found. Aditionally, partial isolation of individual tRNAs or families of them can be performed by specific charging and several rounds of purification by HPLC. In our case, we wanted to test if the frameshifting event on the RF2 mRNAs model is related to the release of the deacylated tRNA ($tRNA^{Tyr}$) from the ribosomal E site or not. For that purpose a tRNA bulk preparation poor in $tRNA^{Tyr}$ content was needed, to which [^{32}P]$tRNA^{Tyr}$ should be added.

The figure 3.7 shows the results obtained on the charging of this $tRNA^{bulk}$ fraction. As we can see, $tRNA^{bulk-Tyr}$ preparation is poor in the $tRNA^{Tyr}$, only a residual charging of 6% is found. About 10 times less amount of $tRNA^{Tyr}$ content was observed as compare with the normal $tRNA^{bulk}$. This preparation showed normal chargeability for the rest of amino acids (not shown). Aminoacylation of purified $tRNA^{Phe}$ and $tRNA^{Tyr}$ showed how efficient the charging of these tRNAs was.

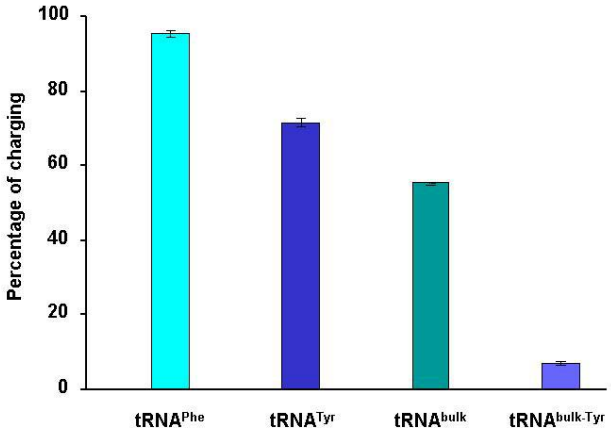


Figure 3.7 Charging reaction on tRNA$^{bulk\text{-}Tyr}$. *Aminoacylation of tRNA$^{bulk\text{-}Tyr}$ (1 A_{260} unit) was performed in order to evaluate the tRNA^{Tyr} content of this preparation. Purified tRNA^{Phe} (30 pmol) and tRNA^{Tyr} (30 pmol) were taken as control. Charging conditions were performed as described in material and methods (section 2.5.2.1, page 42). All charging values represented are minus the background of radioactivity retained on the filters of reactions without tRNA. Percentage of charging was estimated as pmol of tRNA aminoacylated per total pmol in the aminoacylation reaction.*

3.2 Characterisation of the in vitro translation system for the RF2 mRNA model

3.2.1 Binding assay

The binding of labeled N-Acetyl-Phe-tRNA^{Phe} to ribosomes programmed with either pU+SD/RF2 or pU-SD/RF2 messenger was determined. Since both mRNAs contained an $(UUC)_{12}$ sequence , we expected high binding values. In fact, programmed ribosomes with both messengers approached 1.4 molecule of labeled N-AcPhe-tRNA^{Phe} per ribosome (Table 1). Since we observed systematically values above 1 and up to 1.4, a possible reason could be a specific activity higher than indicated by the producer. Such a case has been reported previously (Triana-Alonso *et al*., 2000). The constructed complex is called Pi complex (i for initiation) that could be formed quantitatively.

The high binding efficiency was a clear mRNA effect, i.e., plus the addition of mRNA to empty ribosomes the N-AcPhe-tRNA^{Phe} binding per ribosomes (ν) increased by a factor of 2 (Table 1).

Table 1 Binding test.of Re-associated 70S on different mRNAs.

		mRNAs					
tRNA	**-mRNA**	**pU +SD/RF2**	**pU -SD/RF2**	**+SD/RF2**	**-SD/RF2**	**Poly(U)**	**MF**
Ac[^{3}H]Phe	0.62±0.016	1.38±0.024	1.35±0.02	-	-	1.2 ± 0.02	-
f[^{14}C]Met	0.62±0.02	0.76±0.008	0.77±0.02	0.94± 0.02	1.0±0.011	-	0.9±0.013

Re-associated 70S (5 pmol) were incubated in a total volume of 6,25 µl (1 aliquot) with 6 times excess of different mRNAs. N-Ac[^{3}H]Phe-tRNA^{Phe} (793 dpm/pmol) or N-f[^{14}C]Met-tRNA^{Met} (124 dpm/pmol) was used in order to prime programmed ribosomes (Pi complex). Binding reaction was incubated for 15 min at 37°C and after that all aliquots were filtered through nitrocellulose filters. Binding values represents pmol of tRNA bound per total pmol of ribosomes filtered (ν). Background values (minus ribosomes) were subtracted from all binding values, the background values did not surpass 5% of the tRNA binding.

Less pronounced was the mRNA effect upon the binding of f[^{14}C]-Met-tRNA$_f^{Met}$ on pU+SD/RF2 and pU-SD/RF2, although the relative occupancy of fMet-tRNA$_f^{Met}$ on the ribosomes was good. Binding test comparison using another set of mRNAs, i.e., +SD/RF2 and -SD/RF2 (RF2 mRNAs model without the $(UUC)_{12}$ sequence), showed that almost 100% of ribosomes programmed with these mRNAs carried one molecule of fmet- tRNA$_f^{Met}$ bound (Table 1).

From these results can we conclude that the $(UUC)_{12}$ sequence can be used as an efficient start of protein synthesis also for a heteropolymeric mRNA taking advantage of the fact that it does not contain secondary structure. This mRNA construct promotes a 70S type initiation instead of a 30S type initiation. The high binding obtained in the pre-incubation reaction guarantees a high portion of the active ribosomes engaged in the translational reaction too.

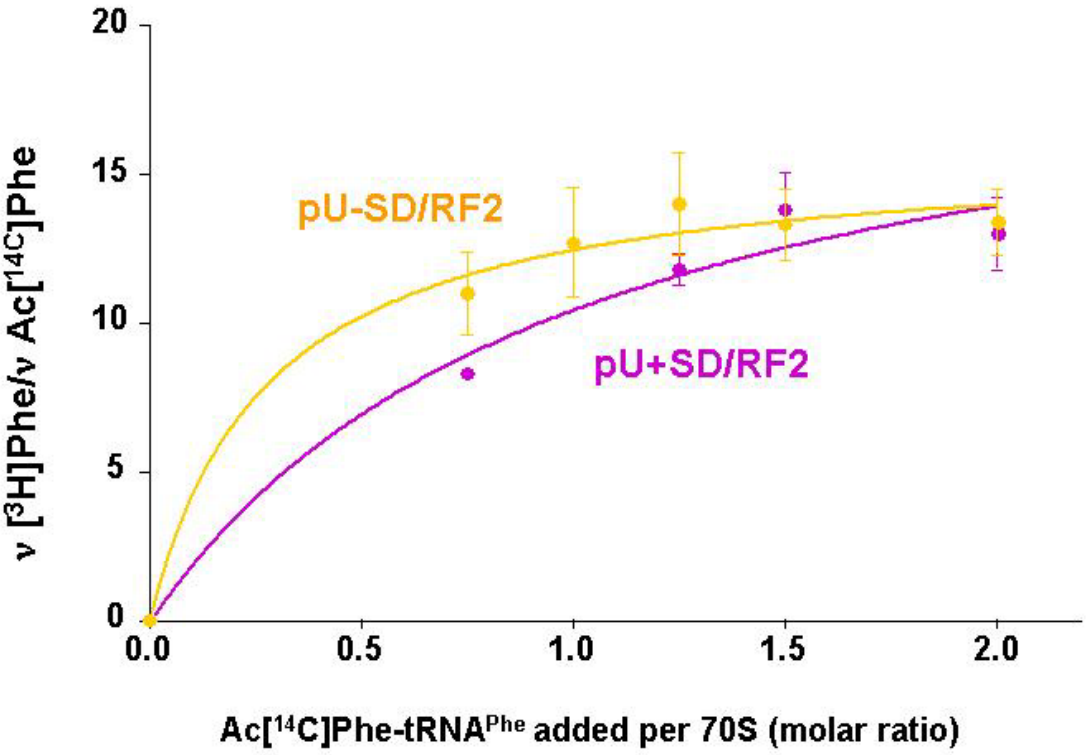


Figure 3.8 Ac[14C]Phe-tRNA^{Phe} titration. *Re-associated 70S programmed with both mRNAs containing the UUC_{12} sequence were primed with increasing amounts of Ac[14C]Phe-tRNA^{Phe} (1064 dpm/pmol). The [3H]phenylalanine incorporation into the polypeptide chain was detected by hot TCA precipitation. ν [3H]Phe and ν Ac[14C]Phe represents the pmol of [3H]phenylalanine and Ac[14C]phenylalanine respectively, normalized per total pmol of 70S precipitated. The fraction ν [3H]Phe/ν Ac[14C]Phe gives the chain length.*

3.2.2 Translational assays

In the next experiment we determined the active fraction of ribosomes *via* the AcPhe residues incorporated into the TCA precipitable chain as well as the absolute length of polypeptide chain following the AcPhe residue.

3.2.2.1 Translation of the $(UUC)_{12}$ sequence

Hot TCA precipitation of ribosomes primed with N-Ac[14C]-Phe-tRNA^{Phe} in the presence of radioactive phenylalanine amino acid ([3H]) showed that around 11 phenylalanine residues were incorporated into the polypeptide chain when 1.25 fold excess of N-AcPhe per ribosomes was used. Adding a molar ratio of N-AcPhe-tRNA^{Phe} per ribosome higher than 1.5 revealed already a saturation of 11 [^{3}H]-Phe

incorporated per ribosome primed with [^{14}C]AcPhe (Figure 3.8). According to this result the total length of the $(UUC)_{12}$ sequence tail is translated completely so that the total phenylalanine codons contained in this tail is equal to 12. On the other hand, no more than one round of translation was made probably because all RF2 mRNAs model contain an UGA stop codon. Kinetics studies using pU+SD/RF2 and pU-SD/RF2 messengers showed that after three minutes of incubation, translation of the poly(Phe) region was already saturated without any tendency to recycling (Figure 3.9).

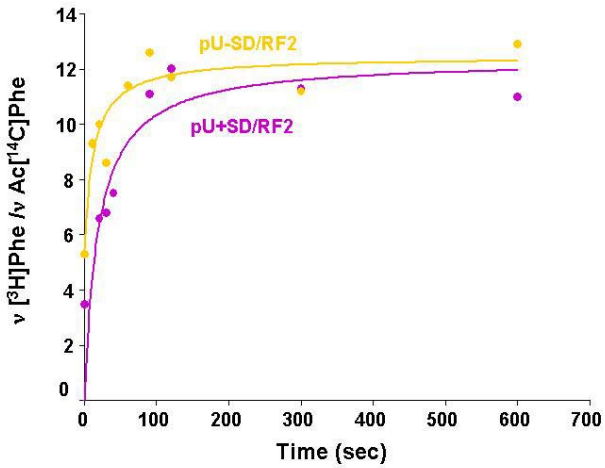


Figure 3.9 Poly(UUC) sequence translation of RF2 mRNAs model. *Re-associated 70S programmed with both poly(U) tailed mRNAs and primed with Ac[^{14}C]Phe-tRNA^{Phe} (1062 dpm/pmol) were subjected to translational reactions. [3H]Phenylalanine incorporation into the polypeptide chain was followed kinetically by hot TCA precipitation.ν [^{3}H]Phe and ν Ac[^{14}C]Phe represents the pmol of [^{3}H]Phenylalanine and Ac[14C]Phenylalanine respectively, normalised per total pmol of 70S precipitated. Fraction ν [^{3}H]Phe/ ν Ac[^{14}C]Phe indicates the polypeptide chain length*

3.2.2.2 Ribosomal Active Fraction

The binding of AcPhe to ribosomes (ν) reflects the active ribosomal fraction that participates in the protein synthesis. Pre-incubation of programmed ribosomes with both $(UUC)_{12}$ sequence containing mRNAs in the presence of N-AcPhe (binding reaction) demonstrated that 100 % of ribosomes were engaged in the binding step. However, upon dilution of the binding mixture with the charging reaction by a factor of 3, the active ribosomal fraction dropped to around 60 and 70% of the initial binding in the case of ribosomes programmed with pU-SD/RF2 and pU+SD/RF2 (Table 2)

Table 2. Binding test of pU+SD/RF2 and pU-SD/RF2 mRNAs

	ν (Ac[^{14}C]Phe-tRNA^{Phe}/70S)	
mRNA	**Pre-incubation**	**After synthesis**
pU+SD/RF2	1.15 ± 0.009	0.31 ± 0.014
pU-SD/RF2	1.14 ± 0.07	0.44 ± 0.034
Poly(U)	1.17 ± 0.09	0.46 ± 0.006

Re-associated 70S (30 pmol) programmed with 6 times excess of either $(UUC)_{12}$ containing mRNAs were primed, in a total volume of 18,75 µl, with 1,5 times excess of N-Ac[^{14}C]Phe-tRNA^{Phe} (1024 dpm/pmol) in order to form Pi complexes. Binding reaction (pre-incubation) was incubated for 15 min at 37°C and then 15 µl was withdrawn and diluted 3 times with 30 µl of charging mix previously-incubated for 2 min at 37°C. Translational reaction was performed in this way in presence of amino acid mixture and 1/3 A_{260} of tRNA^{bulk}. After an incubation of 10 min at 37°C, aliquots were withdrawn and the N-Ac[^{14}C]Phe-tRNA^{Phe} binding was assessed. Binding values were calculated as pmol of tRNA bound per total pmol of ribosomes filtered (ν). The background radioactivity retained on the filters was in every case subtracted from the binding values

Kinetics studies of the ribosomal active fraction evaluated by nitrocellulose filtration and hot TCA precipitation demonstrated that around 30-40% of ribosomes were engaged in the protein synthesis (Figure 3.10); usually an active fraction of not more than 10%, but never more than 30% is reported by colleagues also using near *in vivo* conditions (Karimi *et al*., 1999). A second factor is the buffer conditions that are near those inside the cell. Wintermeyer (Mohr *et al*., 2002) uses ionic conditions far from those in the cells but reports that the ribosomes in his experiments are 100% active in protein synthesis. The problem with these data is that lacking reproducibility by other groups.

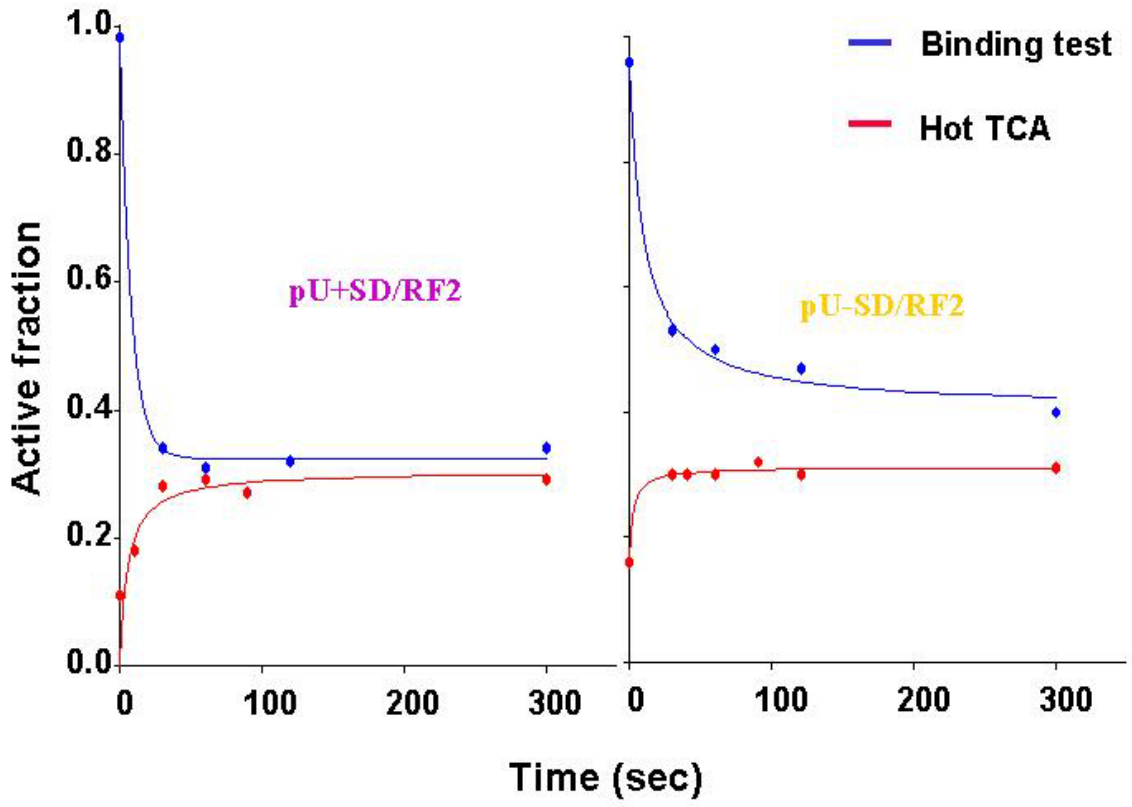


Figure 3.10 Ribosomal active fraction determination. *Active fraction means ribosomes that are engaged in the protein synthesis. Re-associated 70S programmed with either $(UUC)_{12}$ mRNAs were used in the translational reactions and kinetics of the Ac[^{14}C]Phe binding residue (1064 dpm/pmol) were determined by binding to nitrocelulose filters (___) or hot TCA precipitation (__). The pmol of Ac[^{14}C]Phe bound is given as a fraction of the total pmol of 70S present in the assay (ν)*

3.2.2.3 Translation of the heteropolymeric part of the mRNA constructs

Following the same strategy performed in the case of the translation of the $(UUC)_{12}$ sequence, the region corresponding to the RF2 mRNA model was tested. In this experiment glycine was the radioactive amino acid in order to check whether the ribosomes translate into the heteropolymeric region of the mRNA. Glycine was chosen because only one codon for this amino acid is present in the mRNA sequence and, its location is two codons upstream from the UGA stop codon. The incorporation of one glycine residue per nascent chain means that the ribosomes will probably arrive at the frameshifting window after the translation of the $(UUC)_{12}$ sequence.

The expectation is that with a proper translation that one Gly residue should be incorporated per AcPhe residue. Indeed, it was found by means of hot TCA precipitation that in both $(UUC)_{12}$ containing mRNAs about one Glycine residue was incorporated into the polypeptide chain per AcPhe residue. In binding assays using nitrocellulose filtration values slightly above one and in TCA precipitation assays values slightly below one were found (Table 3).

Table 3 Translational of heteropolymeric region of RF2 mRNAs model

	Nitrocellulose filtration				**TCA precipitation**		
	Pre incubation	**After reaction**			**After reaction**		
mRNA	**ν Ac[^{14}C]Phe**	**ν Ac[^{14}C]Phe**	**ν [^{3}H] Gly**	**ν Gly / ν AcPhe**	**ν Ac[^{14}C]Phe**	**ν [^{3}H] Gly**	**ν Gly / ν AcPhe**
pU +SD/RF2	1.28±0.1	0.29±0.005	0.34±0.01	1.2±0.014	0.24±0.02	0.17±0.01	0.72±0.04
pU -SD/RF2	1.3±0.03	0.38±0.005	0.47±0.02	1.24±0.02	0.32±0.02	0.25±0.01	0.8±0.008

The translation of the hetero-polymeric region of both $(UUC)_{12}$ containing mRNAs was tested by the incorporation of glycine polypeptide chain. Pi complexes were formed by pre-incubating 30 pmol of re-associated programmed 70S with 1.5 times N-Ac[^{14}C]Phe-tRNA^{Phe} (1073 dpm/pmol). After 15 min at 37°C of incubation, 15 µl of pre-incubation mixture was mixed with 30 µl of charging mix. Charging mix contained [^{3}H]glycine (1073 dpm/pmol), 1/3 A_{260} of tRNA^{bulk} and amino acid mixture without glycine. Translational reaction was carried out for 3 min at 37°C and after that, aliquots were withdrawn and binding test and hot TCA precipitation was performed.
ν AcPhe and ν Gly represent the pmol of Acetyl-Phe and Gly bound or precipitated per total pmol of 70S present in the assay.

When the same assay was repeated using f[^{14}C]-Met-tRNA$_f^{Met}$ instead of N-AcPhe-tRNA^{Phe}, similar results were obtained, i.e., one glycine residue per polypeptide chain, however the ribosomal active fraction that participated in the reaction was considerably lower, namely 8 to 9% (Table 4). These results confirm those found previously concerning the high ribosomal active fraction when N-AcPhe-tRNA^{Phe} is used.

Table 4 Ribosomal active fraction of ribosomes programmed with RF2 mRNAs model.

	ν f[^{14}C]-Met-tRNA$_f^{Met}$ (Active fraction)		ν N-Ac[^{14}C]-Phe-tRNA^{Phe} (Active fraction)	
mRNA	**+SD/RF2**	**-SD/RF2**	**pU+SD/RF2**	**pU-SD/RF2**
Active fraction	0.09 ± 0.005	0.08 ± 0.001	0.22 ± 0.026	0.41 ± 0.034
ν[^{3}H]-Gly	0.09 ± 0.005	0.09 ± 0.005	0.25 ± 0.015	0.31 ± 0.017
ν[^{3}H]Gly/Activefraction	1.07 ± 0.002	1.2 ± 0.053	1.1 ± 0.026	0.74 ± 0.02

Re-associated 70S ribosomes programmed with $(UUC)_{12}$ containing messengers and normal mRNAs for RF2 (without $(UUC)_{12}$ sequence) were subjected to translational reaction. After 3 min of incubation, aliquots from the samples were taken and precipitated by hot TCA. Newly, the reaction progress was evaluated in function of the [^{3}H]glycine (1073 dpm//pmol) incorporated into the polypeptide chain. The active fraction is estimated by the binding values of N-f[^{14}C]Met (124 dpm/pmol) or N-Ac[^{3}H]Phe (1073 dpm/pmol) normalized to the total pmol of 70S present in the assay.

The glycine incorporation into the growing polypeptide chain was dependent on N-AcPhe-tRNA^{Phe} present in the amino acid mixture (Figure 3.11), revealing that ribosomes had first to translate the $(UUC)_{12}$ sequence before arriving at the glycine codon.

Unexpectedly, when the amino acid mixture did not contain phenylalanine, ribosomes primed with N-AcPhe-tRNA^{Phe} still synthesized the polypeptide chain until the glycine amino acid. It seems that ribosomes could ‘’slide’’ a certain portion of the $(UUC)_{12}$ sequence in the absence of phenylalanine and then translate the heteropolymeric region of the mRNAs.

The polypeptide synthesis evaluated under these conditions stops with the Gly residue, a termination and thus a hydrolysis of the synthesized oligopeptide should not occur. In other words, the peptidyl-tRNA with the incorporated Gly residue should still be bound to the ribosome. In order to test this expectation, sucrose gradient centrifugation was performed in order to test, whether the radioactive peptide comigrates with the ribosome. Fractionation of sucrose gradient showed that ribosomes stalled on the glycine codon in the absence of the amino acids tyrosine

and leucine (next amino acids after glycine in the RF2 mRNA sequence). Hot TCA precipitation of the fractions corresponding to the 70S peak showed stoichiometric incorporation into the polypeptide chain of one ^{3}H-glycine per N-Ac[14C]-Phe residue proving that every nascent chain that carried the initiation AcPhe was translating until the Gly. Shorter peptides are not observed (Figure 3.12).

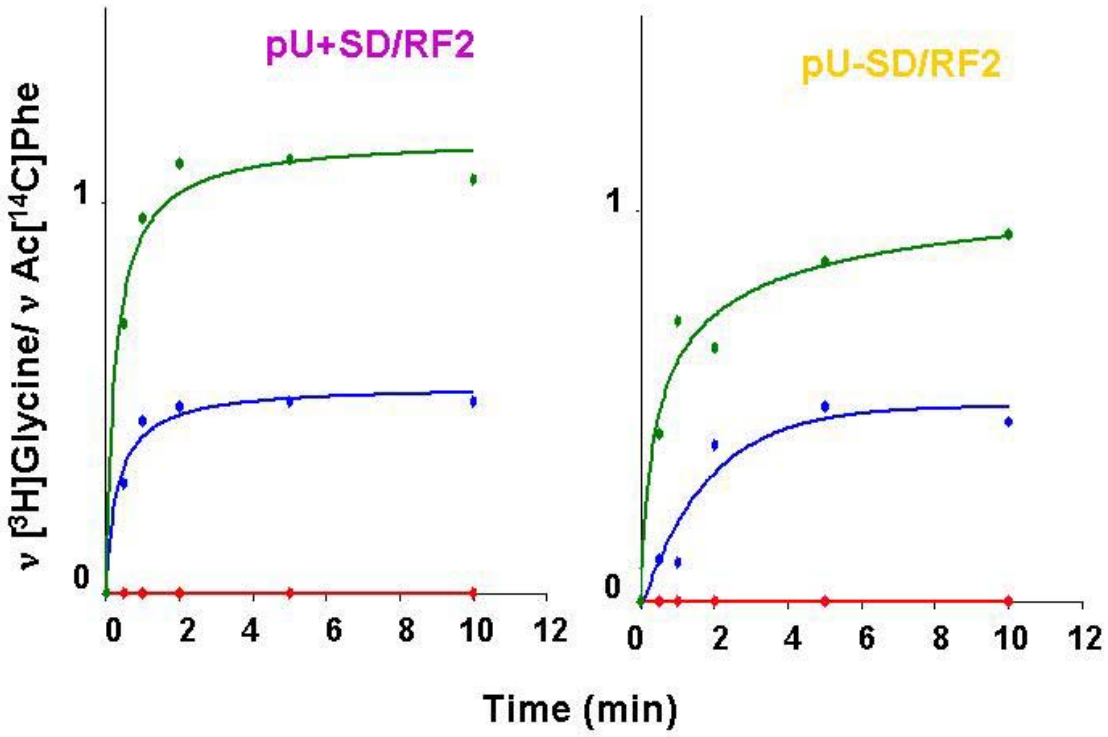


Figure 3.11 AcPhe residue is essential for the complete translation of heteropolymeric region of RF2 mRNAs*. Re-associated 70S were programmed with pU+SD/RF2 and pU-SD/RF2 mRNAs and primmed with Ac[^{14}C]Phe-tRNA^{Phe} (1073 dpm/pmol). Incorporation of [^{3}H]glycine (1073 dpm/pmol) into the polypeptide chain was followed kinetically by hot TCA precipitation. The fraction ν [^{3}H]glycine/ ν Ac[^{14}C]Phe indicates the polypeptide chain length. Translational reactions were performed minus AcPhetRNA plus Phe amino acid (___), plus AcPhetRNA minus Phe amino acid (___) and plus AcPhetRNA plus Phe amino acid (___)*

3.3 Analysis of the frameshift window

Since the S-100 tRNA free fraction was poor in RF2 protein (section 3.1.3, page 77), it can be used to test the frameshifting phenomenon. Taking advantage of the feedback regulation of the RF2 protein, which is dependent on its concentration in the medium, a reproducible system was optimized in function of the exogenous addition of the decoding factor. In this experiment, the amino acid mixture contained aspartic acid as radioactive amino acid marker. Aspartic acid should be the first amino acid incorporated into the polypeptide chain manifesting the +1 new reading frame. Similar to the Gly incorporation that of aspartic acid into the polypeptide chain was normalized relative to the incorporated AcPhe residues.

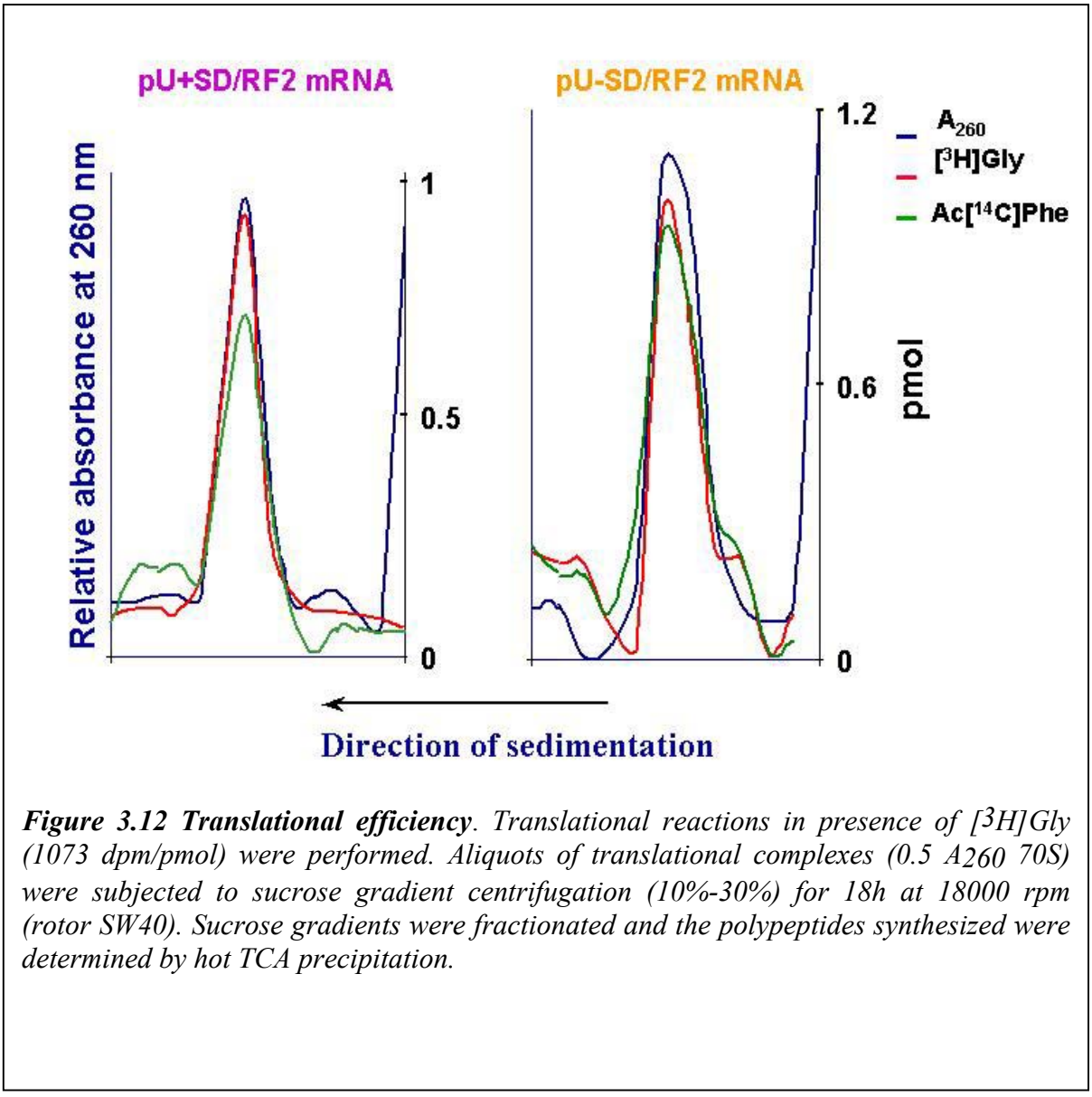


Figure 3.12 Translational efficiency. *Translational reactions in presence of [3H]Gly (1073 dpm/pmol) were performed. Aliquots of translational complexes (0.5 A_{260} 70S) were subjected to sucrose gradient centrifugation (10%-30%) for 18h at 18000 rpm (rotor SW40). Sucrose gradients were fractionated and the polypeptides synthesized were determined by hot TCA precipitation.*

In the first assay the incorporation of the frameshifting signal Asp was slightly exceeding that of AcPhe (molar ratio of incorporated residues about 1.25); demonstrating that every ribosome underwent a frameshift (Table 5). The result confirms that the S-100 tRNA free fraction does not contain functional RF2 protein. Surprisingly, the mRNA lacking the SD sequence was equally efficient in promoting Asp incorporation in the absence of RF2 and thus shows the same degree of +1 frameshifting than the mRNA containing the SD sequence.

Table 5 Frameshifting evaluation

mRNA	ν N-Ac[^{3}H]-Phe (Active fraction)	ν [^{14}C]-Aspartic acid	ν Asp/Active fraction
pU+SD/RF2	0.27 ± 0.012	0.33 ± 0.019	1.27 ± 0.045
pU-SD/RF2	0.33 ± 0.019	0.41 ± 0.012	1.25 ± 0.089

Translational reactions were performed with re-associated 70S primed with Ac[^{3}H]Phe-tRNAPhe (2458 dpm/pmol) in presence of [^{14}C]aspartic acid (460 dpm/pmol) and the frameshifting event was evaluated. Hot TCA precipitation was done and the level of frameshifting corresponded to the fraction of ν [^{14}C] aspartic acid/ ν Ac[^{3}H]Phe.

In order to test a possible dependence of frameshifting on RF2 concentration and, at the same time, to define the best experimental conditions in the study of the translational control of the RF2 protein, frameshifting was studied with increasing amounts of RF2 (T246A) protein.

RF2 titration assays using programmed ribosomes with pU+SD/RF2 resulted in a drastic decrease of the frameshift efficiency on the addition of increasing amounts of release factor 2 (Figure 3.13). Around 50% of the frameshifting was prevented when RF2 was added in a molar ratio of 0.5 (RF2 per ribosomes), this ratio is close to that found *in vivo*. Molar ratios equal or higher than 2 RF2's per ribosomes practically abolished frameshifting.

In order to check if the reduction on the frameshifting level found above was a direct effect of the RF2 dependence termination or due to decreasing efficiency of the translational reactions, the ribosomal active fraction was estimated. The ribosomal active fraction did not change upon the addition of increasing amounts of RF2 (Figure 3.14) but only the incorporation of aspartic acid into the precipitated polypeptide

chain in solution was drastically reduced. This result demonstrated that RF2 induced termination and probably release (see the following section) of the nascent peptide chain on the cost of the frameshifting reaction.

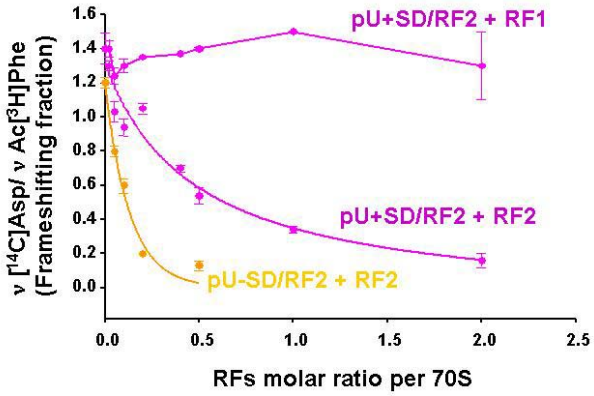


Figure 3.13 Frameshifting fraction in presence of RFs. *Translational reactions in presence of [14C]aspartic acid (460 dpm/pmol) were done. Increasing amount of RF2 and RF1 protein were included in these assays and the effect on the frameshifting level was monitored by the incorporation of [14C]aspartic acid in precipitated fractions of the polypeptide synthesised. ν [14C]Asp and ν Ac[3H]Phe represents the pmol of [14C]aspartic acid and Ac[3H]phenylalanine respectively, normalised per total pmol of 70S precipitated. Fraction ν [14C]Asp/ ν Ac[3H]Phe indicates the polypeptide chain length and frameshifting fraction.*

The same experiment was repeated using the mRNA that lacks the SD sequence (pU-SD/RF2). In this case the RF effect was much more pronounced at low concentration of RF2. At a molar ratio of 0.5 RF2 per ribosome, the frameshifting was practically completely blocked. A 4-fold higher concentration of RF2 was necessary to reach the same effect when pU+SD/RF2 was present (Figure 3.13). This result indicates that our *in vitro* system mimics the *in vivo* situation where at molar ratios below 0.5 RF2 to ribosomes the SD sequence is essential for the frameshifting effect. As a consequence of this result we added RF2 in a molar ratio of 0.3 (RF2: 70S) in the following assays.

In a control assay we tested whether the effects observed in the presence of RF2 are RF2 specific. To this end, the same experiment as described in Figure 3.13 was performed in the presence of the SD containing mRNA (pU+SD/RF2), but now replacing RF2 by increasing amounts of RF1[2]. No influence of the frameshifting efficiency at all was found (Figure 3.13) clearly demonstrating that the effect observed plus the addition of RF2 is specific again underlining the physiological competence of the *in vitro* system applied.

[2] RF1 protein was a kindly gift from Dr. Daniel Wilson, MPI für Molekulare Genetik, Berlin.

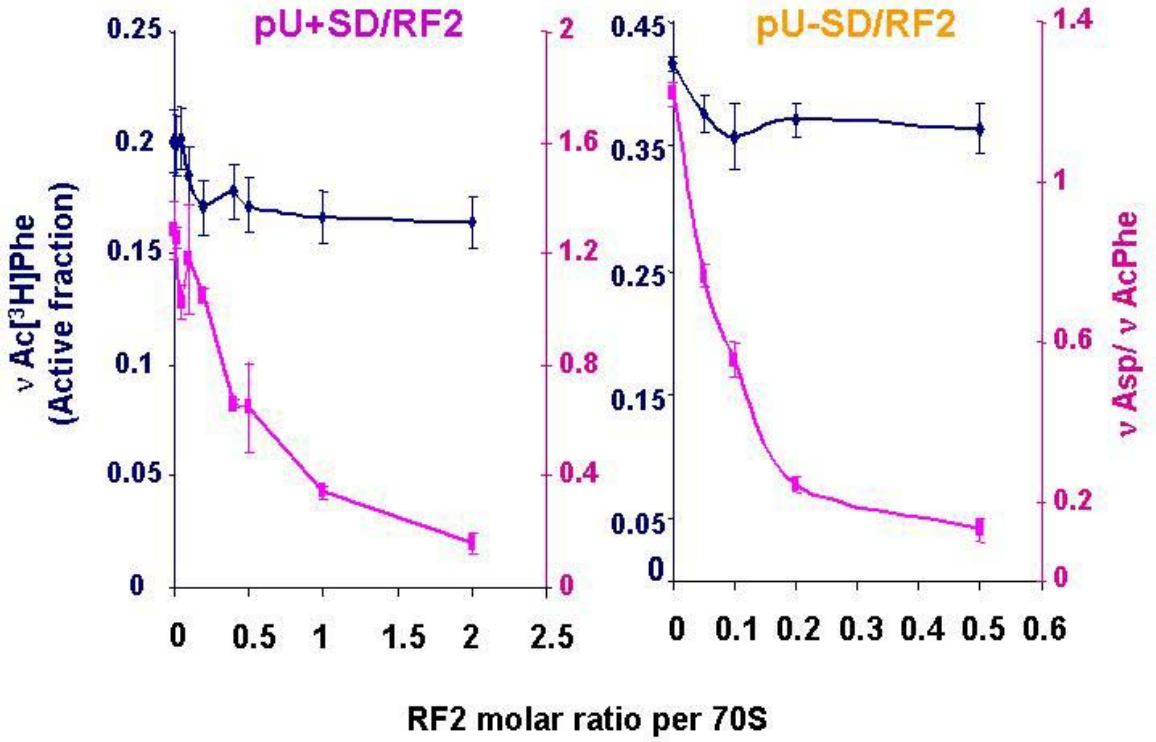


Figure 3.14 Active fraction versus Termination. *Translational reactions were performed as figure 3.13 and the ribosomal active fraction estimated (ν Ac[3H]Phe= Ac[3H]Phe residues/70S). Note the different scales at the x-axes of the two graphs. (--), Asp/AcPhe incorporation; (--), AcPhe incorporation.*

3.4 Protein synthesis termination. An effective in vitro system

The frameshifting assays demonstrated that functional studies on termination of protein synthesis could be carried out *in vitro* efficiently. Additionally, the ionic conditions similar to the physiological ones allow us to draw conclusions concerning the mechanism of termination and frameshifting.

In order to characterise further our system for the RF2 study, the effects on termination, i.e. hydrolysis of the nascent peptide chains, were studied. Translational reactions in the presence of either mRNAs were prepared and aliquots were subjected to sucrose gradient centrifugation. The efficiency of the release of the polypeptide was assessed in the presence or absence of RF2. The translation was measured *via* the incorporation of leucine since this amino acid has four codons in the mRNAs and thus gives a higher signal of the translational reaction.

Two different concentrations of RF2 were tested, and in both the same incorporation of Leu were observed as in the absence of RF2. For example, with the mRNA pU+SD/RF2 an incorporation of about 0.7 Leu was observed under all conditions (Table 6). It follows that with a given mRNA the presence of release factor 2 in translational reactions did not influence the extent of the polypeptide-chain synthesis. Furthermore, since the mRNAs contained four Leu codons and the incorporation of four Leu per Acphe residue was found with both mRNAs, all polypeptide chains were obviously of full length. This means that the internal SD sequence does not influence the quality of the translational process. Note that Asp was not present in these experiments that is used as a signal for frameshifting. Hot TCA precipitation in the experiments described in Table 6 cannot discriminate whether or not the added release factor 2 triggers a termination reaction. This is analyzed in the next experiment. After incubation for protein synthesis the mixture was loaded on a sucrose gradient and the radioactive fraction of the nascent peptide chain still present on the ribosomes was determined in the presence and absence of RF2 by TCA precipitation.

Table 6 Progress of translational reactions in presence of RF2.

mRNA	[RF2]	ν Ac[^{3}H]-Phe (Active fraction)	ν [^{14}C] Leucine	νLeu/Active fraction
pU+SD/RF2	0	0.17 ± 0.001	0.67 ± 0.04	3.9 ± 0.29
	0.3	0.20 ± 0.002	0.72 ± 0.0	3.68 ± 0.03
	0.6	0.15 ± 0.004	0.62 ± 0.046	4.12 ± 0.41
pU-SD/RF2	0	0.51 ± 0.02	1.99 ± 0.058	3.88 ± 0.02
	0.3	0.37 ± 0.04	1.69 ± 0.09	4.61 ± 0.67
	0.6	0.36 ± 0.01	1.55 ± 0.04	4.34 ± 0.03

Translational reactions were performed in presence of RF2 and the [^{14}C]leucine (1000 dpm/pmol) incorporation into the polypeptide chain was evaluated by hot TCA precipitation. Fraction ν [^{14}C]leucine/ν N-Ac[^{3}H]Phe (2458 dpm/pmol) is a measure of the quality of translation, since the mRNAs contain each four codons for Leu.

Hot TCA precipitation in the experiments described in Table 6 cannot discriminate whether or not the added release factor 2 did trigger a termination reaction. This is analyzed in the next experiment. After incubation for protein synthesis the mixture was loaded on a sucrose gradient and the radioactive fraction of the nascent peptide chain still present on the ribosomes was determined in the presence and absence of RF2 by TCA precipitation.

When the mRNA without SD sequence pU-SD/RF2 was present, RF2 hydrolyzed almost 80% of the polypeptide chains, whereas in the presence of both RF2 and the mRNA with SD pU+SD/RF2 only only 30% of the nascent peptide chains were hydrolyzed and thus lost from the ribosome (Figure 3.15).

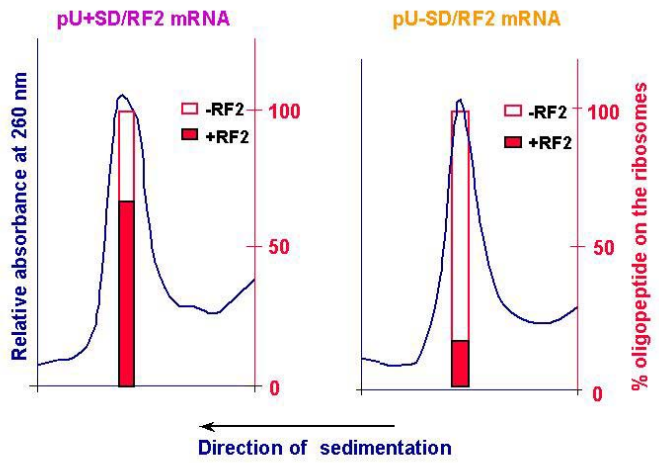


Figure 3.15 Termination efficiency. *Translational reactions in presence of [^{3}H]Leu (1000 dpm/pmol) and RF2 were performed. Aliquots of translational complexes (0.5 A_{260} 70S) were subjected to sucrose gradient centrifugation (10%-30%) for 18h at 18,000 rpm (rotor SW40). Sucrose gradient was fractionated and the polypeptide remained on the 70S was determined by hot TCA precipitation.*

A possible explanation is that the SD-antiSD interaction with its extremely short spacer in front of the P site could foster the +1 frameshifting thus pushing the STOP codon outside the codon window at the A site. The STOP codon at the A site is the signal for RF2 binding and the subsequent hydrolysis of the peptidyl-tRNA at the P site.

3.5 Kinetic evaluation of the frameshifting mechanism

Translational reactions were performed in the absence of RF2, and aliquots were withdrawn at defined intervals of time in order to follow the frameshifting event kinetically.

A +1 frameshifting occurred in ribosomes programmed with both mRNAs. However, in the absence of SD (pU-SD/RF2 messenger) a two times longer period was required in order to obtain the same level of frameshifting as the SD containing mRNA pU+SD/RF2 (Figure 3.16). It follows that the SD sequence promotes or accelerates the frameshifting process.

The frameshifting process looks like to have a lag phase for about 20 sec with both mRNAs (Figure 3.16A), the extent of the frameshift reaction was reached after 2 and 3 min in the presence and absence of SD, respectively.

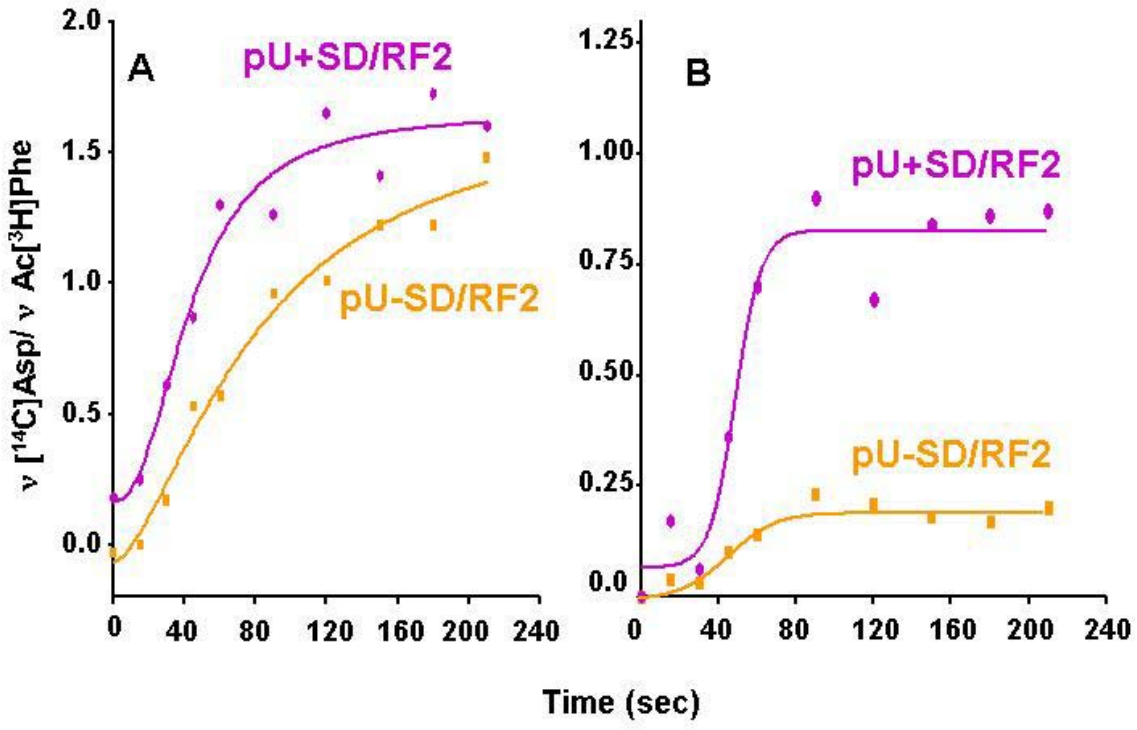


Figure 3.16 Frameshifting kinetics. *Translational reactions were performed as described in Materials and Methods. Aliquots were withdrawn at indicated times and the frameshifting event was evaluated by incorporation of [^{14}C]Asp (460 dpm/pmol) into the recipitated polypeptide chain. Ribosomes were primed with Ac[^{3}H]Phe-tRNA^{Phe} (2458 dpm/pmol) and the frameshift efficiency was determined by the fraction ν [^{14}C]Asp/ ν Ac[^{3}H]Phe.*

Repetition of the same experiments but in the presence of RF2 showed the superiority of the pU+SD/RF2 mRNA over the mRNA lacking the SD sequence concerning the frameshifting efficiency. Concerning the frameshifting event, a 90-

100% efficiency of frameshifting was found in the mRNA contained the SD sequence (Figure 3.16B). However, the lag phase was prolonged to about 40 seconds. We note that in the absence of RF2 the molar ratio of incorporated Asp/AcPhe was exceeding one, whereas in the presence of RF2 the expected maximal level of one was approached.

3.6 The effects of SD on the E site tRNA, termination process and frameshifting

The essential feature of the allosteric three site model is a negative cooperativity between the A and E sites, i.e., an occupied E site leads to a low A-site affinity and *vice versa*, an occupied A site induces a drastic drop of the E site affinity for deacylated tRNA. During the elongation cycle the ribosomes oscillates between PRE and POST states defined by tRNAs at the A and P sites and at the P and E sites, respectively.

Our hypothesis on the mechanism of RF2 regulation uses the fact that the interaction between the SD sequence and the antiSD sequence found at the 3' end of 16S rRNA overlaps with the base-pairing between the first base of E-site codon of the RF2 mRNA and the anticodon of the deacylated tRNA at the E site. We propose that the SD: antiSD interaction sterically clashes with the base pairing at the E site tRNA promoting the release of this tRNA from the ribosome and thus favouring the +1 frameshifting.

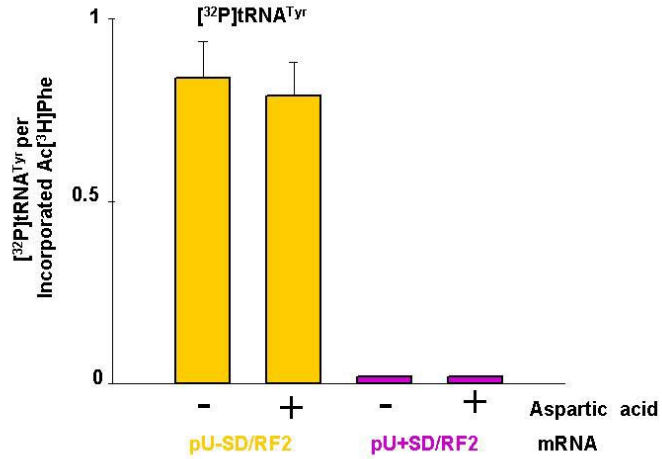


***Figure 3.17 [32P]tRNA^{Tyr} binding**. Translational reactions were done with re-associated 70S programmed with both mRNAs and primed with N-Ac[^{3}H]Phe-tRNA^{Phe} (2458 dpm/pmol). The bound [^{32}P]tRNA^{Tyr} (593 dpm/pmol) was determined via nitrocellulose filtration in the presence and absence of cold aspartic acid. Binding values are given per incorporated Ac[^{3}H]Phe residue.*

In the next experiment we want to test whether indeed the SD sequence of the pU+SD/RF2 mRNA is able to trigger the release of deacylated tRNA^{Tyr} at the

ribosomal E site when the STOP codon is present at the A site. The experiments were conducted in the absence of RF2 factor.

For that purpose, $tRNA^{Tyr}$ was specifically removed from a $tRNA^{bulk}$ preparation that contained all tRNAs present in our mRNAs construct (see section 3.1.4, page 79). This tRNA preparation was supplemented with $[^{32}P]$-$tRNA^{Tyr}$.

Nitrocellulose filtration assays after a translation of the mRNAs (in the absence of aspartic acid thus preventing the manifestation of a frameshift event) should demonstrate to what degree $[^{32}P]tRNA^{Tyr}$ was present at the E site. The results showed binding values of -$[^{32}P]$-$tRNA^{Tyr}$ up to 0.8 per ribosome when pU-SD/RF2 mRNA was present. In sharp contrast, no binding of $tRNA^{Tyr}$ at all was found in the presence of SD in the mRNA (Figure 3.17).

The next point to be addressed was the relationship of $tRNA^{Tyr}$ binding and the frameshifting mechanism. Translational reactions were done in the presence of amino acid $[^{14}C]$ aspartic acid and $[^{32}P]tRNA^{Tyr}$ (Figure 3.18). Incorporation of the frameshifting signal $[^{14}C]$ aspartic acid was determined by hot TCA precipitation. A reciprocal appearance of $tRNA^{Tyr}$ at the E site and Asp incorporation became evident: A high $tRNA^{Tyr}$ presence at the E site was related with a low Asp incorporation in the presence of pU-SD/RF2 mRNA (no SD), whereas in the presence of pU+SD/RF2 mRNA the $tRNA^{Tyr}$ at the E site was completely released combined with a high Asp incorporation In conclusion, SD: antiSD interaction removes deacylated $tRNA^{Tyr}$ from the ribosomal E site and facilitates ribosomes to move +1 nucleotide downstream thus allowing the incorporation aspartic acid.

3.7 Location of Shine-Dalgarno sequence. Effect on frameshifting

Normally the distance between the initiator AUG and the SD sequence is 4 to 12 nucleotides, most common is a distance of 5 to 7 nucleotides measured from the A nucleotide of the AUG at the P site. However, upstream of the frameshift window of the RF2 mRNA the distance between the SD sequence and the P site tRNA comes to only two nucleotides. The short distance is a characteristic for the mRNA for RF2, and previously the importance of the SD: antiSD interaction for the translational regulation of RF2 was shown (Weiss *et al*., 1988). The next experiment addresses the importance of the distance of the SD sequence from the peptidyl-tRNA residing at the P site. To this end, a set of messengers were constructed, where the SD

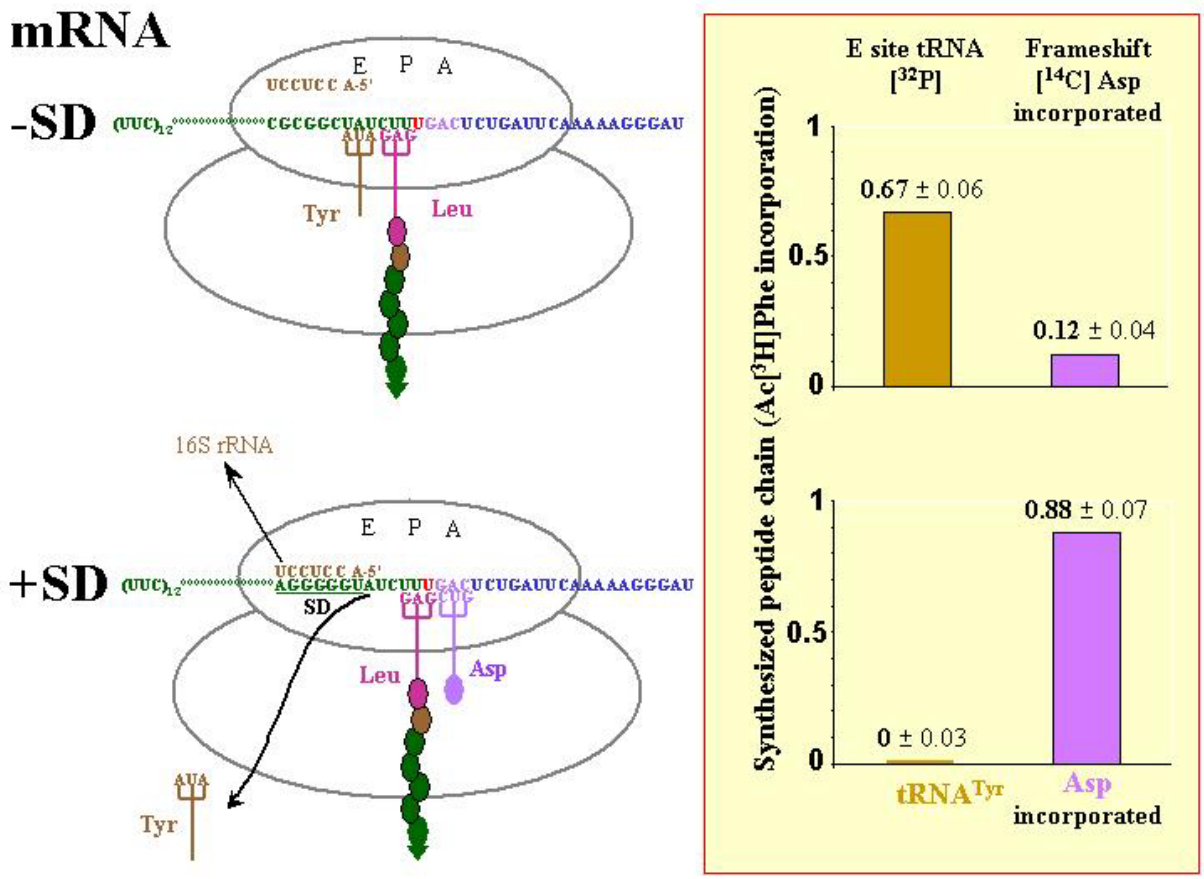


Figure 3.18 Frameshifting and [32P]tRNA^{Tyr} binding evaluation. *Re-associated 70S programmed ribosomes with both $(UUC)_{12}$ mRNAs and, primmed with Ac[3H]Phe-tRNA^{Phe} (2458 dpm/pmol) were subjected to translational reactions in presence of [32P]tRNA^{Tyr} (590 dpm/pmol) and [14C]Asp (460 dpm/pmol). Binding assays and hot TCA precipitation were performed . Values on the bars represents the fraction of active ribosomes that bound tRNA^{Tyr} at their E site or incorporated an Asp residue thus demonstrating a +1 frameshifting.*

sequence was shifted by 2 and 6 nucleotides upstream from the wild type position, pU+SD(-2)/RF2 and pU+SD(-6)/RF2, respectively.

Figure 3.19 compiles the results. Hot TCA precipitation of translational reactions of ribosomes programmed with pU+SD(-2)/RF2 or pU+SD(-6)/RF2 in the presence of RF2 showed no incorporation of [^{14}C]aspartic acid into the nascent peptide chain. It follows that frameshifting is abolished if the SD sequence is displaced from its right position in the upstream direction.

Nitrocellulose filtration assays of the translational mixture demonstrated that [^{32}P]tRNA^{Tyr} occupied quantitatively E-site in the case of pU+SD(-6)/RF2

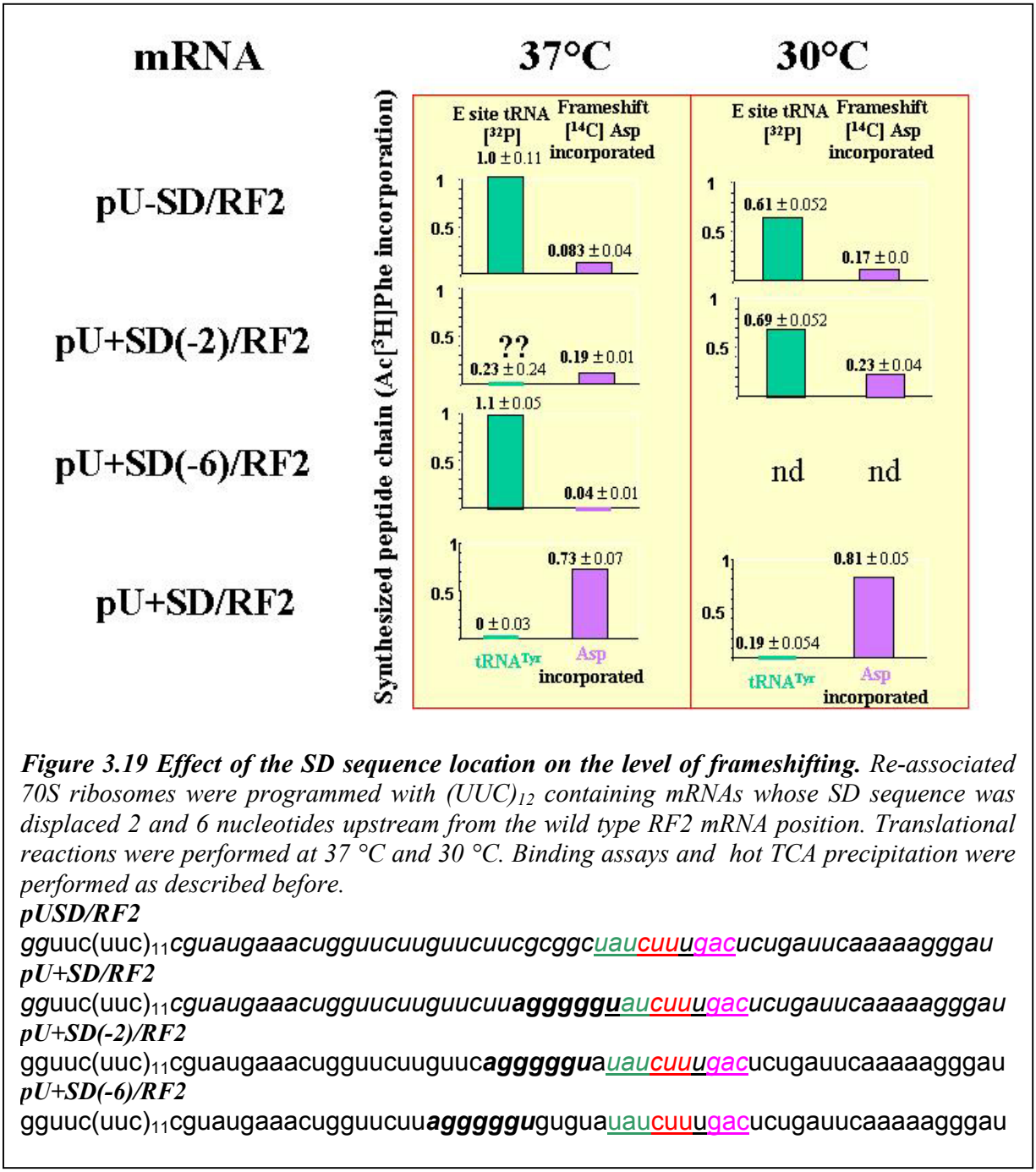


Figure 3.19 Effect of the SD sequence location on the level of frameshifting. *Re-associated 70S ribosomes were programmed with $(UUC)_{12}$ containing mRNAs whose SD sequence was displaced 2 and 6 nucleotides upstream from the wild type RF2 mRNA position. Translational reactions were performed at 37 °C and 30 °C. Binding assays and hot TCA precipitation were performed as described before.*

pUSD/RF2

*gg*uuc(uuc)$_{11}$*cguaugaaacugguucuuguucuucgcggcuaucuuugacucugauucaaaaagggau*

pU+SD/RF2

*gg*uuc(uuc)$_{11}$*cguaugaaacugguucuuguucuu****aggggguaucuuugac****ucugauucaaaaagggau*

pU+SD(-2)/RF2

gguuc(uuc)$_{11}$cguaugaaacugguucuuguuc***aggggguaucuuugac***ucugauucaaaaagggau

pU+SD(-6)/RF2

gguuc(uuc)$_{11}$cguaugaaacugguucuu***aggggguguguauaucuuugac***ucugauucaaaaagggau

messenger, i.e., the presence of the E-site tRNA accompanied the absence of frameshifting. However, in the case of pU+SD(-2)/RF2 mRNA, an unexpected result was observed: no [^{32}P]tRNA^{Tyr} at the E site was combined with the lack of [^{14}C]aspartic acid incorporation (Figure 3.19). Because in the latter mRNA the SD sequence is only two nucleotides displaced from the wild type position, we wondered whether the E-site tRNA is present during translation thus preventing frameshifting, but the antiSD-SD interaction represents still a relevant hindrance. The latter thus

might remove the deacylated $tRNA^{Tyr}$ from the ribosomal E site during the time that is taken for the filtration process. If so, this kinetic effect would possibly be overcome by a reduction of the incubation temperature. Therefore, the experiment was repeated at 30 instead of 37°C.

Indeed, figure 3.19 showed that at 30°C ribosomes programmed with pU+SD(-2)/RF2 mRNA still bound a good complement of [^{32}P]-$tRNA^{Tyr}$. It is clear that during protein synthesis this tRNA fully occupied the E site thus preventing a frameshift but allowing a termination event. However, the stability of the E site tRNA was weakened and thus lost during the processing of the samples for filtration at 37 °C.

3.8 Di-peptide formation

For the next experiments a model reaction was designed to shed more light onto the frameshifting mechanism. We wanted to known, whether the presence of two tRNAs on ribosomes during the elongation cycle is the main factor that determines maintenance of the correct reading frame for the ribosomes. Would the presence of only one tRNA on the ribosome allow frameshifting? Therefore, a di-peptide system was developed.

The principle is the following: an AcPhe-tRNA was bound to the P site with or without a $tRNA_f^{Met}$ at the E site (POST and Pi complex, respectively). At the A site is a STOP codon, and in the +1-frame the codon for aspartic acid. The formation of the di-peptide AcPhe-Asp was measured *via* HPLC with and without an occupied E site.

New RNAs were constructed, propagated, transcribed and purified for this purpose.

Table 7. mRNA sequences

	mRNA name	Sequence
SEE FIGURE 3.2 SECTION 3.1.2.1, PAGE 73	YFstopC	**gggaaaacaaaacaaaacUAC-UUC-UGAC-aaaacaaaacaaaac**
	SDYFstopC	**ggaaaaacaaaac*aggggg*UAC-UUC-UGACaaaacaaaacaaaac**
	MFstopC	**gggaaaacaaaacaaaacAUG-UUC-UGAC-aaaacaaaacaaaac**

aggggU: SD sequence. Uppercase sequences: mRNA coding region

3.9.1 Di-peptide formation on non-programmed ribosomes?

Spirin et al. (Belitsina *et al*., 1981; Belitsina *et al*., 1982) have described that di- and oligo-peptides can be formed on empty ribosomes. In order to prove this

assumption, control experiments were performed and a di-peptide analysis was done as described in Materials and Methods.

Ribosomes were programmed with YFStopC mRNA. Binding tests of Pi complexes before addition of the ternary complex for the +1 frame showed an occupancy of N-Ac[^{3}H]Phe-tRNA^{Phe} of around 40-50%. Puromycin reaction revealed that 90% of the N-AcPhe-tRNA^{Phe} was at the ribosomal P site (Table 8). Under these experimental conditions the yield of AcPhe-puromycin exceeded the binding values.
It was shown previously that only non-programmed 70S ribosomes show this behavior: AcPhe-tRNA at the P site react with puromycin, the resulting deacylated tRNA at the P site can be chased by an AcPhe-tRNA that also can undergo a puromycin reaction. In this way a ribosome can perform more than one puromycin reaction (A. Potapov and K.H. Nierhaus, unpublished). After the addition of ternary complexes, the AcPhe-tRNA binding values decreased for about 40%, but non-programmed ribosomes lost completely the N-AcPhe-tRNA bound initially without any traces of dipeptides. These observations demonstrate that non-programmed AcPhe-tRNA is bound in an unstable fashion and is completely chased by the ternary complexes. Obviously, ribosomes require an mRNA template in order to catalyze a peptide bond (Figure 3.20A). On the contrary, Pi complexes made in the presence of mRNA showed a peak corresponding to the di-peptide N-Ac[^{3}H]Phe-[14C]Asp acid (Figure 3.20C), since in this fraction equimolar pmol amounts of N-Ac[^{3}H]Phe and [^{14}C]Asp were found. Remarkably, on nitrocellulose filters no traces of [^{14}C]aspartic acid were found with programmed ribosomes.

This means that the di-peptide fraction formed after addition of ternary complex quantitatively dropped off the ribosome so that only N-Ac[^{3}H]Phe-tRNA^{Phe} remained on the filters.
The dipeptide fraction formed (around 10-16% of the AcPhe present on Pi complexes) seems to be equal to the difference between the bound N-Ac[^{3}H]Phe-tRNA^{Phe} before and after the addition of ternary complex. For example, Pi complexes without EF-G in Table 8: 0.13 (dipeptides) + 0.3 (AcPhe after ternary-complex addition) ≈ 0.49 AcPhe before ternary-complex addition. This result indicates that the di-peptide formed with the frame-shifted Asp-tRNA quantitatively dropped off the ribosome supporting the nitrocellulose-filtration results reported in the previous paragraph.

Table8 Di-peptide analysis on empty re-associated 70S and ribosomal Pi and Post-complexes

		Binding Test				
		Before addition of ternary complexes	**After addition of ternary complexes**	**Puromycin before addition of ternary complexes**	**% P site Occupation**	**Di-peptide Fraction**
Ribosomal State	**EF-G**	**νAcPhe**	**ν AcPhe**	**ν AcPhe**		**ν**
Empty 70S	-	0.33±0.005	0.007±0.005	nd		0
	+	0.32±0.04	0.006±0.003	nd		0
Pi complex	-	0.49±0.003	0.30±0.02	0.60± 0.04		0.13
	+	0.50±0.03	0.29±0.003	0.67±0.002	89.6	0.17
Post complex	-	0.58±0.005	-	0.09±0.006		-
	+	0.70±0.093	0.55±0.005	0.82±0.0316	0	0.16

Empty 70S and Pi and Post complexes of ribosomes programmed with YFstopC mRNA were prepared and binding tests were performed before and after the addition of ternary complexes [^{14}C]Asp-tRNA·EF-Tu·GTP. The ratio of puromycin reaction (minus EF-G/ plus EF-G) gives the percentage of P site occupation. Di-peptide fractions were calculated as pmol dipeptides per totally bound AcPhe residues. The stoichiometry of [^{14}C]Asp (460 dpm/pmol) and N-Ac[^{3}H]Phe (2458 dpm/pmol) was equal to one in the dipeptide fraction

POST complexes were also prepared and analyzed for di-peptide formation. In these experiments deacylated tRNA^{Tyr} was used to block the P site in a first incubation, N-Ac[^{3}H]Phe-tRNA^{Phe} was bound to the A site and then the tRNA·mRNA complex was translocated.

Before the addition of the ternary complexes, higher binding values (0.6 to 0.7) of AcPhe-tRNA were found than in the preceding experiment (see Table 8). 90% of the ribosomes carrying an AcPhe-tRNA were at the pre-translocational state. Pre-translocational states became POST states upon addition of EF-G.

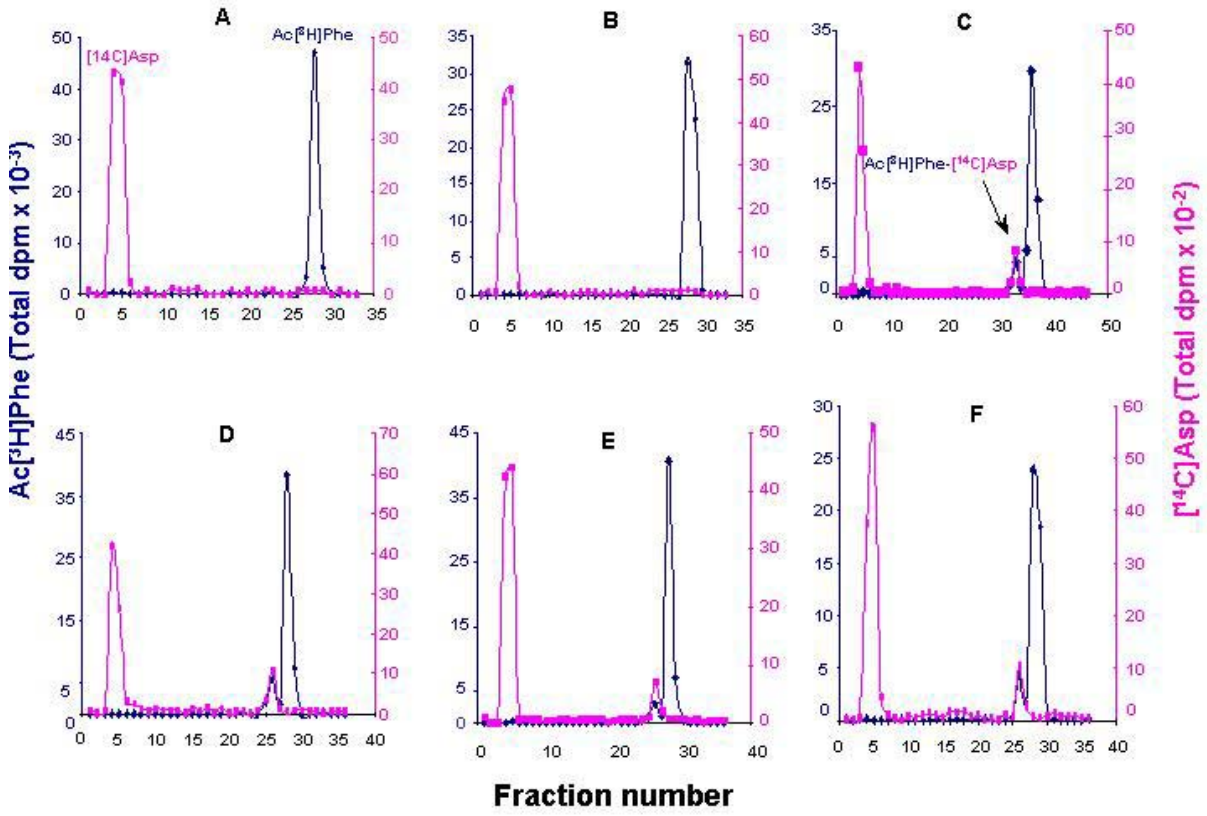


Figure 3.20 Di-peptide analysis. *Di-peptide formation was performed on: Empty ribosomes minus EF-G (A), and plus EF-G (B). Pi ribosomal complex programmed with YFstopC mRNA minus RF2 (C). Post-ribosomal complex programmed with YFstopC mRNA minus RF2 (D). Pi ribosomal complex programmed with MFstopC mRNA minus RF2 (E). Post-ribosomal complex programmed with MFstopC mRNA minus RF2 (F).*

Translocation efficiency was practically 100%. A slight puromycin overreaction 0.82 as compared to 0.70 bound AcPhe (Table 8) indicates that obviously a small fraction of the ribosomes did not carry an mRNA.

Di-peptide analysis on post-translocational complexes did not show significant differences as compared to Pi complexes, i.e., practically the same amounts of di-peptide around 0.15 were obtained (Table 8). Newly, the amount of AcPhetRNA before the addition of the frameshifting Asp-tRNA corresponded to the amount of AcPhe-tRNA after ternary complex addition plus the amount of di-peptides (0.70 ≈0.55 + 0.16) again suggesting that the formed dipeptides fell quantitatively from the ribosome.

Our results indicate that in absence of RF2 the same amounts of di-peptide were formed in both ribosomal complexes P_i and POST. Does this mean that di-peptide formation is independent of the functional state of the ribosome?

In these assays RF2 was not present. In order to approach better physiological conditions, we repeated the experiments, but now in the presence of RF2.

3.9.2 Di-peptide formation in the presence of RF2: Pi versus POST complex

Ribosomes programmed with YFstopC or MFstopC mRNA were analyzed for their capabilities to form di-peptides. RF2 was mixed with the ternary complex and then added to the reaction mix.

In the previous experiments, where no RF2 was added, di-peptide analysis from ribosomal P_i- and POST-complexes prepared with either YFstopC or MFstopC messengers showed practically the same amounts of di-peptides (about 0.15; Table 9). With the addition of RF2 (0.3 molar ratio RF2:70S), a remarkable reduction of di-peptide formation of about 55% (0.06 versus 0.13) was observed on Pi complexes (Table 9), whereas the dipetide formation under the same conditions was reduced for only 25% on POST complexes (0.12 versus 0.16). Decreased di-peptide formation was accompanied by a strong reduction of the bound AcPhe-tRNA obviously due to the presence of RF2 (the binding of AcPhe decreased with increasing addition of RF2, Table 9). This reduction of AcPhe-tRNA binding was more pronounced on Pi complexes than Post-complexes (75% on ribosomes programmed with YFstopC and MFstopC mRNAs, respectively; e.g. with YFstopC mRNA: 0.36 versus 0.09). Obviously, RF2 hydrolyzes more effectively the peptidyl-tRNA, if the ribosomal E site is free. Interestingly, there was more hydrolysis of AcPhe residues than reduction of di-peptide formation, suggesting that RF2 recognizes the UGA stop codon faster than the dipeptide formation with the +1 frame Asp-tRNA that requires a sliding of the ribosome.

The maximum concentration of release factor 2 used in the dipeptide system was the same as in the translational reaction for the RF2 frameshifting study, however this concentration was not enough to hydrolyze the N-AcPhe-tRNAs bound at the P site completely in the POST states, whereas almost quantitatively the AcPhe-tRNA was hydrolyzed on the P_i states.

The extent of dipeptide formation here and in Table 8 is about 0.12 up to 0.2 and thus much less than the frameshift observed with the RF2 mRNAs (section 3.6, page 96). One reason might be that in the latter case the peptidyl-tRNA can change the frame in the +1 direction without losing codon-anticodon interaction, whereas in the dipeptide assays reported here the AcPhe-tRNA at the P site will loose its capability of codon-anticodon interaction if it would slide downstream by one nucleotide for dipeptide formation. This inability of the AcPhe-tRNA might be also the main reason why here the dipeptide falls quantitatively from the ribosome. Table 9 also shows that the amounts of [^{32}P]-labeled tRNA^{Tyr} or tRNA$_f^{Met}$ bound to the E sites of POST complexes maintained constant upon increasing amounts of RF2 added. It follows that release factor 2 does not remove the deacylated tRNA from the E site. The almost stoichiometric binding values of the E site labeled tRNAs and Ac[^{3}H]Phe residues in both POST ribosomal complexes before the ternary complex addition in the absence of RF2 (for example 0.92 for the E site tRNA and 0.80 for the P site tRNA in the YFstopC POST complexes), indicate that indeed two tRNAs are simultaneously bound on the ribosomes. Furthermore, the puromycin reaction showed quantitative translocation of PRE complexes. After addition of the ternary complex the binding of E site [^{32}P]tRNA^{Tyr} decreased about 60% (from 0.92 to 0.41), whereas the Ac[^{3}H]Phe-tRNA^{Phe} binding decreased by only 30% (0.80 to 0.56) in the absence of RF2 (see Table 9). It seems to be that an active mechanism operates on the ribosomes that provokes more release of the E site tRNA than the loss of the AcPhe label due to dipeptide formation.

After the addition of the ternary complex and in the absence of RF2 the loss of E-site tRNA exceeds the decrease of the AcPhe label. With the YfstopC mRNA the E site reduction is 0.51 (=0.92-0.41) corresponding to a decrease of the AcPhe label at the P site of 0.24 (= 0.80-0.56). The corresponding numbers with the MfstopC mRNA are 0.53 (0.64-0.11) and 0.33 (0.73-0.4), whereas peptide bond formation is only 0.22. One explanation is that the ternary complex provokes E site release even if peptide bond formation does not occur. Since peptide bond formation requires an accommodation of the tRNA at the A site, the conclusion is that the allosteric interplay between A site and E site is triggered BEFORE the aminoacyl-tRNA is full adapted at the A site ready for peptide-bond formation.

Table 9 Di-peptide analysis

			Binding (before)			Binding (after)			
			E site	P site			E site	P site	
Ribosomal state	**mRNA**	**EF-G**	**ν [^{32}P]-tRNA^{Tyr}**	**ν Ac[^{3}H]Phe**	**Puromycin**	**RF2**	**ν [^{32}P]-tRNA^{Tyr}**	**ν Ac[^{3}H]Phe**	**Dipeptide fraction**
P_i complex	YFstopC	-	-	0.67±0.02	0.87±0.0	0	-	0.36±0.005	0.18
						0.1	-	0.28±0.004	0.16
						0.3	-	0.087±0.003	0.07
Post complex	YFstopC	+	0.92±0.04	0.80±0.0	0.79±0.004	0	0.41±0.013	0.56±0.012	0.16
						0.1	0.42±0.024	0.46±0.017	0.15
						0.3	0.39±0.008	0.31±0.018	0.13
P_i complex	MFstopC	-	-	0.46±0.008	0.69±0.0	0	- 0.16±0.006		0.15
						0.1	-	0.05±0.029	0.10
						0.3	-	0.023±0.003	0.06
Post complex	MFstopC	+	0.64±0.022	0.73±0.011	0.73±0.035	0	0.11±0.003	0.4±0.007	0.22
						0.1	0.10±0.008	0.32±0.01	0.18
						0.3	0.11±0.003	0.12±0.016	0.12

Ribosomal Pi and Post- complexes were formed as described in material and methods. Enzymatic A site binding was performed in presence of [^{14}C]Asp-tRNA^{Asp}:EF-Tu:GTP. Di-peptide was analyzed by HPLC and aliquots were withdrawn and the binding test was done. Post-complexes were prepared with their E site tRNA labelled with [^{32}P]. Binding ***before*** *and* ***after*** *ternary complex addition was analyzed. Puromycin reaction indicated the specific tRNA location*

3.10 Translocation efficiency

Classical elongation-cycle assays were performed on ribosomes programmed with the three mRNAs YFstopC, MFstopC and SDYFstopC. PRE complexes were prepared in the following way: [^{32}P]-labeled tRNA^{Tyr} or tRNA$_f^{Met}$ were used to block the P site in a first incubation, then Ac[^{3}H]Phe-tRNA^{Phe} was added and a translocation reaction was performed with the help of EF-G·GTP. Neither RF2 nor ternary complex were present in the reaction mix.

Control experiments with MFstopC mRNA did not show a repetitive puromycin reaction (repetitive reaction means that one ribosome made more than one puromycin reaction with the consequence that the values of the puromycin reaction could be exceed those of the binding). This finding indicates that the ribosomes were successfully programmed with this mRNA. Around 40% of ribosomes bound one Ac[^{3}H]Phe-tRNA^{Phe} at the A site with a high specificity (90%)[3]. Stoichiometric binding values between [^{32}P]tRNA^{Met} and Ac[^{3}H]Phe-tRNA^{Phe} were obtained indicating that the fraction of ribosomes carrying tRNAs contained almost exclusively two tRNAs simultaneously per the ribosomes.

Ribosomes programmed with YFstopC mRNA showed a similar behavior as that seen with the control MFstopC mRNA. Here, better Ac[^{3}H]Phe-tRNA^{Phe} binding was obtained (about 0.7 per ribosome) and the binding of [^{32}P]tRNA^{Tyr} exceeded that of the former one by a factor of 1.4-1.6. The puromycin reaction ± EF-G indicated a good homogeneity of the preparation (about 82%; (0.1:0.55)x100) and no release of tRNAs was observed in the course of translocation (Table 10). Ribosomes were successfully programmed with YFstopC mRNA since no repetitive puromycin reaction was observed as in the control experiment.

We called the third mRNA SDYFstopC, since it is identical to the YFstopC mRNA except that it contained a SD sequence at the same position as the RF2 model mRNA pU+SD/RF2 (see figure 3.2, page 72). The results obtained with this mRNA strikingly deviated from those of the previous assays described before. [^{32}P]tRNA^{Tyr} added in the first incubation of ribosomes programmed with this mRNA did not block the P site properly. Highly repetitive puromycin reaction was observed, as practically nothing of the bound Ac[^{3}H]Phe-tRNA^{Phe} was found at the A site (Table 10). Therefore, in the presence of this mRNA a PRE complex with tRNA^{Tyr} and

AcPhe-tRNA cannot be formed. Obviously, the SD sequence near to the codons for Tyr and Phe prevents *via* steric hindrance the binding of these tRNAs. As a consequence, these mRNA and tRNAs bind mutually exclusive to the ribosome.

Table 10. Translocation efficiency.

		Binding			Puromycin
		E site binding		P site binding	
mRNA	**EF-G**	**ν [^{32}P]tRNA^{Tyr}**	**ν [^{32}P]tRNA$_f^{Met}$**	**ν AcPhe**	**ν AcPhe**
YFstopC	-	1.08 ± 0.008		0.67 ± 0.03	0.10 ± 0.002
	+	1.05 ± 0.002		0.72 ± 0.01	0.55 ± 0.06
MFstopC	-		0.5 ± 0.007	0.41 ± 0.004	0.019 ± 0.0006
	+		0.37 ± 0.03	0.36 ± 0.04	0.19 ± 0.004
SDYFstopC	-	0.39 ± 0.02		0.11 ± 0.01	0.68 ± 0.01
	+	0.40 ± 0.04		0.13 ± 0.01	0.71 ± 0.003

Pi and Post complexes of re-associated 70S ribosomes programmed with different mRNAs were prepared and the non-enzymatic A site binding of N-Ac[^{3}H]Phe-tRNA^{Phe} (2458 dpm/pmol) was evaluated. Pre-translocational complexes were prepared with [^{32}P] labelled tRNA (Tyr or fMet) and translocation was done plus the addition of EF-G. Ratio of puromycin reaction minus EF-G per plus EF-G gives the percentage of P site occupation.

[3] Ratio of puromycin reaction minus EF-G over plus EF-G gives the P site occupation