The bacterial ribosome:
Testing a new concept for translational initiation

APPENDIX: Solving preparation problems for tmRNA•70S complexes

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vorgelegt von Daniela Wittek

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Gutachter:

1. Prof. Dr. Rupert Mutzel

2. Prof. Dr. Knud H. Nierhaus

Summary

Protein synthesis encompasses three universal steps initiation, elongation and termination. The standard model for initiation is that the small 30S subunit finds the initiation signals on an mRNA with the help of three monomeric factors IF1, IF2 and IF3. IF1 and IF3 are believed to bind exclusively to the 30S subunit rather than to 70S ribosomes.

This 30S-binding mode as standard initiation type cannot easily be reconciled with a number of observations. We developed a hypothesis according to which these inconsistencies are resolved, namely the 70S-scanning mode saying that a 70S does not dissociate after the translation of a cistron, but is able to scan in both directions on the mRNA checking for another initiation signal of a downstream cistron. In this thesis we present *in vitro* evidence that support this hypothesis:

1. First evidence that beyond the standard 30S initiation mode another mode might exist was provided with a fully synthetic *in vitro* system for the translation of GFP. We could demonstrate that the optimal effect for GFP synthesis was obtained with tightly coupled 70S ribosomes rather then with isolated subunits, IF3 was essential and IF1 important, the latter one stimulated GFP synthesis by more than 3-fold.
2. We constructed a bicistronic mRNA coding for Renilla and Firefly luciferase, respectively, without any secondary structure in the intercistronic region. Blocking translation of the first *via* antisense-DNA or binding antisense-DNA to the intercistronic region blocked seriously the translation of the second one, whereas the monocistronic Firefly mRNA was hardly effected demonstrating that a 70S leaving the first cistron is involved in the translation of the second one.
3. Next we synthesized a model mRNA containing Phe-stop codons and downstream Met-Lys codons with a Shine-Dalgarno sequence before. With this mRNA we could construct a post-termination complex carrying a deacylated $tRNA^{Phe}$ in the P site with a stop codon at the A site. Adding fMet-tRNA triggered a movement towards the downstream AUG. This result was obtained not only with re-associated 70S ribosomes but also with crosslinked 70S, which could not anymore dissociate. This result proves the 70S can scan downwards from the UUC codon to the next cistron start site.

4. We overexpressed and purified five factors and proteins and together with the factors, which were available in the lab a complete analysis of the components essential for scanning could be performed. Surprising results were obtained: (i) fMet-tRNA alone could provoke scanning of the 70S down to the initiation site. (ii) Under more physiological conditions in the presence of all factors the initiation factors were essential and the elongation factors and RRF improved the scanning effect.

These results establish a new initiation mode for bacterial translation, a mode that we termed 70S-scanning mode.

Zusammenfassung

Die zelluläre Proteinsynthese besteht aus drei universellen Schritten: Initiation, Elongation und Termination. Das Standardmodell der bakteriellen Initiation beschreibt die kleine ribosomale Untereinheit, die mit der Hilfe der monomeren Faktoren IF1, IF2 und IF3 Initiationssignale findet und die Translation einleitet. Diese Initiationsfaktoren sind als 30S spezifische Proteine bekannt, die nicht an 70S Ribosomen binden.

Es ist schwierig diese 30S-Initiation mit einer Anzahl von Beobachtungen in Einklang zu bringen. Daher haben wir eine Hypothese entwickelt, die es erlaubt diese Unstimmigkeiten zu klären. Wir schlagen ein Modell vor, das davon ausgeht, dass 70S Ribosomen nach der Translation eines Cistrons nicht Dissoziieren und die mRNA verlassen, sondern in der Lage sind in beide Richtungen - upstream und downstream des Stoppkodons – zu scannen bis sie ein neues Initiationssignal erreichen und dort erneut Proteinsynthese einleiten. Diesen Modus nennen wir 70S-Scanning-Initiation. In dieser Doktorarbeit präsentieren wir mehrere Indizien aus *in vitro* Versuchen, die diese Hypothese stützen:

1. Den erste Anhaltspunkt, das jenseits der 30S-Modus eine andere Form der Initiation existiert, konnten wir mit einem synthetischen *in vitro* System für die Translation von GFP erbringen. Wir konnten zeigen, dass optimale GFP Synthese nur mit 70S Ribosomen, nicht aber mit Untereinheiten, möglich war. IF1 war für diesen Vorgang wichtig (stimulierte GFP-Synthese 3x) und IF3 sogar essentiell.
2. Des Weiteren konstruierten wir eine bi-cistronische mRNA, die sowohl für Renilla- als auch für Firefly-Luziferase kodiert und keine Sekundärstruktur in der intercistronischen Region (IR) besitzt. Durch die Blockade des ersten Cistrons oder der IR durch Bindung von “anti-sense DNA” konnten wir die Translation des zweiten Cistron dramatisch reduzieren. Dies war mit einer monocistronischen mRNA nicht möglich und wies darauf hin, dass 70S Ribosomen, die für die Translation des ersten Cistrons verantwortlich sind, auch an der Translation des darauf folgenden, zweiten Cistrons beteiligt sind.
3. Ferner synthetisierten wir eine mRNA, die im ersten Cistron ein Kodon für Phe als auch ein Stoppkodon besaß und downstream im zweiten Cistron ein Kodon für Met und Lys, mit einer vorangehenden Shine-Dalgarno-Sequenz. Mit dieser mRNA waren wir in der Lage Posttermination skomplexe

herzustellen, die eine deacylierte $tRNA^{Phe}$ in der P-Stelle besaßen und in der A-Stelle ein Stoppkodon aufwiesen. Durch die Zugabe von fMet-tRNA waren wir in der Lage eine Bewegung des Ribosomens zum „downstream“ AUG auszulösen. Dieses Ergebnis konnten wir nicht nur mit reassoziierten 70S sondern auch mit chemisch modifizierten Ribosomen, die nicht dissoziieren können, erzielen. Dieses Resultat beweist, dass 70S in der Lage sind nach der Termination eines Cistrons zum Startkodon des nächsten zu scannen.

4. Letztendlich überexpremierten und reinigten wir fünf Proteine, die wir mit den in der Gruppe bereits vorhandenen Faktoren auf Ihrer Funktion im 70S-Scanning-Modus testeten. Einige überraschende Ergebnisse wurden erbracht: (i) fMet-tRNA ist in der Lage das Scannen eines 70S Ribosomens auszulösen (ii) unter physiologischen Bedingungen, d.h. in der Präsenz aller an der Proteinsynthese beteiligten Faktoren, scheinen die Initiationsfaktoren essentiell für das 70S-scannen zu sein. Darüber hinaus haben wir auch eine wichtige Funktion von Elongationsfaktoren und RRF in diesem neuen Initiationsmodus nachweisen können.

Die Resultate führten zu Aufklärung eines neuen Initiationsmechanismus in Bakterien, diesen Mechanismus nennen wir 70S-Scanning-Initiation.

Abbreviation

Translation specific

30S	small ribosomal subunit of *E. coli*
50S	large ribosomal subunit of *E. coli*
70S	*E. coli* ribosome
aa	amino acid
aa-tRNA	aminoacyl-tRNA
Acaa-tRNA	N-Acetyl-Aminoacyl-tRNA
AcPhe-tRNA^{Phe}	N-Acetyl-Phenylalanyl-tRNA^{Phe}
A - Y	amino acids in the „one letter code”
BSA	bovine serum albumin
E. coli	*Escherichia coli*
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
GTP	guanosine-5’-triphosphate
GDPNP	guanosine-5’-(β-γ-Imino)-triphosphate
GDPCP	guanosin-5’-(β-γ-Methylen)-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
dNDP	deoxy-Nucleosid-5’-diphosphate
dNMP	deoxy-nucleosid-5’-monophosphate
dNTP	deoxy-nucleosid-5’-triphosphate
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
EF-Ts	Elongation factor Ts
h1-h45	helices of *E. coli* 16S rRNA
H1-H101	helices of *E. coli* 23S rRNA
HPLC	High Performance Liquid Chromatography
IF	Initiation factor
cryo-EM	cryo-electron microscopy
L1-L36	*E. coli* proteins of the large ribosomal subunit
Lys	lysine
mRNA	messenger RNA
NDP	Nucleosid-5'-Diphosphate

NMP	nucleoside-5'-monophosphate
NTP	nucleosid-5'-triphosphate
nt(s)	nucleotide(s)
ORF	open reading frame
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
Phe	phenylalanine
PK	pyruvate kinase
poly(U)	homopolymer Uridine-RNA
poly(A)	homopolymer Adenosine-RNA
POST	post-translocational state of the ribosome (tRNAs in P and E-site)
PRE	pre-translocational state of the ribosome (tRNAs in A and P-site)
PTC	peptidyl transferase center
RNA	ribonucleic acid
RNase	ribonuclease
RF	Release Factor
RBS	ribosome binding site
RRF	Ribosome Recycling Factor
rRNA	ribosomal RNA
S1-S21	*E. coli* proteins of the small ribosomal subunit
S-30	Lysate of *E. coli* cells after removal of cellular debris
S-100	Supernatant after centrifugation of S-30 at 100000 g
tRNA	transfer RNA
$tRNA^{bulk}$	tRNA mix
$tRNA^{aa}$	amino acid specific tRNA
$tRNA_f^{Met}$	Formyl-Methionin specific tRNA
fMet- $tRNA_f^{Met}$	Formyl-Methionin-$tRNA_f^{Met}$; Initiator-tRNA

Chemicals

AA	acrylamide
AcOH	acetic acid
Amp	ampicillin
APS	ammoniumperoxodisulfate
BAA	N,N'-Methylen-Bisacrylamide

DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylendiamin-tetra acetate
EtOH	ethanol
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethan-sulfonic acid
IPTG	isopropyl-thio-ß-D-galaktosidose
MeOH	methanol
MQ	salt free, sterile, RNase free water (*via* Milli Pore)
NaOAc	sodium acetate
PAA	polyacrylamide
PMSF	phenylmethansulfonylfluorid
SDS	sodiumdodecylsulfat
Sp	spermine
Spd	spermidine
TCA	trichloracedic acid
TEMED	N,N,N',N'-tetramethyl-ethylendiamin
Tris	tris-hydroxymethyl-aminomethan

Units, other abbreviations

n	number
A_x	extinction of a substance in a volume of 1 ml in a cuvette of 1 cm width at a wavelength of λ= x nm
DPM	disintegrations per minute
f.c.	final concentration
x g	g-force
h	hour
min	minute
rpm	rounds per minute
RT	room temperature
S	Svedberg unit as sedimentation coefficient (1 S = 10^{-13} s)
s	second
ON	over night; longer than 10 hours
% v/v	concentration in ml per 100 ml solvent
Vol	volume
% w/v	concentration in g per 100 ml of solvent

1.Introduction

1.1 Protein synthesis at a glance

The ribosome is a ribozyme (Cech 2000), a molecular machine able to synthesize proteins dependent on the information flow which is best described in the principle named as the as the central dogma of molecular genetics.

“DNA makes RNA makes protein”, this sentence has been subjected to certain modifications, but its essentials are still true and have become the central point of interest of scientific research, proving indeed the that “RNA makes protein”; yet revolutionizing the idea entirely from what was thought of a template driven mechanism, where RNA had no active role in the process of “making proteins”.

Today we know that the RNA itself is the central key player and catalytic compound to link amino acids (aa) to an active protein (translation summary given in Figure **1.1-1**). Doing so the ribosome has an unimaginable fast rate with about 20 aa/s (Dennis and Nomura 1974) and is at the same time super precise, an error occurring only every 3000 codons.

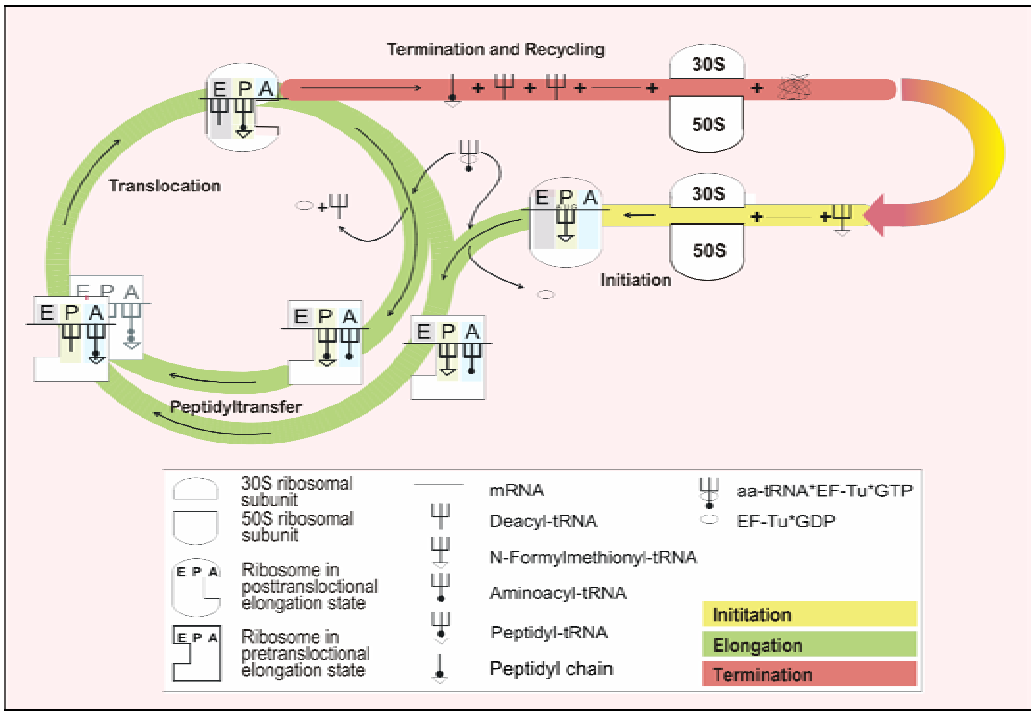


Figure 1.1-1: Steps of ribosomal translation, encompassing factor driven initiation, elongation and termination

In all cells the ribosome is built out of two subunits, the small one is called 30S and the large one the 50S subunit in bacteria. To be actively engaged in protein synthesis they are brought together in the cell to form a 70S particle, the ribosome.
Both subunits consist out of protein and RNA, the large subunit of about 33 proteins and two ribosomal RNAs, 5S and 23S rRNA, and the small subunit consisting of one 16S rRNA and 21 proteins (Wilson and Nierhaus 2003).
Next to its catalytic site, which is able to synthesise polypeptides, driving the formation of peptide bonds (PTC = peptidyl transferase centre), it possesses more interesting sites which play important roles in the process of translation.
Summarized in Figure **1.1-2** some of these residues are highlighted. Next to PTC, the GTPase associated centre (GAC) should be mentioned. Here proteins such as EF-Tu and EF-G, GTPases that foster elongation - bringing tRNAs to and translocating them through the ribosome – come in contact with the 70S, to receive signals which are transmitted through the overall structural changes and ribosomal movements that trigger the cleavage of GTP and promote their dissociation after their job is done.
GTP cleavage of G-proteins is also driven by another structural entity of the ribosome, the longest most conserved stretch in rRNA that can be found in all kingdoms of life, the sarcin-ricin loop (SRL).

In addition to serving as an entity for synthesis and protein interaction the rRNA provides a large contact surface to the tRNAs that travel through the ribosome during the elongation of a peptide chain. On the far left hand corner of the 50S close to the protein binding site of L1 we find an important nucleotide for this job - A2394. It is needed to fix the E-site tRNA in place that is allowed to leave the ribosome only after a ternary complex delivers a new charged tRNA to the A-site and is therefore essential to the decoding process. Ribosomes that lose the tRNA in the E-site too early create a situation unfavourable for the decoding process. This correlation is explained with the allosteric three-site model (Nierhaus 1990), which suggests that E and A-site influence each other. A tRNA filled E-site leads to a low affinity A-site so that not all tRNAs have the same binding chances. This is crucial to keep the fidelity and accuracy of protein-synthesis that would otherwise slow down dramatically and produce considerably more errors.
The small subunit has a group of interesting features as well. First of all the decoding centre which is essential for ribosome function, because it ensures the precision of protein synthesis (residues A1492/93 and G530). The mRNA anticodon defines the

tRNA able to be delivered to the A-site and thus the correct building block of a protein. With the help of these three important nucleotides the ribosome helps in defining right from wrong. When a new tRNA is delivered the mRNA and tRNA form a codon-anticodon pair; only if this pair is properly matched the bases can form a network of interactions and allow the tRNA to move into the A-site. Structurally interesting for a coordinated movement of tRNAs are the nucleotides A790, U1341 and G1338. They form a rim and ensure that only during translocation a codon-anticodon complex at the P-site can travel to the E-site (Schuwirth, Borovinskaya et al. 2005). To make proper contacts to the mRNA the 16S rRNA possesses a defined sequence forming contacts to the Shine Dalgarno region (SD) at the 5' terminus of messenger RNAs. This sequence is about 7 nt long and called the anti-Shine-Dalgarno region starting with A1534.

Ribosome research has mostly been able to make a connection between conservation levels and function. The higher the conservation level the more

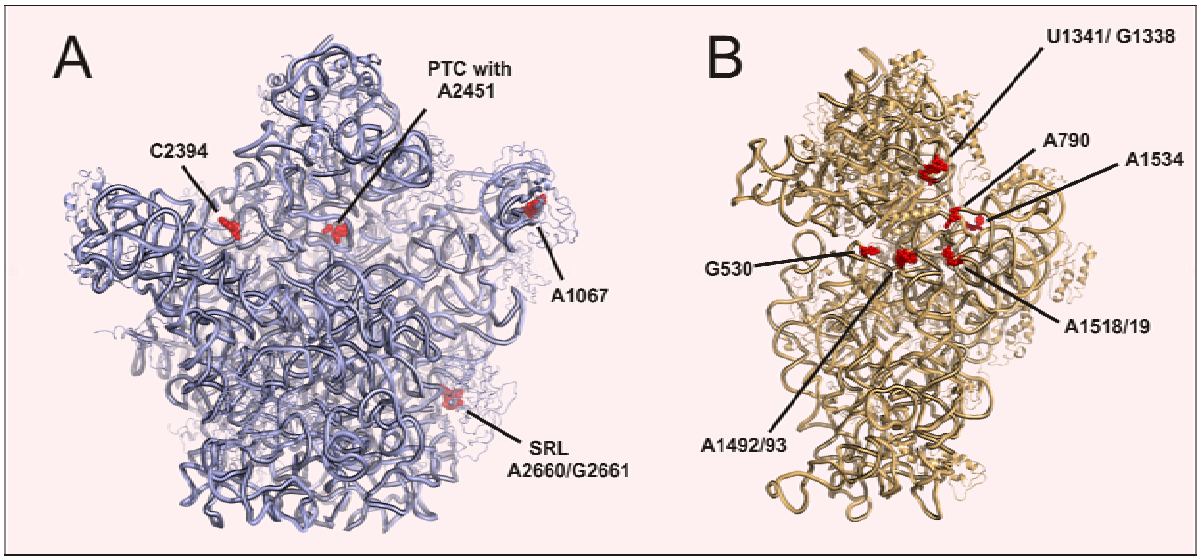


Figure 1.1-2: Important residues for initiation and the elongation cycle within the large **A** and small **B** ribosomal subunit (shown from the inter-subunit side) are coloured in red (modelled with PDB 1VOR, 2AW7) their function explained within the proceeding paragraphs

important is the residue. The modified nucleotide pair A1518/19 which is conserved among all bacteria yet has given us a riddle to solve, for the deletion of this modification has absolutely no effect on cell growth or protein synthesis, a rather surprising finding. The only known function is that the lack of methylation confers rise

to kasugamycin resistance, a drug that functions as an initiation inhibitor, disturbing the path of the mRNA on the 30S subunit (Schuwirth, Day et al. 2006) though literature mentions possible involvement in 30S subunit formation as well (Micura, Pils et al. 2001)

Even if the ribosome possesses numerous intrinsic functions essential for translation, accessory factors are needed to make initiation, elongation and termination happen.

1.2 Initiation factors

Initiation, the first step of protein synthesis, needs a large set of about 12 initiation factors in eukaryotes (Sonenberg and Hinnebusch 2007), some of which consist of many peptides presenting a huge protein complex, e.g. the eIF3 has a molecular weight of 900 kDa, as much as the small subunit of bacterial ribosomes! In contrast, bacteria just have three monomeric initiation factors.

To understand their precise function and the diverse initiation pathways they are involved in, a short explanation of each bacterial factor along with the prevalent understanding of initiation *via* 30S subunits follows.

1.2.1 IF1

IF1 is essential for cell *via*bility (Cummings and Hershey 1994) it is the smallest of the initiation factors and has a remarkable beta barrel structure (Sette, vanTilborg et al. 1997). It has a notable similarity with the cold shock protein CspA and, like this factor, a pronounced function in cold shock response (Giuliodori, Brandi et al. 2004). It possesses the capability to function as helicase and RNA chaperon and can act as a transcriptional anti-terminator (Howe and Hershey 1983). As to present knowledge IF1 is exclusively acting on 30S, since it is ejected from 70S during subunit association (Celano, Pawlik et al. 1988). It has been found to crosslink to proteins of the small subunit S1 and S12 (Langberg, Kahan et al. 1977) as well as to IF2. Its function as a 30S factor has been underlined by crystal structure studies (Carter, Clemons et al. 2001) (Figure **1.2.1-1**). IF1 binds in the proximity of the A-site and leads to a defined morphological rearrangement within the 30S moving h44 and leading to a flipped out orientation of A1492/93 which have a famous function during decoding checking the proper fit of codon-anticodon (Figure **1.4-2**). In its orientation close to loop 539 and ribosomal protein S12 it covers (but does not block) the

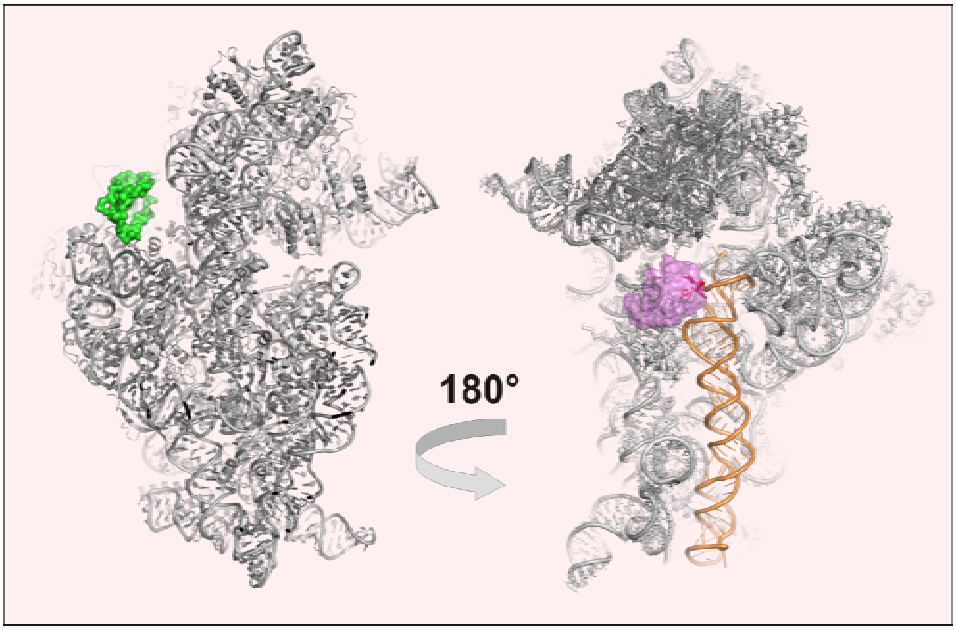


Figure 1.2.1-1: Positions of IF3 N-Terminus (green) binding to the solvent site and IF1 (pink) to the interface of the small subunit. To underline the special character of IF1 binding to the small subunit h44 is coloured in orange and the bases 1492, 1493 are marked in red (modelled with PDB 1HRO, 1I95)

channel at the base of the A-site through which the mRNA passes (Lata, Agrawal et al. 1996). It has been argued that IF1 might act as a tRNA analog to prevent premature binding of ternary complexes to the 70 initiation complex (70SIC) (Moazed, Samaha et al. 1995). The true nature of the indispensability of IF1 is yet undetermined and is one topic of this thesis. Most described functions are more stimulatory like: enhanced affinity of IF2-fMet to 30S, enhanced activity of dissociation capability of IF3, improved formation of 30S IC (Pon and Gualerzi 1984) and cannot account for cellular lethality after a knock-out of IF1. Further it has been reported that together with IF3 it promotes splitting (Pavlov, Antoun et al. 2008) and together with IF2 it can lead to the drop-off of small peptides form the ribosome itself (Karimi, Pavlov et al. 1998).

1.2.2 IF3

IF3 binds tightly to 30S and inhibits subunit reassociation (Kaempfer 1972). Its dissociation activity which can force 70S particles apart is one of the best described features of this protein (Subramanian and Davis 1970), though this function has also

let to some controversy and has been in some cases discussed as a simple *in vitro* artefact (Umekage and Ueda 2006) due to non *in vivo* like conditions of spermidine and an extreme excess of factors over ribosome concentration in many experimental setups.

The importance of the protein for initiation was also observed *in vivo* (Olsson, Graffe et al. 1996). Depletion of polysomes are a direct result of its removal from the cell, hinting to the profound importance to initiation of protein synthesis. The Ueda group recently speculated that IF3 might help in adjusting 70S and mRNA in proper position within an initiation complex, before the ribosome resumes translation (Takahashi, Akita et al. 2008). They had proven that even crosslinked ribosomes can contact SD-sequences without dissociating into subunits, here IF3 would efficiently facilitate initiation without subunit dissociation.

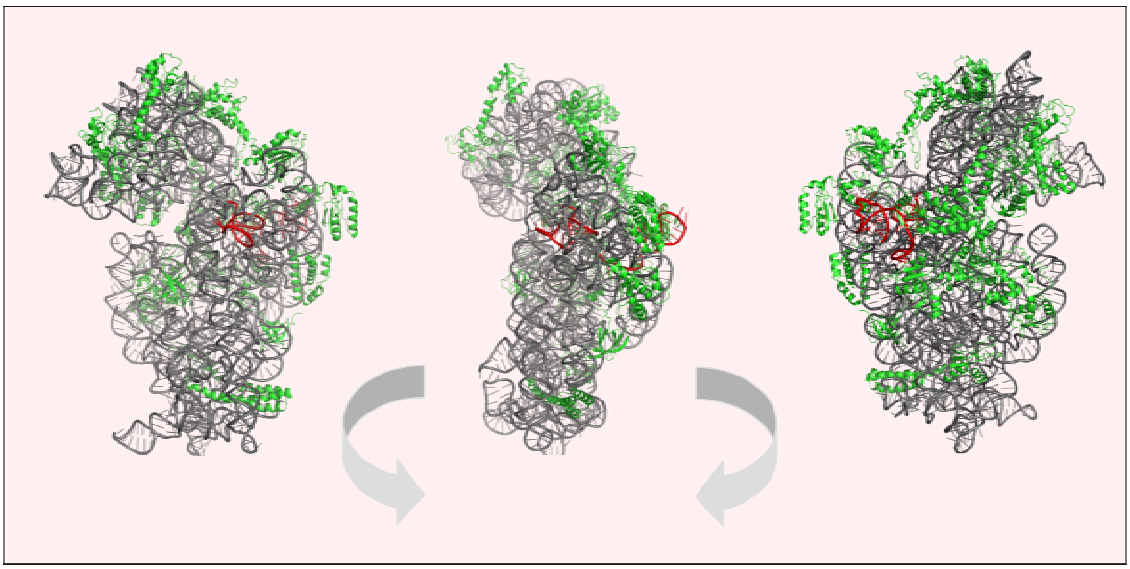


Figure 1.2.2-1: Reflecting the possible binding modes of this factor to the small subunit and possibly the ribosome (modelled with PDB 2AW7) ; IF3 crosslink sites (red) to 16S RNA of 30S (ribosomal proteins in green) (Ehresmann, Moine et al. 1986)

Though IF3 is found to bind to 30S IC as reported by (Allen, Zavialov et al. 2005) the exact mode of binding remains elusive, since information derived from cryo-EM (McCutcheon, Agrawal et al. 1999) and crystallography (Pioletti, Schlünzen et al. 2001) (Figure **1.2.1-1**) as well as biochemical studies for binding (Dallas and Noller 2001) and crosslinking (Ehresmann, Moine et al. 1986) (Figure **1.2.2-1**) seem to identify two possible interaction hotspots of the factor on the small subunit, but agree on the fact that the protein binds to the E-site region.

One of them is considered to be the major contact point and is found on the intersubunit side; the other one far away on the solvent side. Whether or not both are possible we can not argue about. Yet it clearly states that our understanding of the function of IF3 is still most probably incomplete and needs revision.

1.2.3 IF2

Among the three initiation factors IF2 is not only the largest but has the best understood function. Thus it is an all over accepted fact, that the protein forms a ternary complex with initiator tRNA and GTP (Gualerzi, Severini et al. 1991) and delivers it to the 30S subunit (Myasnikov, Marzi et al. 2005) to form the 30S IC (Simonetti, Marzi et al. 2008) (see Figure **1.2.3-1**).

It binds solely to initiator tRNA and ensures delivery to the ribosomal P-site an event leading to the consequence that 30S forms contacts with a 50S subunit to yield functional 70S ICs that enter translation elongation after the factor probably together with IF1 has left the ribosome and GTP cleavage has occurred. No clear understanding of the exact order of events following 70S formation has been established, yet the mechanism *per se* is clear and unquestionable. More unclear are

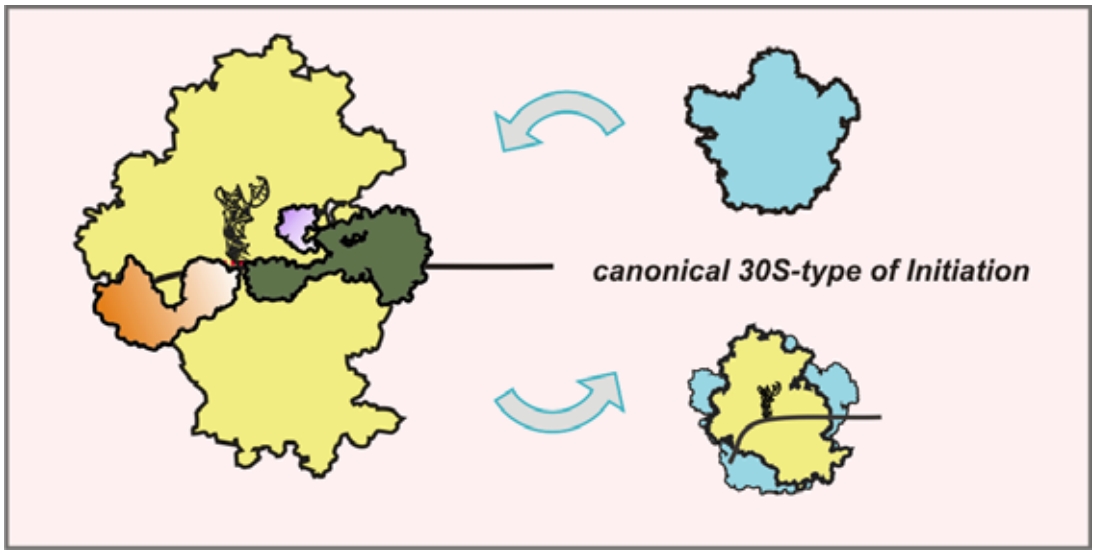


Figure 1.2.3-1: The 30S IC with IF1(pink), IF2 (green) and IF3 orange forming an arc-like structure across the small subunit (yellow), a snapshot probably just prior to dissociation of IF3 and just before association with the large subunit (modelled after (Simonetti, Marzi et al. 2008))

functions of IF2 that have been associated to leaderless translation (Grill, Lodei et al. 2000) and translational coupling (Yoo and RajBhandary 2008), two initiation pathways that shall be explained in detail in the following paragraphs.

1.3 Initiation Modes

We should clearly rationalize the fact that the translation of proteins is a complex controlled mechanism that reaches out to the transcriptional level. However, equimolar synthesis of proteins organized in a polycistronic mRNA - as necessary for ribosomal proteins transcribed from one polycistronic mRNA - can't be achieved this way. Ribosomal proteins are translated in a defined 1:1 stoichiometry (Howe and Hershey 1983) yet most of them are organized in polycistronic mRNAs. A defined ratio of translation cannot be explained by 30S initiation from mRNAs with more than one cistron without complicating the model by adding a further control element, protein or RNA of nature. Since such control elements must be highly abundant in the cell, for the occurrence of polycistronic mRNAs and their translation is frequent for ribosomal proteins, it is highly unlikely that they exist and have not been discovered hitherto. The idea is intriguing that one ribosome just after translating an upstream ORF slides on to the next downstream ORF until the end of the mRNA is reached, so that a controlled ratio of proteins coded in this sequence becomes likely (Figure **1.3-1**), arguing that a yet undefined mode of initiation exists, that we call in the following sections as 70S-scanning type of initiation.

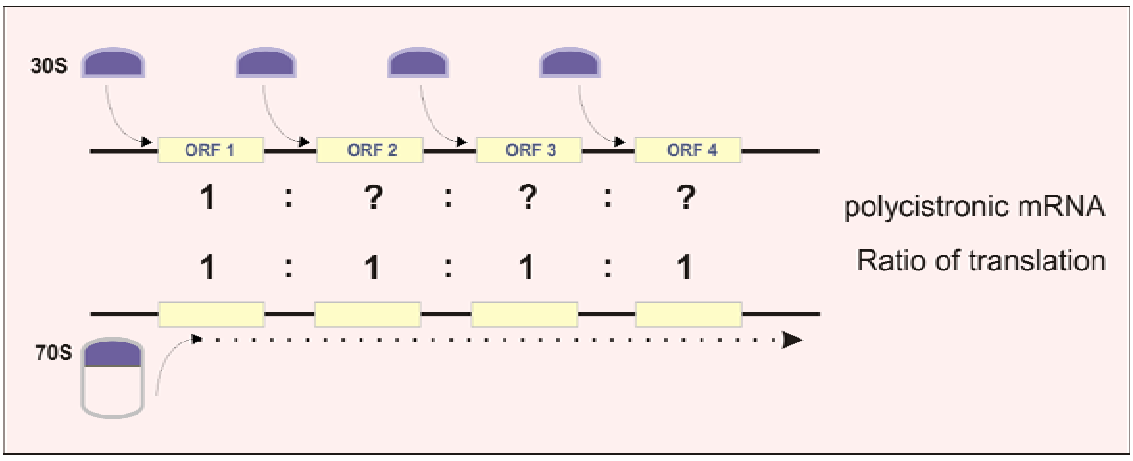


Figure 1.3-1: Outlining the essential idea why a novel initiation pathway must be considered for bacterial translation

1.3.1 30S binding type of initiation

For a new round of initiation to start a pool of free 30S and 50S subunits needs to be continuously present in the cell at all times. In the initial step the SD-element of the mRNA makes contacts to the anti-SD sequence of 16S rRNA. To precede further from this point the three initiation factors IF1, 2 and 3 as well as the initiator fMet-tRNA$_f^{Met}$ are needed. IF3 is believed to play the role of an anti-association factor, while IF2 acts analogously to EF-Tu as a transfer factor bringing the initiator tRNA in complex with GTP to the small subunit. The function of the two is believed to be stimulated by IF1, which is situated in the location of the A-site. The result is the 30S initiation complex (30S IC). As soon as 50S and 30S subunits join together IF3 is no longer needed as anti-association factor and leaves the ribosome. In a yet unknown order GTP is cleaved and IF2 most probably together with IF1 leave the 70S ribosome in a concerted fashion yielding the 70S initiation complex (70S IC). (for a complete overview please refer to Figure **1.3.3-1 A**).

1.3.2 70S types of initiation

In the present literature only a minority of initiation events is believed to occur with 70S ribosomes. This subgroup of initiation events is involved in translation of leaderless mRNAs (Moll, Grill et al. 2002) (Figure **1.3.3-1 C**), that have no 5'UTR and especially no SD-sequence, normally found in this region. Subunits cannot support translation from leaderless mRNAs; IF2 has been observed to stimulate this reaction, in contrast IF3 is proven to be strongly inhibitory to it (a function of IF1 has not been discussed here). Recent studies showed that a special ribosome type, the 61S ribosomes lacking certain proteins from the small subunit, tend to exclusively translate leaderless mRNA and seem to give us a glance about translation at ancient times about three billion years ago (Kaberdina, Szaflarski et al. 2009).

Translational coupling or re-initiation is another scenario, where initiation with an intact ribosome is believed to take place (Figure **1.3.3-1 D**). Primary example is the tryptophan operon (Oppenheim and Yanofsky 1980). Translation of a downstream ORF is prerequisite for the translation of the following within the operon. Coupling is usually observed when both reading frames overlap, or the stop codon of a preceding ORF and start of the next lie in close proximity (~ 20 nucleotides (Karamyshev, Karamysheva et al. 2004)). Here the second AUG is hidden in a

secondary structure, which is melted during the termination phase of ORF1 making it possible for 30S subunits or 70S ribosomes to approach and initiate at this otherwise hidden position.

Translational coupling has been suggested for an equimolar expression of ribosomal proteins (Nomura 1984) and was particularly explained for L11-L1 (Sor, Bolotin et al. 1987) as well as the alpha operon (Thomas, Bedwell et al. 1987), yet the mechanism doesn't in all cases lead to a "1:1" expression of proteins within the complete cistron. Besides, once the second start codon is exposed either a 70S can slide on, or a free 30S subunit can start with translation, disturbing equimolar protein levels, that we must assume for ribosomal proteins. Such a steady synthesis level is observed *in vivo,* and can hardly be anticipated by random 30S initiations from "opened" ORFs due to coupling.

We suggest that 70S initiation is not only involved in exceptional cases but is frequently found in the cell as "70S-scanning type of initiation" (see Figure **1.3.3-1 B**), that is found in translation of the majority of polycistronic mRNAs of the bacterial cell, this way guaranteeing a defined protein ratio for ribosomal proteins (Figure **1.3-1**).

1.3.3 Further evidence on the road of defining a new initiation mode

Additional evidence for the predominance of an initiation pathway with 70S ribosomes is the fact that they tend to bind to mRNAs with SD sequences without a required dissociation event (Takahashi, Akita et al. 2008). Together with the prevailing 30S model the common understanding that a ribosome has to fall apart in its subunits before being able to start a new round of translation is widely accepted. This view must now be modified. Results from translation of homopolymeric mRNA show that 70S can not only form a 70S initiation complex without a pool of free subunits but also start translation and reinitiate as such (Szaflarski, Vesper et al. 2008). Additional facts strengthening the view that 70S initiation is a widespread form in the cell, are due to the observation that under physiological conditions ribosomes are in fact the prevailing form in the cell and not subunits (Kohler, Ron et al. 1968). This is also observed in RTS, a reconstituted *in vitro* translation system of the company Roche based on *E. coli* cell extracts. Analysis on sucrose gradients of the extracts used for *in vitro* protein synthesis has led us to the surprising finding that there is only a minor sub-fraction of ribosomes present in dissociated subunits as well (Gupta).

Opting for a new initiation mechanism that is carried out with the help of IF1 we could find additional evidence for our model in the literature. A scanning model was already proposed by Adhin et al (Adhin and van Duin 1990): their *in vivo* study resulted in the finding that a ribosome would tend to reinitiate within a region of 40 nt from the last encountered stop codon stimulated by the presence of an SD element close to the next translation start. Another important literature finding is the fact that 30S cannot distinguish formylated from unformylated species of fMet-$tRNA_f^{Met}$, only 70S is able to discriminate them both with and without factors leading to the assumption that the initiator tRNA is solely important for initiation with 70S ribosomes (Petersen, Danchin et al. 1976) (Petersen, Danchin et al. 1976) which then must be the main initiation mode in bacteria. A further argument in favour of a 70S like mechanism supported by IF1 is seen in the paper of Hartz and Gold (Hartz, McPheeters et al. 1989). IF1 is shown to make the selection of the right initiator tRNA through IF2 and IF3 on 70S happen and finally several publications hint toward the fact that has been widely ignored by the scientific community saying that both IF1 and IF3 are found on 70S ribosomes. Various papers have been published citing that initiation factors can be purified from 70S ribosomes (Nagel and Voigt 1992) (Voigt and Nagel 1990) (Ganoza, Aoki et al. 1996) . Such a finding significantly alters the view of the 30 initiation pathways that regards IF3 as an anti-association / dissociation factor and IF1 as a stimulatory protein that leaves the ribosome soon after 70S IC has formed.

These ambiguous data prompted us to set out studying a yet undefined translation initiation pathway that has been overlooked and extends our understanding of the functions of IF1 and IF3 in translation initiation.

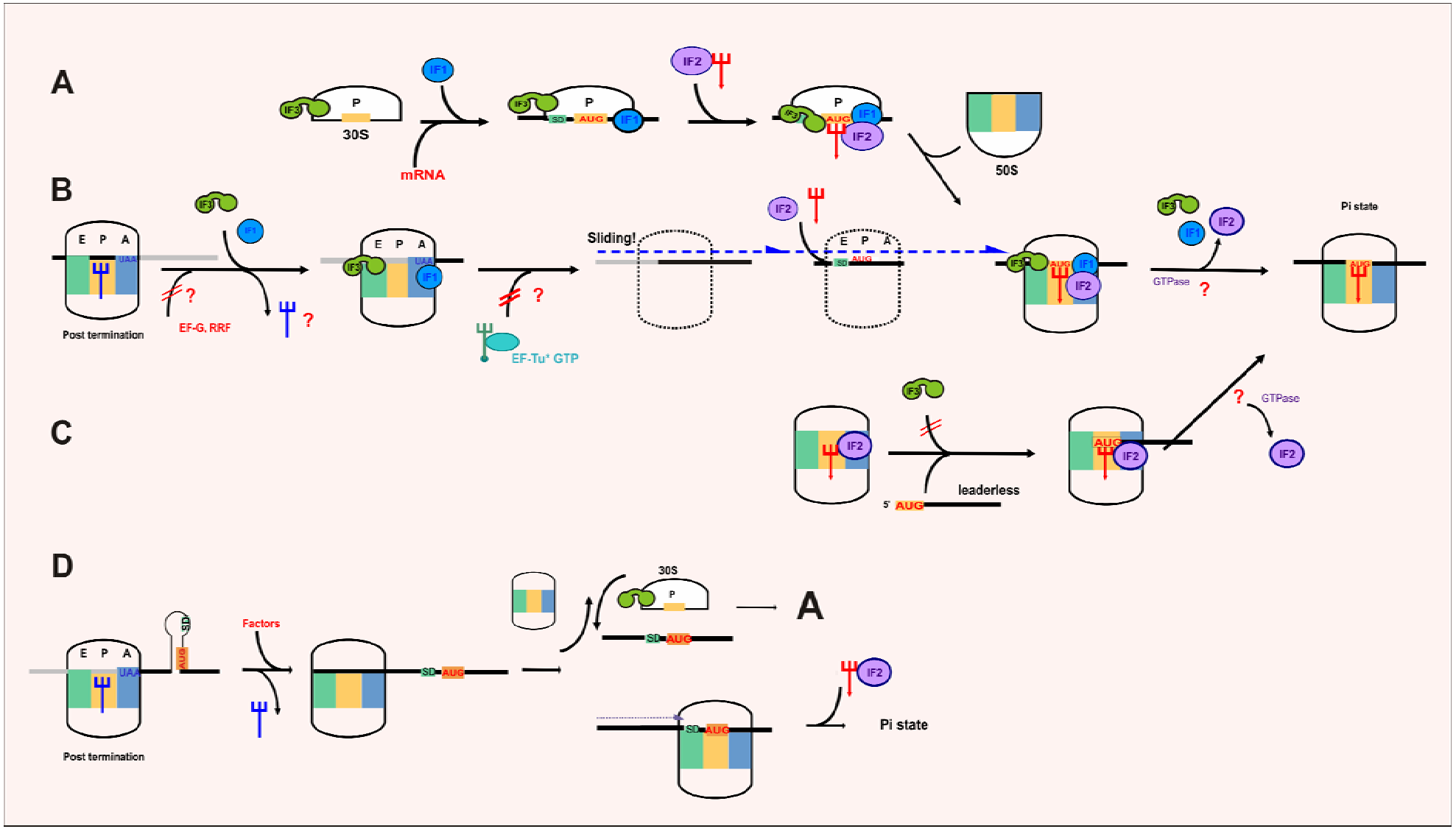


Figure 1.3.3-1: Describing prevalent and new initiation models – **A.** depicting the 30S-binding initiation pathway; **B.** outlining the suggested new model of 70S scanning type of initiation; **C.** showing to one of the established 70S initiation modes, the leaderless initiation pathway; **D.** portraying the second accepted 70S initiation model during translational-coupling

1.4 Initiating the next step –

tRNA shuttling to the ribosome, Decoding and Accommodation

Important progress has been achieved concerning our understanding of the molecular mechanisms involved in the step just following the initiation. I will here briefly outline some principles.

Initiation leaves the ribosome with one tRNA bound to the P-site. Lengthening the peptide chain follows in the subsequent step "elongation". This process is carried out by EF-Tu and EF-G. EF-Tu brings charged tRNAs carrying amino acids (aa) to the ribosome. The aa's are incorporated into the growing peptide chain, EF-G translocates the mRNA•$tRNA_2$ complex through the ribosome. Thus uncharged tRNAs can leave the ribosome and give space to the new incoming ones. Not every tRNA carrying an aa can be accepted to move into the A-site, only a cognate/near cognate tRNA (codon of mRNA and anticodon of tRNA match) is accepted. The ribosome possesses various controlling entities ensuring that exactly this is happening. Since the tRNA doesn't come alone, the process is even a bit more complicated. After the ribosome has made sure that the tRNA is correct, it also has to give signal to the carrier protein to let go, otherwise the process cannot be completed.

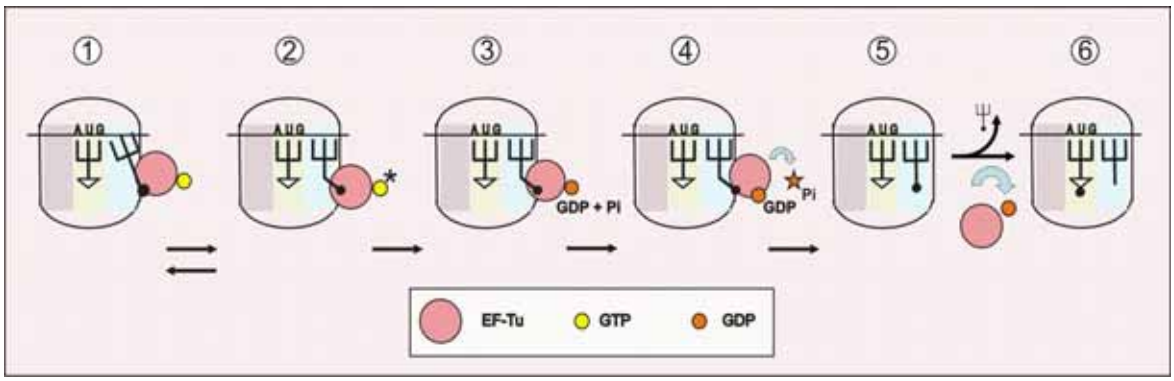


Figure 1.4-1: Schematic view of tRNA delivery, accommodation and peptide bond formation (modified from (Rodnina 2009) for detailed explanation refer to the text; A-site (light blue), P-site (light green), E-site (light purple).

Figure **1.4-1** shows an overall presentation of the complete process until peptide bond formation is achieved. In the first step aa-tRNA is presented to the ribosome in ternary complex with EF-Tu-GTP (**1.4- 1,** step 1). This binding is rapid and reversible, a non-cognate tRNA will be immediately rejected (Marshall, Aitken et al. 2008). This

A-site sampling allows a transient probing. Correct codon anticodon interaction is monitored by the ribosome, several universally conserved nucleotides check whether or not Watson crick base paring could be established between the tRNA and mRNA. The ribosome possesses - so to say – a molecular ruler. Nucleotides A1492, A1493 flip out of place in h44 and establish, what is called an A-minor motif, forming contacts to the minor groove side of the first and second base pair of the codon-anticodon helix (figure **1.4-2**).

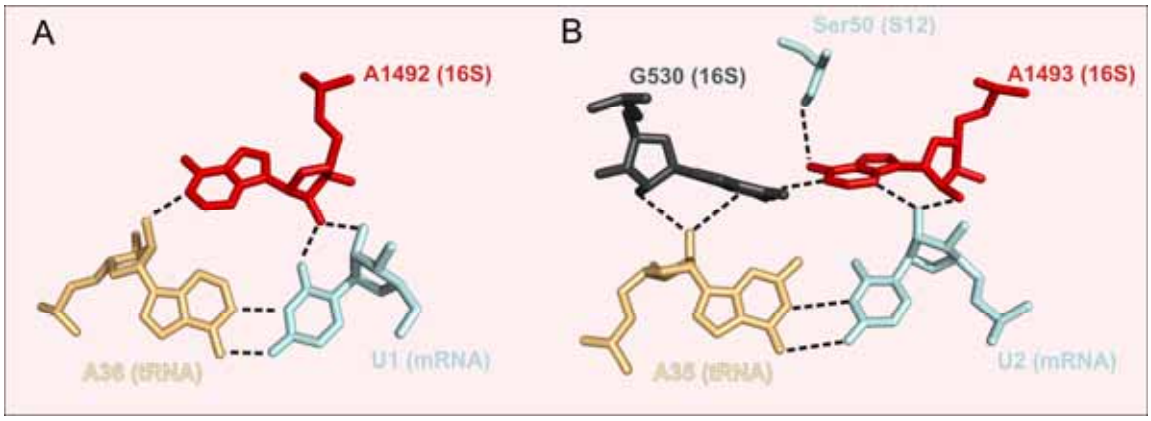


Figure 1.4-2: Interaction of A1492/ 93, G530 with the codon-anticodon complex leading to the formation of A-minor motif type II in **A** and A-minor motif type I in **B (**after **(Ogle, Brodersen et al. 2001)**

G530 switches from syn to anti conformation making together with the aforementioned nucleotides strong contacts with the minor grove of the codon anticodon helix. A1492 then interacts closely with G530 as well as protein S12. Therefore this ribosomal protein which is located in the shoulder domain of the 30S links this region *via* the codon-anticodon duplex to h44 leading to a so called domain closure of the 30S shoulder. This complex mechanism is called “decoding” and involves both RNA and protein. S12 is thought to hold most probably a relay function having contacts with the decoding centre as well as with the tRNA signalling conformational changes to EF-Tu (**1.4- 1,** step 2). These events lead to a deformation of tRNA depicted as a kink in Figure **1.4-1** (step 2 – 4) happens when the ASL (anticodon stem loop) is forced into the A-site while the CCA end is still held by EF-Tu (Valle, Sengupta et al. 2002). The energy that results out of decoding and then is transferred into domain closure (Ogle and Ramakrishnan 2005), where head and the shoulder of the 30S move towards each other most probably activate the GTPase activity of EF-Tu (**1.4- 1,** step 3). The factor must cleave the GTP that it

holds so that energy becomes free and leads to an overall structural rearrangement of the three domains of EF-Tu (Domain 1 is the GTPase domain which holds the hydrophobic gate), so that EF-Tu can let go of the tRNA and leave the ribosome. A conserved residue of EF-Tu – histidin 85 – is needed to activate a water molecule which cleaves a phosphoanhydrid bond of GTP. This histidin is usually shielded by two other residues Ile 60 and Val 20 so that a GTP cleavage does not happen sporadically without a decoding event on the ribosome (Figure **1.4-3 A**). After decoding has happened the ribosome makes contacts to the factor in such a way that the gate is opened (Schuette, Murphy et al. 2009; Villa, Sengupta et al. 2009). The shoulder of 30S interacts with the switch one region (in close proximity to Val 20 in Domain I of EF-Tu), opening one wing of the gate. The SRL on the other hand fixes the other wing, so that switch two relocates (near His 85) and the histidin can approach GTP leading to cleavage → GDP + Pi (Figure **1.4-1** step 4 and **1.4-3 B**). The energy out of this cleavage turns the rearrangement of the domain of the elongation factor in motion, once this has happened the factor leaves the ribosome and lets go of the tRNA that can now fully accommodate into the A-site (Figure **1.4-1** step 5). Now a final check happens, due to proofreading a near cognate

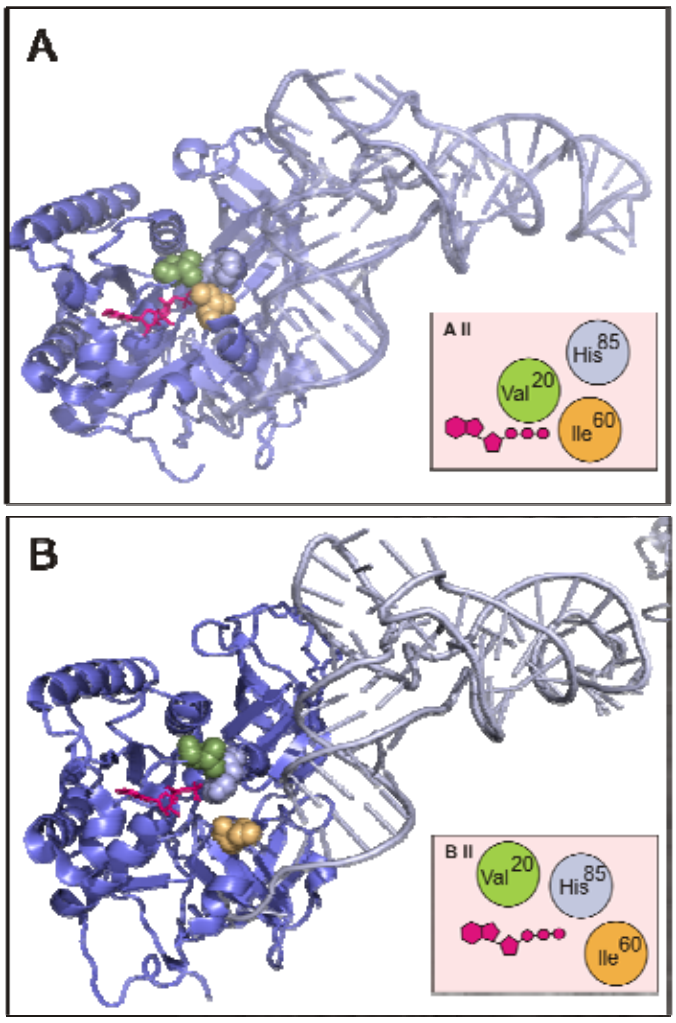


Figure 1.4-3: EF-Tu in its GTP bound form both off (A) and on the ribosome (B).Tu depicted in dark-blue, the residues of the hydrophobic gate in green, light-blue and orange, 30S in gray; A/B II detailed residue positioning within the hydrophobic gate

tRNA which might have up to this point of time been able to sneak through the tight control of the ribosome will now dissociate (Figure **1.4-1** step 5) near cognate meaning that the last (wobble) or less frequent the first position in codon anticodon helix mismatches; whereas non-cognate mismatch in the second position. Some people have described this step as kinetic proof-reading (Ehrenberg, Andersson et al. 1986) or “induces fit” mechanism. The model concludes that near cognate tRNAs have a lower binding energy than cognate ones resulting them to have less chances to trigger the conformational changes needed to accommodate in the A-site after EF-Tu has left the ribosome (Rodnina, Daviter et al. 2002) However, if the tRNA has passed, peptidyl transfer catalysed by the PTC on the 50S happens and the aa-chain of the P-site bound tRNA is transferred to the tRNA in the A-site resulting in a PRE-state harbouring tRNAs in the A and P-site (**1.4-1** step 6). To complete one elongation step EF-G binds to the ribosome and translocates the tRNA from A- and P- to P- and E-site resulting in a POST-state. Now a new ternary complex has the chance to bring a new aa-tRNA to the ribosome.

2. Material and Methods

2.1 Chemicals

1,4-Dithiothreitol (DTT) (Roche Pharmaceuticals # 1 583 786)

1,4-Dithioerythritol (DTE), (Carl Roth # 8814.1)

6x Loading Dye Solution (Fermentas # R 0611)

Agarose (Ultra pure) Gibco-BRL

Albumin, bovine (Sigma # A7906)

Ampicillin (Carl Roth # K029.2)

ATP disodium salt (Roche # 519987)

Bacto agar® Difco, Detroit (USA)

Bacto tryptone® Difco, Detroit (USA)

Chloroform (Carl Roth # AX984.2)

Complete mini, EDTA-Free (Roche # 118360001)

Coomassie® Brilliant Blue G-250 (Serva # 17524)

Coomassie® Brilliant Blue R-250 (Serva # 17525)

CTP Disodium salt (Sigma # C1506)

GeneRuler™ 100bp DNA Ladder RNA ladder (Fermentas # SM 0241)

GeneRuler™ 1kb DNA Ladder (Fermentas # SM 0311)

Goat anti rabbit HRP conjugate (Santa Cruz Biotechnology, USA)

GTP Tris salt (Sigma # G9002)

Filter Count (Szintillation liquid) (Perkin Elmer # 6013149)

HEPES (N-(2-Hydroxyethyl)-piperazin-N`-(2-ethansulfonsäure) (AppliChem # A1069, 1000)

Imidazole (Sigma # I0125)

Isopropylthiogalaktosid IPTG 25g (Carl Roth # 2316.4)

Lysine (Serva # 28195)

Phenol (Carl Roth # A980.1)

Phenylalanine (Sigma # P2126)

PMSF (Roche # 837091)

Puromycine (Serva # 33835.01)

ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma # P-4600)

Roti-Mark STANDARD 1 ml (Carl Roth # T851.1)

Rotiphorese® Gel 30 (37,5:1), (Carl Roth # 3029.1)

Rotiphorese® Gel 40 (19:1), (Carl Roth # 3030.1)

Rotiszint eco plus (Carl Roth # 0016.2)

Sodium-dodecyl-sulfate (SDS), (Sigma # 161-0301)

Spermidine (Fluka, Neu-Ulm # 82501)

Spermine (Fluka, Neu-Ulm # 85605)

Sucrose Ultrapure (Invitrogen #)

TEMED (NNN`N`-Tetramethylethylendiamin) (Invitrogen # 15524-010)

Trichloroacetic acid (TCA), (Carl Roth# 8789.1)

Urea (Fluka # 02493)

Yeast extract Difco, Detroit (USA)

Other bio chemicals which are not mentioned in this list were purchased from Merck such as 2-Mercaptoethanol # 8.05740.0250 etc.

2.2 Columns for Protein and RNA purification

HisTRAP HP 1 ml (**GeHealthcare**)
MonoQ (**Pharmacia**)
MonoS (**Pharmacia**)

Nucleosil C4/C6/C8 (**Machery Nagel**)

Quick Spin Columns for radiolabeled RNA purification (**Roche #** 11273990001)

Superose 12 (**Pharmacia**)

2.3 Enzymes

EcoRI and all other restriction enzymes (New England Biolabs)

Pyruvate kinase (PK) (**Sigma)**

Phosphatase, alkaline (AP) from calf intestine (**Roche Pharmaceuticals #**713 023)

Polynucleotide kinase (**Roche Pharmaceuticals #** 174 645)

T4 DNA Ligase, (**Fermentas #** EL 0015)

T1 RNase (**Fermentas #** EN0541**)**

RevertAid™ H Minus (**Fermentas #** EP0452**)**

AMV Reverse Transcriptase (**Roche#** 10109118001)

2.4 Kits for molecular biology

ECL Plus Western Blotting Detection Kit (**Ammersham #** RPN2132)

High Pure Plasmid Isolation Kit (**Roche #** 11754785001)

Invisorb Spin DNA Extraction Kit (**Invitek**, **Berlin)**

Jena JBS-Methylation Kit (**Jena Bioscience #** CS-510)

Ni-NTA Agarose (100 ml), (**Qiagen, Hilden #** 30 230)

Qiagen Maxi Prep® Tip 500 **Qiagen, Hilden**

Qiagen Midi Prep® Tip 100 **Qiagen, Hilden**

QIAquick® PCR Purification Kit **Qiagen, Hilden**

T7 RiboMax Express Large Scale RNA Production System (**Promega #**P1320)

RTS 100 E. coli HY Kit (**Roche #**03186148001)

Dual-Glow Luciferase Assay System (**Promega #**E2920)

2.5 Labware

Ultracentrifuge tubes (Ultra-Clear) SW 60, SW40 and SW28, (Beckman #344060, # 344058)

Filters 0.45 µm for sterile filtration (**Millipore #** HAW02500)

Nitrozellulose Filter (**Sartorius, Göttingen #** 11306)

Glass Fiber Filters ∅23 mm (**Schleicher and Schüll #** 10 370 021)

Folded Filters 595 ½ (**Schleicher and Schüll #** 10311647) Φ=185 mm

2.6 Laboratory technical devices

Biocomp Gradient Master 107ip

FPLC System Äcta 900 (P/UV/C)

HPLC System Pharmacia LKB (Gradient Pump 2249, Fraction collector Super Frac)

Phosphoimager (Ammersham)

Wallac 1409 Scintilation counter

RTS ProteoMaster (Roche #3 265 650)

Centro Mikroplatten-Luminometer LB 960

Sucrose gradient fractionation and collection device was made up out of the following components: Pico ADC-16 module

LKB-Bromma 2138 uvicord

LKB-Bromma 2211 Superfrac

2.7 Radioactive compounds

[γ ^{32}P]-Adenosine-5'-triphosphate (**Amersham-Bioscience #** PB 10218)

[γ ^{32}P]-Guanosine-5'-triphosphate (**Hartmann Analytic #** FP 402)

L-[U-^{14}C]-Alanine (**Amersham-Bioscience #** CFB 62)

L-[U-^{14}C]-Lysine (**Amersham-Bioscience #** CFB 69)

L-[4-^{3}H]-Phenylalanin (**Amersham-Bioscience #** TRK 204)

L-[U-^{14}C]-Phenylalanin (**Amersham-Bioscience #** CFB 70)

L-[methyl-^{3}H]-Methionine (**Amersham-Bioscience #** TRK583**)**

L-[^{35}S]-Methionine **(Hartmann Analytik#** KSM01**)**

Formaldehyde [^{14}C] (**Perkin Elmer #** NEC039H250UC**)**

2.8 Software

Image Quant 5.2

Unicorn 5.01 (Äcta Software)

Pico Log Recorder (Software for the Pico ADC-16 module)

Mfold (Zuker 2003) (online http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi)

PyMOL v1.1

CorelDRAW X4

2.9 tRNA and homopolymeric mRNAs

poly(A), (27-4110-01) **Amersham (GE Healthcare)**

poly(U), (27-4440-02) **Amersham (GE Healthcare)**

tRNAs (acceptor specific for methionine, lysine etc.) **Chemical Block**

$tRNA^{bulk}$ from *E. coli* MRE 600 (RNase negative), (109 550) **Boehringer Mannheim**

tRNA^{bulk} from Yeast (109525) **Boehringer Mannheim**

2.10 Bacterial strains and plasmids

2.10.1 Bacterial strains

XL-1 Blue: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]

BL21(DE3): F- dcm ompT hsdS(rB- mB-) gal λ(DE3),

CAN20: derived from *E. coli* K12; deficient in RNases BN, II, D (Deutscher, Marlor et al. 1984)

2.10.2 Plasmids

The following plasmids were used conducting the work of this thesis:

pQEtuf in Dh5α (EF-Tu of *T. thermophilus* cloned in the BamHI site of pQE12 (Ribeiro, Nock et al. 1995); a kind gift of Dr.Sprintzel)
pQE_infA,B,C in *Dh5α* (*E. coli* initiation factors cloned in pQE60 to produce a C-Terminal His-Tagged version of the protein (Shimizu, Inone et al. 2001), a kind gift of Dr. Ueda)
RF1,2 in pET 3a in BL21(DE3) (Nierhaus group) (Wilson 1999)
pET15bAlaRS in XL-1 blue (*E. coli* Alanine-tRNA synthetase was cloned into the pET15b vector; Nierhaus group)
pGEMEX-2smpB in BL21DE3 (*E. coli* SmpB was cloned in pGEM-X2 to produce a C-Terminal His Tagged version of the protein; a kind gift of Dr. Muto (Hanawa-Suetsugu, Takagi et al. 2002)
pGEMEX-2ssrA in BL21DE3 (*E. coli* tmRNA was clones in the pGEMEX-2; a kind gift of Dr. Muto (Tadaki, Fukushima et al. 1996)

pET22bcca (*E. coli* CCA-adding enzyme was cloned into the pET22b a kind gift of Dr. Ya-Ming Hou) → purification after: Evolution of tRNA nucleotidyltransferases: A small deletion generated CCA-adding enzymes (Neuenfeldt, Just et al. 2008)
pMBP-MS2 (PCR fragment containing the MS2 ORF was cloned in the *StuI HindIII* sites of pMalc (New England Biolabs); a kind gift of Dr. Vilardell (Macias, Bragulat et al. 2008)
pSP65 subcloning of mRNAs was generally carried out in this plasmid *pGEM®-T Easy Vector system* Subcloning of other constructs was done in this vector *pGL3-Control* was used as a template for cloning of Firefly luciferase pRL-TK was used as a template for subcloning of Renilla luciferase *http://www.promega.com/vectors/allvectors.htm*

2.11 Buffers, Solution and media compositions for microbiology

2.11.1 Buffers

2.11.1.1 Agarose gels

10X TBE-Buffer	Tris	108 g
	Boric acid	55 g
	EDTA	9.6 g
	MQ	Ad 1000 ml
50X TAE-Buffers	Tris	242 g
	Glacial accetic acid	57.1 ml
	EDTA 0.5M	100 ml
	MQ	ad 10 ml
Agarose solution (n %)	10X TBE	10 ml
	Agarose	(n) g
	Ethidium bromide (1 %)	5 µl
	MQ	100 ml
6XSample Buffer (DNA)	Ficoll 400	15 % (w/v)
	Bromphenolblau	0.25 (w/v)
	Xylencyanol	0.25 (w/v)

Electrophoresis Buffer	10X TBE/TAE	100 ml
(1X TBE/TAE)	water (MQ)	ad 1000 ml

2.11.1.2 Protein SDS Polyacrylamide gels

Acrylamide solution	Acrilamide	375 g
(37.5:1) or Roti 30	Bisacrylamide	10 g
	MQ	1000 ml
Stacking gel buffer	Tris	60 g
(0.5 M Tris-HCl, pH	MQ	800 ml
6.8)	adjust to pH=6.8 with HCl	
	MQ	ad 1000 ml
Separating gel buffer	Tris	180 g
(1.5 M Tris-HCl, pH	MQ	800 ml
8.8)	adjust to pH=8.8 with HCl	
	MQ	ad 1000 ml
5X Sample buffer	SDS	0.2 g
	0.5 M Tris-HCl pH=6.8	1.8 ml
	Glycerol (85 %)	1 ml
	β-Me (14.3 M stock)	20 µl
	0.2 % (w/v) Bromphenolblue	50 µl
	MQ	ad 10 ml
Electrophoresis buffer	Tris	30 g
(10X Stock)	Glycine	144 g
	SDS	10 g
	MQ	1000 ml
Electrophoresis buffer	Electrophoresis buffer (10X)	100 ml
(1X)	MQ	ad 1000 ml
Coomassie staining	Coomassie Blue G250	0.2 g
solution	Ethanol	25 ml
	HCl 37%	50 ml
	MQ	Ad 40 ml

2.11.1.3 Polyacrylamide gels denaturating conditions (RNA)

Acrylamide solution	Acrilamide	380 g
(38:2) or Roti 40	Bisacrylamide	20 g
	water	1000 ml
2X RNA Sample Buffer	Tris HCl 1 M, pH 7.5	100 µl
	EDTA 0.5 M, pH 8.0	20 µl
	Xylencyanol	0.05 %
	Bromphenol blue	0.05 %
	Urea (f.c. 8 M)	4.8 g
	MQ	ad 10 ml
Electrophoreses buffer	1X TBE	100 ml
(1X TBE)	MQ	1000 ml
Toluidine blue	Glacial acetic acid (100%)	100 ml
(Staining solution)	Toluidine blue	1 g
	MQ	ad 1000 ml

2.11.2 Buffers for microbiological preparative methods

2.11.2.1 Buffers for plasmid isolation

P1 (Resuspension Buffer)	Tris-HCl pH=8.0	50 mM
	EDTA	10 mM
P2 (Cell Lysis Buffer)	NaOH	200 mM
	SDS	1 % w/v
P3 (Neutralisation Buffer)	Potassium acetate, pH=5.5	3 M
QBT (Equilibration Buffer)	MOPS-KOH	50 mM
	pH=7.0	1000 mM
	NaCl	15% v/v
	Ethanol	
QC (Wash Buffer)	MOPS-KOH pH 7.0	50 mM
	NaCl	1250 mM
	Ethanol	15% v/v
QF (Elution buffer)	Tris-HCl, pH=8.5	50 mM
	NaCl	1250 mM
	Ethanol	15% v/v
TE	Tris-HCl, pH =8.0	10 mM
	EDTA	1 mM

2.11.2.2 Buffer for Ni-NTA Spin Kit

Lysis Buffer, pH=8.0	Sodium dihydrogenphosphat	50 mM
	NaCl	300 mM
	Imidazole	10 mM
Wash Buffer, pH=8.0	Sodium hydrogenphosphat	50 mM
	NaCl	300 mM
	Imidazole	20 mM
Elution Buffer, pH=8.0	Sodium dihydrogenphosphat	50 mM
	NaCl	300 mM
	Imidazole	250 mM

2.11.2.3 Buffers for Protein purifications other than Ni-NTA Kit CCA-adding Enzyme (E. coli)

Cell Lysis Buffer	Tris-HCl	20 mM
Running Buffer A	NaCl	500 mM
	Imidazole	5 mM
	HCl 38 %	1.5 ml
	Add to 1 L with MQ	
Running Buffer B	Same as A but with	
	Imidazole	500 mM
	HCl 38 %	25 ml
Dialysis Buffer/	Tris-HCl	20 mM
Storage	NaCl	500 mM
	$MgCl_2$	5 mM
	DTT	5 mM
	HCl 38 %	1.5 ml
	Glycerol	10 %
	Add to 1 L with MQ	

EF-Tu (*Thermus thermophilus*)

Cell lysis Buffer	Tris-HCl, pH=7.5	50 mM
	NH_4Cl	50 mM
	KCl	50 mM
	$MgCl_2$	10 mM
	β-Me	10 mM
	GDP	50 µM
	PMSF	1 mM
	Add to 1 L with MQ	
Dialysis Buffer	Hepes-KOH, pH=7.5	20 mM
	KCl	150 mM
	$MgCl_2$	6 mM
	DTE	1 mM
	GDP	50 µM
	Glycerol	10 %
Dialysis Buffer 2	Same as above but with 50 %	
Storage	Glycerol	
Ni-NTA Buffer	Same as lysis buffer but without	
	PMSF	
	with Imidazole	500 mM

Initiation Factors (*E. coli*)

Cell lysis Buffer	Hepes-KOH, pH=7	20 mM
	Magnesium acetate	4.5 mM
	Ammonium acetate	150 mM
	β-Me	4 mM
	Spd	2 mM
	Spm	0.05 mM
	Mini protease blocker (Roche)	
Ni-NTA Buffer	Same as lysis buffer without	
	Protease blocker	
	with imidazole	500 mM

Dialysis/Storage	Same as lysis buffer but with the following changes:	
	- Protease blocker	10 %
	Glycerol	250 mM
	Ammonium acetate	

SmpB (*E. coli*)

Cell lysis Buffer	Hepes-KOH, pH=7.5	50 mM
	KCl	1 M
	DTT	1 mM
	Glycerol	10 %
	PMSF	100 µg/ ml
Ni-NTA Buffer	Same as lysis buffer without PMSF	
	with Imidazole	500 mM
Storage/Dialysis	Same as lysis but without PMSF	

RelE (*E. coli*)

Lysis Buffers	NaH_2PO_4, pH=8.0	50 mM
	NaCl	300 mM
	Imidazole	10 mM
	β-Me	5 mM
Ni-NTA Buffer A (wash buffer)	NaH_2PO_4, pH=8.0	50 mM
	NaCl	300 mM
	Imidazole	35 mM
	β-Me	5 mM
Ni-NTA BufferB (Rel B elution)	NaH_2PO_4, pH=8.0	100 mM
	Tris-HCl	10 mM
	Urea	9.8 M
	β-Me	1 mM
Ni-NTA BufferC (Rel E elution)	NaH_2PO_4, pH=8.0	100 mM
	Tris-HCl	10 mM
	Urea	9.8 M

	Imidazole	250 mM
	β-Me	1 mM
Dialysis Buffer	3x PBS, pH=7.4	
	10 % Glycerol	
	5 mM DTT	

2.11.3 Buffers for methods in molecular genetics

10X Transcriptions buffer	Tris-HCl, pH= 8.0	400 mM
	$MgCl_2$	220 mM
	Spermidine	10 mM
RNA extraction buffer	Tris-HCl, pH=7.8	10 mM
	Dithioereithrol	1 mM
	SDS	1 % w/v
	NaCl	100 mM
HPLC running buffer A	NaCl	400 mM
	$Mg(Ac)_2$	10 mM
	NH_4Ac	20 mM
	bring with glacial acetic acid to pH=5.0	
HPLC running buffer B	NaCl	400 mM
	$Mg(Ac)_2$	10 mM
	NH_4Ac (pH 5,0)	20 mM
	Methanol	60 %
	bring with glacial acetic acid to pH=5.0	
VD Buffer (crosslinking)	Tris-HCl, pH=7.4	10 mM
	NH_4Cl	60 mM
	$Mg(Ac)_2$	10 mM
	β-Me	6mM
VD (-) (crosslinking)	same as VD but	
	$Mg(Ac)_2$ →	1mM

2.11.4 Buffers and solutions for western blotting

Transfer buffer (1x methanol)	Tris base	25 mM
	Glycine	192 mM
	Methanol	20 %
	pH adjusted to 8.1 - 8.4	
5x PBS (phosphate buffered saline)	Na_2HPO_4	57.5 g
	NaH_2PO_4	14.8 g
	NaCl	29.2 g
	pH adjusts to 7.1	
	with MQ to 1L and autoclave	
1x PBS-T (PBS and Tween 20)	5xPBS	50 ml
	Tween	100 µl
	MQ	200 ml
Blocking buffer, 3 %	PBS-T	50 ml
	milk powder	1.5 g

2.11.5 Microbiological Media

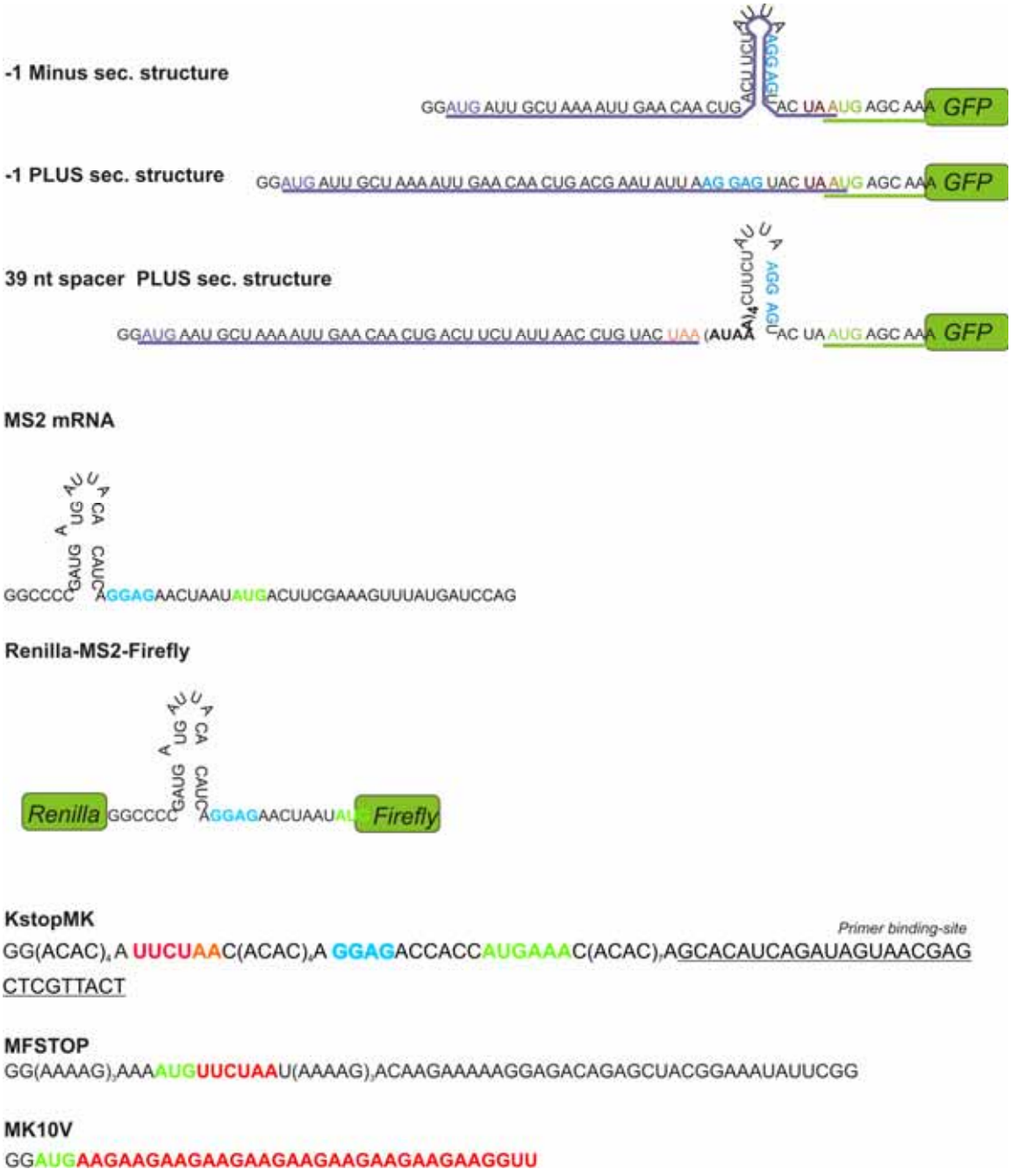
LB (Luria-Bertani)Liquid Medium	Bacto-Pepton	10 g
	Yeast Extract	5 g
	NaCl	10 g
	Water (MQ)	ad 1000 ml

After solving all components completely the pH of the medium was adjusted to 7.4-7.5 with NaOH. Thereafter the medium was sterilised *via* autoclaving at 120 °C and 2 bar for 20 min (steam sterilization).

LB-Agar Plates

For making agar plates the above mentioned medium was used before sterilization by adding bacto agar to a concentration of 1.5- 2 % (w/v). After the steam sterilization process the solution was slowly steered till it reached a moderate temperature and selective antibiotics were added.

2.11.6 List of mRNAs used in this study



KstopMK

Primer binding-site

GG(ACAC)$_4$A **UUCUAA**C(ACAC)$_4$A **GGAG**ACCACC**AUGAAA**C(ACAC)$_7$AGCACAUCAGAUAGUAACGAG CTCGTTACT

MFSTOP

GG(AAAAG)$_7$AAA**AUGUUCUAA**U(AAAAG)$_7$ACAAGAAAAAGGAGACAGAGCUACGGAAAUAUUCGG

MK10V

GG**AUG**AAGAAGAAGAAGAAGAAGAAGAAGAAGAAGGUU

2.11.7 List of cloning primers and important oligos

-1 PLUS sec.struct. A (together with B to produce construct for mRNA transcritpion from cycle GFP)
5'-GGA TGA ATG CTA AAA TTG AAC AAC TGA CTT CTA TTA AGG AGT ACT AAT
GAG CAA AGG AGA AGA ACT TTT CAC TGG AGT TGT CC

-1 PLUS sec.struct. B
5'-CCC CAG ATC TCG ATC CCG CGA AAT TAA TAC GAC TCA CTA TAG GAT
GAA TGC TAA AAT TGA ACA ACT GAC

-1 MINUS sec.struct. A (together with B to produce construct for mRNA transcritpion from cycle GFP)
5'-GGA TGA ATG CGA AAA TTG AAC AAC TGA CGA ATA TTA AGG AGT ACT
AAT GAG CAA AGG AGA AGA ACT TTT CAC TGG AGT TGT CC

-1 MINUS sec.struct. B
5'-CCC CAG ATC TCG ATC CCG CGA AAT TAA TAC GAC TCA CTA TAG GAT
GAA TGC GAA AAT TGA ACA ACT GAC

39nt PLUS sec.struct. (together with -1 PLUS B to produce construct for mRNA)
5'-GGA TGA ATG CTA AAA TTG AAC AAC TGA CTT CTA TTA ACC TGT ACT
AAA TAA AAT AAA ATA AAA TAA ACT TCT ATT AAG GAG TAC TAA TGA
GCA AAG GAG AAG AAC TTT TCA CTG GAG TTG TCC CAA TTC TTG TTG

αRenilla (anti-sense)
TTTGTCGGCCATGATTGGGGTGCTTGTTTG

αIR (anti-sense)
GCAGGATCGAGCGCAGACTG

αFirefly (anti-sense)
ATTTCGCAGCCTACCGTGGTGTTCGTTTCC

Toe-printing primer
AGTAACGAGCTCGTTACTATCTGATGTGCT

Renilla A (together with B to subclone Renilla from pRL-TK)
5'-GAAGATCTTAATACGACTCACTATAGGCCCCCCACCCCACCCCACCCCAGGAGA
ACTAATATGACTTCGAAAGTTTATGATCCAGAACAAAGG

Renilla B
5'-GTTGAGCGAGTTCTCAAAAATGAACAATAAACCCCACCCCACCCCACCCCACCC
CGATGATGATTACACATCACCCCACCCCACCCCACCCCACCCCcAGGAGAACTAAT

Firefly A (together with B to subclone Firefly from pGL3-Control)
ATGGAAGACGCCAAAAACATAAAGAAAGGC

Firefly B
GCGGAAAGATCGCCGTGTAAGAATTCAAAAA

psp65forward (sequencing primer - to check subcloning results of mRNA in pSP65)
CTTTATGCTTCCGGCTCG

2.11.8 Buffers and Solutions for *in vitro* systems

2.11.8.1 Non enzymatic site specific tRNA-Binding Assay (Watanabe-Assay)

The Watanabe-Assay is a modular built *in vitro* system. The following buffer modules were used:

Tico-Buffer	Hepes-KOH, pH=7.5	20 mM
$H_{20}M_6N_{30}SH_4$	Magnesium acetate	6 mM
	Ammonium acetate	30 mM
	ß-Mercaptoethanol	4 mM
HMK Buffer	Hepes-KOH, pH=7.5	20 mM
$H_{20}M_6K_{150}SH_4$	Magnesium acetate	6 mM
	Potassium chloride	150 mM
	ß-Mercaptoethanol	4 mM
Mix I	Hepes-KOH, pH=7.5	60 mM
$H_{60}M_{10.5}N_{690}SH_{12}Spd_{10}Spm_{0.25}$	Magnesium acetate	10.5 mM
(Watanabe Mix I)	Ammonium acetate	690 mM
	ß-Mercaptoethanol	12 mM
	Spermidine	10 mM
	Spermine	0.25 mM
Mix II	Hepes-KOH, pH=7.5	100 mM
$H_{100}M_{22.5}N_{750}SH_{20}Spd_{10}Spm_{0.25}$	Magnesium acetate	22.5 mM
(Watanabe Mix II)	Ammonium acetate	750 mM
	ß-Mercaptoethanol	20 mM
	Spermidine	10 mM
	Spermine	0,25 mM
Binding Buffer	Hepes-KOH, pH=7.5	20 mM

($H_{20}M_{4.5}N_{150}SH_4Spd_2Spm_{0.05}$)	Magnesium acetate	4.5 mM
	Ammonium acetate	150 mM
	ß-Mercaptoethanol	4 mM
	Spermidine	2 mM
	Spermine	0.05 mM

All buffer solutions were set up in volumes of 10 ml from stock solutions. They are aliquotized, shock frozen in liquid nitrogen and stored at -80 °C.

Composition of the stock solutions are listed below in Table 1 :

Table 1: Concentration of stock solutions

Stock	concentration [M]
HEPES-KOH pH=7.5 / 0 °C	1
Magnesium acetate	1
Ammonium acetate	4
ß-Mercaptoethanol	14,3
Spermidine	0.5
Spermine	0.1

2.11.9 Buffers and Solutions for *primer-extension*

10x RT buffer (+/- Mg^{2+})	Tris-HCl pH 8.3 (20°C)	500 mM
	KCl	500 mM
	$MgCl_2$	100 mM/ -
	DTT	50 mM
5xdNTP	dGTP	3.75 mM
	dATP	3.75 mM
	dTTP	3.75 mM
	dCTP	3.75 mM
	in 1x RT + Mg^{2+}	

5xddNTP **4 diffrent must be prepared for each ddNTP !**	ddNTP (A ,G, T or C) in 1x RT + Mg^{2+}	1mM
5x VD (+/- Mg^{2+})	Tris-HCl pH 7.4 (4°C)	50 mM
	NH_4Cl	300 mM
	ß-Mercaptoethanol	30 mM
	Magnesium acetat	50 mM/ -
MuMLV-Mix	5x VD +Mg^{2+}	2 µl
	BSA in VD + (1 mg/ml)	6 µl
	dNTP in VD +	6 µl
	MQ	6 µl
	MuMLV (200 U/µl)	4 µl
Formamide dye (Stop solution)	Formamide	95%
	Bromphenolblue	0.05 %
	Xylenxeanol	0.05 %
	EDTA	20 mM
AMV-RT Mix	AMV (20 U/µl)	0.5 µl
	10x RT (+)	1 µl
	MQ	8.5 µl
Sequence ladder Mix **4 diffrent must be prepared for each ddNTP !**	1x RT (+)	1 µl
	Anneal Mix (oligo:mRNA)	2 µl
	5x dNTP	1µl
	5xddNTP	4 µl
	AMV-RT-Mix	4 µ

2.12 Analytical Methods

2.12.1 Concentration measurement for nucleic acids

Photometrical concentration measurement can be carried out for DNA, RNA as well as ribosomal particles such as 50S, 30S and 70S at a concentration yielding and absorbance between 0.1 and 0.8 at a wavelength of 260nm. The measurement is carried out in a micro cuvette (Beckman Coulter) with a volume of 100 µl the optical path has a length of 1cm.

This simplifies the Beer–Lambert law

$A = -\lg (E/E0) = c * \varepsilon * d \rightarrow A_{260} = c * \varepsilon_{260}$

According to (Sambrook, Fritsch et al. 1989) one can use the following approximation for easy calculation of the concentrations of bio-molecules listed below:

1 A_{260}	= 50 µg/ ml double stranded DNA
	= 40 µg/ ml single stranded DNA oder RNA
	= 20 µg/ ml oligo nucleotide

For ribosomal particles and tRNA

1 A_{260}	= 24 pmol 70S ribosomes (ε= 4.2 x 107 M-1 x cm –1)
	= 36 pmol 50 S subunits (ε= 2.8 x 107 M-1 x cm –1)
	= 72 pmol 30 S subunits (ε=1.4 x 107 M-1 x cm –1)
	= 1500 pmol tRNA

For precise calculation which is needed to determine the concentration of individual transcripts the molecular extinction coefficient of nucleotides and ribonucleotides can be used.

Adenine: ε_{260} [A] = 1.5 x 10^4 M^{-1} cm^{-1}

Guanine: ε_{260} [G] = 1.2 x 10^4 M^{-1} cm^{-1}

Uridin : ε_{260} [U] = 1.0 x 10^4 M^{-1} cm^{-1}

Cytidine: ε_{260} [C] = 0.8 x10^4 M^{-1} cm^{-1}

Thymidine: ε_{260} [T] = 1.0 x 10^4 M^{-1} cm^{-1}

The calculation is carried out by summing up the molecular extinction coefficients after multiplication of each with their according number. Due to the hyperchromic effect the calculated amount should be deduced by ~10% when dealing with DNA samples.

2.12.2 Radioactivity Measurements

All measurements concerning radioactive samples were carried out in the Szintillationcounter Wallac 1409.

2.12.3 Electrophoresis with Agarose Gels

Agarose gel electrophoresis can be used to separate, purify and identify DNA and RNA. The charged nucleic acid is migrating in the electric field towards the anode. The separation is mainly dependent on the molecular weight and the conformation of the molecule. Depending on the desired resolution power a percentage of 0.4 % and 2 % agarose can be used. For preparation of agarose gels 100 ml of 1xTBE or TAE Buffer are mixed with the proper amount of agarose (e.g. 1 g for 1 %) and shortly heated in a microwave, this should be repeated till there are no agarose pieces left in the solution. To the slightly cooled solution 5 µl of a 1 % ethidium bromide solution are added. The mixture is then poured into a gel chamber and a comb is inserted. When the gel is cooled and solid, the pocket forming comb is removed and the chamber is being filled with buffer. Note that a system in TBE should be used for quick analysis and TAE should be strictly used when purification and cloning procedures are carried out afterwards. Boric acid can be present in the DNA-sample after purification and result in a block of enzyme activity especially seen with ligase!

Table 2: Resolution power of Agarose gels

Agarose (%)	Resolution power in the range of
0.4	30 – 2.5 kb
0.8	15 – 1 kb
1	10 – 0.5 kb
1.25	6 – 0.4 kb
1.5	3– 0.2 kb
2	2.5 – 0.1 kb

The electrophoretic separation is carried out at 5-10 V centimetre. After separation the gel is analysed under UV-light. Ethidium bromide intercalates in DNA double strands and is visible *via* fluorescent excitation. The detection of fluorescence is carried out in a Herolab gel documentation system.

Again if any continuative cloning procedures are carried out with the DNA, it should be kept for very short times under UV light. When long UV exposures can not be avoided, only long-wavelength UV-light should be used. In general one should load a small amount of the sample in one pocket and the rest into another. When exposing to short length UV the gel part with less material should be cut off, the band of interest excised and under day light the rest excised using the exposed small piece

as a template, in this way DNA damage can be minimized and cloning results will be successful.

2.12.5 Electrophoresis of DNA/RNA and Proteins

2.12.5.1 Polyacrylamide Gel electrophoresis under denaturing conditions (RNA Gels)

Denaturing polyacrylamide (PAA) gel electrophoresis under urea condition was carried out to separate, identify and purify transcripts from *in vitro* transcriptions. For transcripts between 40 and 100 nucleotides a 10 % PAA gel in TBE buffer was used.

Table 3: Concentrations of acrylamide Giving Optimum Resolution for Purification of DNA Fragments Using Denaturing PAGE

Acrylamide (%)	Fragment sizes separated (bases)	Migration of bromophenol blue (bases)	Migration of xylene cyanol (bases)
30	2-8	6	20
20	8-25	8	28
10	25-35	12	55
8	35-45	19	75
6	45-70	26	105
5	70-300	35	130
4	100-500	~50	~230

The gel composition includes 8 M urea, to denature RNA and resolve secondary structures which might distort the result. The detection of RNA can be achieved *via* Toluidine staining. In general the Table 4 can be used for the identification of appropriate PAA percentage to give best resolution of transcripts:

For analytical PAA gel electrophoresis Biorad's Mini-Protean II System can be used. The glass plates are backed at 180 °C for 3 h to destroy RNases. Plastic parts are cleaned thoroughly with RNaseZap an RNase Decontamination Solution from Ambion and rinsed with MQ water; also 10 % SDS solutions are possible for decontamination purposes.

Table 4: Composition of denaturing RNA gels

Reagent	**Acrylamide concentration**			
	4 %	6 %	8 %	10 %
Urea (ultrapure; g) f.c. 7 M	25.2	25.2	25.2	25.2
38 % acrylamide / 2 % bisacrylamide (ml)	6.0	9.0	12.0	15.0
10× TBE buffer (ml)	6.0	6.0	6.0	6.0
H_2O (ml)	27	24	21	18.0
Total volume (ml)	60	60	60	60

Gel solutions can be stored at 4 °C in the dark for longer times (1-2 month), but should not be kept at room temperature. For preparative electrophoresis a larger apparatus is needed (MPI, electronic workshop, self build). Glass plates of the size 160 mm x 140 mm are used together with a spacer of 1 mm. A special gel pocket former is used to create a single pocket of the size of 10 mm x 100 mm. For such a gel usually a volume of 25 ml is needed.

After gel polymerisation the gel is placed into an electrophoretic device and the pocket former is being removed, then TBE buffer is added and a pre-run is conducted using 280 V for 30 min. Such a pre run is strictly required, since an operating temperature of ~50 °C or more has to be reached, if not the samples have the possibility to renature leading to double bands and smear.

Sample preparation:

From analytical transcriptions 3 µl, 7 µl and 15 µl are mixed with 2xRNA loading dye and brought to the same end volume with MQ. Samples should be heated to 95 °C 2 min and then put on ice immediately to avoid formation of secondary structures. Before loading the samples to the pockets, they should be flushed with TBE buffer intensively, since urea tends to diffuse into the wells and disrupt sample loading.

For preparative electrophoretic separation the sample of large transcription volumes should be lyophilized before loading and then dissolved in loading dye. This way sample loss can be minimized and a sharper band separation of transcription products or labelled RNA can be achieved. The run is conducted at 280 V for 2 h, or until the bromophenol front has reached the end of the gel.

Staining of the gel is done with toluidine solution. The gel is submerged in the dye for 10 min and slowly shaken. For destaining the gel is put into MQ, the RNA will remain coloured.

2.12.5.2 Polyacrylamide gel electrophoresis under denaturing conditions (Sequencing gels)

For the separation and purification of P^{32}-labelled tRNAs and mRNAs as well as testing RelE activity with comparative alkali ladders and T1 cleavage 0.4 mm thick PAA gels containing urea were prepared. The gel solution for sequencing gels (8-10% PAA, 8M UREA in TBE) should always be made fresh and not stored over longer periods, since this can result in a decay of urea and oxidising of the PAA making acrylic acid. Solving such large amounts of urea takes approximately 60min to speed up this process the solution may be heated up to 50 °C but not above since this also increases the decay of the components. Afterwards the solution should be filtrated (folded filter Schleicher and Schüll 595 ½ #10311647 Φ=185 mm) and should also be degassed.

To prepare glass plates for large sequencing gels they should be layered with Dimethylsilan (Fluka), this way the very thing gels can be easily transferred to filter paper and dried, without this treatment gels might stick to glass plates resulting in gel breakage. A film of 5 % dimethyldichlorosilane in $CHCl_3$ to one side of each plate should be applied by wetting a Kimwipe with the solution and wiping carefully. After the film dries, the plate should be wiped with 70 % ethanol or isopropanol and dry with a Kimwipe. Subsequently the plates should be cleaned with acetone until they become “squeaky”. Check plates for dust and other particulates before assembling the gel sandwich, which should be assembled in such a fashion that the silanized surfaces are facing inward.

Use 0.2 - to 0.4 mm uniform-thickness spacers (Biorad 165-3818) and large book-binder clamps, making side and bottom spacers fit tightly together. Instead of bottom spacers also Whatman paper can be used to seal the bottom of the gel. Rest of the paper remains inside after pouring of the gel and does not disturb the run.

60 ml of the desired denaturing acrylamide gel solution is mixed with 60 µl TEMED, then 0.6 ml of 10 % ammonium persulfate and gently pulled into a 60 ml syringe, avoiding bubbles. With short plate on top, raise the upper edge of gel sandwich to

45° angle from the benchtop and slowly expel acrylamide between plates along one side. Adjust angle of plates so gel solution flows slowly down one side.

When the solution reaches the top of the short plate, the gel sandwich should be lowered so that the top edge is ~5 cm above benchtop. Then an empty disposable pipet-tip rack or stopper has to be put underneath the sandwich to maintain the low angle. The flat side of a 0.2 to 0.4 mm shark's-tooth comb is inserted into the solution below top of short plate, being very careful to avoid bubbles. Book-binder clamps again can be used to pinch combs between plates so that no solidified gel forms between combs and plates. Extra acrylamide gel solution can be layered onto the comb to ensure full coverage. For full polymerisation the gel should be kept at least for 2 h at room temperature but can also be kept overnight in a bag to avoid complete drying out.

Sample preparation should be carried out according to 2.4.3.3. Pre-run of the gel has to be carried out for ~30 min by setting power supply to 45 V/cm, 1700 V, 70 W constant power. Then reinsert teeth of cleaned shark's-tooth comb into gel sandwich with points just barely sticking into gel. Using a Pasteur pipette, wells are rinsed thoroughly with 1×TBE buffer to remove stray fragments of polyacrylamide and urea. 2 to 3 µL sample per well are loaded. Gels are run at 45 to 70 W constant power. A gel temperature of ~65 °C should be kept. To determine length of electrophoresis migration of marker dyes has to be observed, see table. After the gel run is finished buffer from upper and lower reservoirs of apparatus has to be drained the liquid discarded as radioactive waste.

The gel sandwich is then removed from apparatus and laid flat on paper towels with short plate up. Then excess liquid and remaining clamps or tape are removed. Side spacer are also removed and long metal spatula is insert between glass plates where spacers had been. The plates are pried apart by gently rocking spatula.

Once the plates are separated lay paper (Whatman) on top of the gel, care should be taken to prevent air bubbles from forming between paper and gel. The blotting paper is peeled up and gel should come off the plate with it. Afterwards paper with the gel on can be placed on a preheated gel dryer. Before, it has to be covered with saran wrap. Then the gel is dried thoroughly 20 min to 1 h at 80 °C. Then the gel can be put to a phosphorimager plate (Amersham) and exposure is carried out ON (12-16 h) for sharper bands and background reduction up to 4days. When preparative isolation of RNA is performed the gel is not removed from the glass plate and dried! It is rather kept on the plate and put in a plastic bag or wrapped with seran sheet before

exposure with x-ray film which is then used as a template to excise the isolated RNA from the gel.

2.12.5.3 Protein-SDS-Polyacrylamide gel electrophoresis

The SDS polyacrylamide gel electrophoresis (SDS-PAGE) after Laemmli (Laemmli 1970) can be used to separate protein mixtures under denaturising conditions. The separation of the proteins in the discontinuous system is achieved in a biphasic gel. The Stacking gel is used to concentrate the protein-SDS complexes; this results in a sharp separation of proteins in the gel matrix of the separating gel, in this phase of the gel proteins are separated according to their mass, since the native charge of the protein is shielded by SDS molecules and the structure has become comparable due to denaturation. The number of bound SDS molecules is depending on the number of amino acids, thus the resulting charge of the SDS protein complex is dependent on the molecular weight of the protein. For the SDS-Page a Mini Protean II System from Biorad was used. To make gels solutions are combined according to Table 6 and 7.

Table 5: Resolution power of SDS-PAGE

Range of Separation of Proteins in SDS-Page	
Gel %	Range (kDa)
6	60-200
8	40-140
10	20-80
12	15-70
15	10-15

Table 6: Composition chart of a 12 % Separation Gel (for different % simply change amount of 30 % acrylamide stock)

Number of gels	**1**	**2**	**3**	**4**
30 % Acrylamide	2 ml	4 ml	6 ml	8 ml
1.5 M Tris, pH 8.8	1.3 ml	2.6 ml	3.9 ml	5.2 ml
H_2O	1.6 ml	3.2 ml	4.8 ml	6.4 ml
10 % SDS	50 µl	100 µl	150 µl	200 µl
10 % APS	20 µl	40 µl	60 µl	80 µl
TEMED	2,5 µl	5 µl	7.5 µl	10 µl

Table 7: Composition chart for stacking gels (4 % Acrylamide)

Number of gels	**1**	**2**	**3**	**4**
30 % Acrylamide	0.2 ml	0.4 ml	0.6 ml	0.8 ml
0.5 M Tris, pH 6.8	0.18 ml	0.36 ml	0.54 ml	0.72 ml
H_2O	1.1 ml	2.2 ml	3.3 ml	4.4 ml
10 % SDS	15 µl	30 µl	45 µl	60 µl
10 % APS	7.5 µl	15 µl	22.5 µl	30 µl
TEMED	1.25 µl	2.5 µl	3.75 µl	5 µl

First the separating gel is poured and over layered with butanol or isopropanol, to produce a smooth, completely levelled surface on the upper edge of the separating gel. Upon polymerisation and removal of the over layer the stacking gel can be poured and a comb inserted.

Samples are prepared with 5x loading dye and all filled with MQ to the same volume. The samples are heated to 85 °C for 5 min for complete denaturation and prior to loading quickly cooled on ice.

Staining of gels was achieved using the quick staining protocol. Here the gel is simply put into staining solution and left submerged for 5-15 minutes, older solution usually needs longer staining periods. The solution can be used until it starts to loose the dark brown colour meaning that HCl is not present anymore. Then the gel is washed in water and kept in it. Destaining is carried out in microwave boiling the gel for 2-5 minutes. The process usually needs to be repeated 1-2 times always adding fresh water. Gel can be kept in water for long periods without loss of band intensity.

2.12.6 Analytical density gradient centrifugation

For the analysis of ribosomal complexes as well as the integrity of isolated 70S, 50S and 30S analytical density centrifugation is used. A linear sucrose gradient between 10 % - 30 % (w/v) is applied. This gradient is formed using the Biocomp Gradient Master 107ip. The separation of sample over the gradient is achieved *via* ultracentrifugation in an appropriate swinging bucket rotor (SW60, SW40 or SW28; Beckmann). One should choose between the three by taking into account the amount of material which should be loaded onto the gradient. SW60 should be loaded with 0.5 A_{260} – 2 A_{260}, SW40 should me loaded maximally with 10 A_{260} units and SW28 with material which is ranging high above 10 A_{260} units.

Running time and rpm were optimized for ideal separation:

Subunit profile in SW60, 38,000 rpm and 240 minutes
Polysome profile in SW40, 18,000 rpm and 16 hours
Subunit profile in SW40, 22,000 rpm and 18 hours

Analysis was carried out by pumping out the gradients with a "flow-through" photometer which continuously monitors the optical density measured at A260. Data could be further analyzed with the help of the pico log recorder.

2.12.7 High Performance Liquid Chromatography (HPLC) fort he purification of aminoacyl tRNAs

The reversed-phase chromatography, in its HPLC mode, is a powerful tool for the separation of the different tRNA forms. Although the classical low-pressure reversed-phase as well as the Benzoyl-DEAE cellulose chromatography involves hydrophobic interactions as a separation parameter, a large portion of the energy involved in the binding of the tRNA to such matrices is due to ionic interactions. When the reversed-phase chromatography is performed in presence of a constant and relatively high salt concentration, these interactions are weakened, and using an increasing buffer-methanol gradient the separation becomes more dependent on the hydrophobic of the sample. Following this idea, Odom et. Al. (Odom, Deng et al. 1988) developed an efficient reversed-phase HPLC system in which the hydrophobicity of the aminoacyl group, its N-acetyl derivative or a covalent attached organic groups, are the main factors in the separation of different tRNA species. A modification of such a system was used here for the purification of acetylated tRNAs. An aliquot of 1-2 ml comprising 20-50 A_{260} units of Acetyl-tRNA (labeled with [^{3}H] or [^{14}C]), prepared as described in the previous section, was spun down 5 min at 15,000 x g in order to pellet any solid residue in suspension. The clear supernatant was then applied to a Nucleosil 300-5 C8 column (250x4 mm, 5 µm bead size, 300 Å pore diameter) equilibrated in buffer A at a flow rate of 0.5 ml/min, and at a working pressure of 40-50 bars. The eluate was collected in 1 ml fractions and the absorbance at 260 nm was continuously monitored. The column was washed during 5-10 min (depending on the size of the sample) and a programmed binary gradient of buffers A and B was applied for elution. The free nucleotides (mainly ATP and AMP remaining from the

aminoacylation step) and the free amino acids eluted during the washing step with buffer A and the different forms of the tRNA eluted sequentially when the percentage of buffer B increased. The deacylated tRNA elutes first and the charged form elutes later.

2.12.8 Western Blot and immunodetection of proteins

Prior to analysis proteins were separated on a SDS-PAGE. The transfer of proteins to a PVDF membrane was carried out in a Mini Trans Blot system (Biorad) in transfer buffer (25 mM Tris pH 8.2; 200 mM Glycine; 20 % Methanol) at constant voltage of 100 V for one hour in the cold room or at room temperature using an appropriate cooling system in the tank. Prior to transfer the membrane had to be activated by wetting it in methanol for some 3-5 seconds then washing it in MQ for 5 minutes and equilibrating it in transfer buffer before the blotting procedure. Gels from SDS-PAGEalso should be equilibrated in running buffer before for about 20 min. The transfer was checked with staining of membrane with Ponceau and staining of the gel with Coomassie blue. To prepare the membrane for binding of the antibody and immunodetection unspecific binding sites for the antibody need to be blocked therefore the membrane should be incubated in a small tray with 1xPBS-T with 3 % milk powder (blocking buffer). The milk powder has to resolved in PBS-T and centrifuged for 10 min at 10,000 rpm to remove any non-solving particles and other impurities which might distort the result of the immunoblot. The blocking in such a “blocking buffer” can be either carried out overnight at 4 °C with gentle shaking in the cold room or 1-2 h at room temperature. Afterwards the membrane should be washed 3x with wash buffer (1x PBS-T) at room temperature (RT) for 10 min, the amount of washing buffer used varies with the size of the container and the size of membrane. In general the membrane should swim in the buffer taking 20-50 ml; this is true for all washing and incubation steps. After washing membrane is then incubated with the primary antibody, which means the antibody that detects the a given protein blotted to the membrane. The antibody is supplemented in an optimized dilution for protein detection in 1 x PBS-T, 3 % milk powder (e.g. anti-S7 1:100,000). This incubation is carried out at RT for 2 h. If the primary antibody is not already coupled to a horse radish peroxidase (conjugated primary antibody), which is used for luminescence detection, now again 3x washing of the membrane is being performed in 1x PBS-T. Afterwards the secondary antibody is supplied in a concentration of 1:10000 in PBS-T, 3 % milk powder. The membrane is submerged

in this solution for 1 h at RT. Again three washing steps with PBS-T for 10 min follow. The membrane is then quickly dried, be aware that over drying is not good and will impair good results. To develop the membrane an ECL-Kit for western blotting detection from GE Healthcare is used according to the manual of the supplier. chemiluminescence detection is then performed with a LAS-1000 camera (Fuji Film) which makes it possible to store the results digitally.

2.12.9 Stripping and reprobing membranes for immunodetection

The complete removal of primary and secondary antibody for reprobing membranes with an antibody specific for another antigen is only possible for PVDF membranes, since the so called “stripping” procedure is to harsh for nitrocellulose membranes and destroys them. PVDF membranes may be stripped of fluorescent signals and bound antibodies and then reprobed several times - keep in mind the important fact, that with each stripping procedure the loss of antigen from the membrane occurs as well. Thus antibody dilutions for primary antibodies generally have to be shifted up, to still have good signals when deciding for reprobing. For stripping, the membrane should be submerged in stripping buffer (sealed in a small plastic bag) and heated to 60 °C for 45 min. Then the membrane should be washed with large volumes of PBS-T for 2 x 10 min and can then again be blocked with blocking buffer, restarting the protocol mentioned in section 2.12.8.

2.13 Methods of microbiology and molecular genetics

2.13.1 Growing and preserving E. coli

As a standard medium for growing *E. coli* cells LB was used throughout this study. To select specifically for growth of plasmid carrying strains an appropriate antibiotic as a selective agent was added to the medium. The plasmid encoded resistance then allowed growth of the selected cells only. Incubation if not indicated otherwise was carried out at 37 °C in a shaking incubator at 160 rpm or higher.

Single colonies can be picked when growing cells on solid LB-Agar plates, these also can be used for short term conservation of *E. coli* strains or for reactivation of glycerol stocks (several weeks at 4 °C; when Tetracycline was the selective antibiotic, plates should be kept in the dark since Tet is light sensible).

Glycerol stocks are used for long term conservation of *E. coli* strains. For making a glycerol stock 1 ml of an ON culture grown in LB-Medium is mixed with 333 µl of sterile 50 % glycerol (v/v). After mixing thoroughly the stock is shock frozen in liquid nitrogen and stored at -80 °C.

2.13.2 Production of electro-competent *E. coli* cells

For the generation of transformation competent cells for electroporation 500 ml LB were inoculated with the appropriate strain and grown at 37 °C to the absorption of 0.7 at 560 nm in a shaking incubator. Cells are then harvested in sterile centrifuge bottles at ~6,000 rpm for 15 min. The cells are then resuspended in 100 ml of cold demineralised water or MQ, and centrifuged again at 6,000 rpm for 15 min. This washing step is repeated twice. After that the cells are dissolved in 4 ml of cold 10 % (v/v) glycerol and aliquotized in volumes of 40 µl. Aliquots are shock frozen in liquid nitrogen and stored at -80 °C. All steps of this procedure should be carried out quickly at 4 °C.

2.13.3 Transformation *via* electroporation

Cells for electroporation are slowly thawed on ice for 10minutes. The ligation mixture or plasmid (0.1 to 1 ng) is added to the cells and mixed. This is then transferred to a cooled electroporation cuvette (0.2 cm gap size; Biorad #165-2092). The electroporation is carried out in an electroporation device (Biorad Gene Pulser/ Pulscontrol System) with the following settings: Voltage (U)=1.5 kV, Resistance 200 Ω and capacitance C=25 µF. The time constant for a successful transformation lies between 3 to 4 ms. Directly after administering the pulse LB-Medium without the selective antibiotic is added to the cells and incubation at 37 °C in an incubator shaker follows (~60 min). 10-200 µl are plated on LB agar plates with the selective antibiotics and incubated ON in an incubator.

2.13.4 Digestion of DNA with Endonucleases

Sequence specific digestion of DNA can be carried out with special restriction endonucleases. In general endonucleases of Type II are used - such enzymes can produce either 3’ or 5’ overhangs (so called sticky ends) or smooth ends (also called as blunt ends). The choice of the restriction enzyme in general is done according to cloning problem.

Programs such as the “NEBcutter” http://tools.neb.com/NEBcutter2/index.php can be used to find the fitting enzyme. Normally enzymes are selected that produce sticky ends, since they allow choosing for specific orientation when ligating the subcloned fragment (insert) into the destination plasmid (vector). The activity of the endonucleases is described in units (U). One unit is defined as the amount of enzyme which can digest standard DNA (the genome of the phage λ is usually used as a reference) at 37 °C completely. This phage carries a genome of 48 kb that shows a random distribution and number or restriction sites for different enzymes, when choosing the amount of enzyme this fact should be kept in mind.

For fast analytical restriction a rule of thumb can be applied: 1 µg of plasmid needs 1 Unit of restriction enzyme. The reaction is conducted at 37 °C in the supplied buffer for 1-2 h. Recently superfast enzymes have been put on the market which allow much shorter incubation times of 5 minutes and less.

For small DNA fragments (e.g. PCR products or inserts which are a result of oligo annealing) a precise calculation of enzyme units is reasonable. The following formula is taking the size of the DNA fragment to be digested as well as the number of restriction sites into account.

$$\text{Unit per µg and hour} = \frac{\text{Size of standard DNA (bp)} * \text{number of restriction sides (Fragment)}}{\text{Size of fragment DNA (bp)} * \text{Number of restriction sites (Standard-DNA)}}$$

The ligation of DNA fragments is conducted with the help of T4 DNA-Ligase (NEB). The insert and the vector are digested with the appropriate nucleases and purified *via* gelextraction. The purified products (insert and vector) are mixed in molecular ratios of 3:1 and 5:1 (insert to vector ratio). The ligase reaction is incubated at 16 °C for 16 hours (ON). Following the reaction the ligase should be heat-inactivated at 65 °C for 20 min and the whole ligase reaction filter dialysed. Here a nitrocellulose filter (membrane filters # VMVP02500; Millipore) is put in a Petri-dish swimming on water, to remove the salts of the enzyme buffer the reaction is pipetted onto the filter and left there for ~ 20-30 min (if salt is not removed this can result in lowering electrical resistance during electroporation and “sparking” where cells are killed and failing transformation occurs!!!). The resulting ligation reactions can be transformed in *E. coli* cells.

2.13.5 *In vitro* transcription with T7-Polymerase

Transcriptions are carried out with DNA dependent RNA-polymerases (transcriptases). The transcriptases from phages like T7, T3 and SP6 are good for in vitro transcriptions, since their subunits are relatively easy to purify. Phage transcriptases have high processivity, they can use plasmid DNA or PCR-transcripts with their suitable promoters as templates. In the in vitro transcriptions used in this work the run-off transcription is used as a general method, which uses linearised plasmid DNA as a template (Bommer, Burkhardt et al. 1996). The plasmid is cut with a restriction enzyme directly after the transcribed sequence. To optimize the best quantity of linearized plasmid for the transcription protocol an analytic reaction is carried out in 50 µl using 0.5-1 pmol/ µl). 1.8 µl of ATP, GTP, UTP and CTP of 100 mM are mixed with 5 µl 10xtranscription buffer and appropriate amount of MQ on ice. Then everything is brought to room temperature and the linearised plasmid as well as 2.1 µl BSA (3 mg/ ml, RNase free) and 1.4 µl RNasin (40U/ µl) is added. The reaction is started after administering 0.25 Units pyrophosphatase and 20 pmol of T7-transcriptase. Everything is incubated at 37 °C. The success of the transcription is monitored on a denaturing PAA gel. When the optimal amount of plasmid has been identified an analytical scale transcription can follow which is between 300-1000 µl. Such a reaction is usually incubated overnight at 37 °C. The isolation of an mRNA with correct size is carried with a preparative PAA gel electrophoresis with urea. The mRNA containing band is excised from the gel. To define the area of excision, a small part of the gel should be cut of and stained with toluidine blue then the stained strip shows the band. This strip is put next to the gel and used as a template to perform exact excision of the mRNA band. Do not stain the complete gel, this would result in possible contamination with RNases. The gel is then crushed with a sterile blade or by pushing it through a syringe and the RNA is extracted from the Gel with extraction buffer and phenol being vortexed ON.

If the mRNA is tricky to translate or very long mRNAs of over 200 nucleotides length should be transcribed the T7 RiboMax Express Large Scale RNA Production System was used according to the manual of the supplier.

2.13.6 Overexpression of His-tagged proteins

The analysis of overexpressed protein was carried out with SDS-PAGE. For expression of a given protein an ON culture was prepared and diluted to 0.1 OD_{560}. in medium. The culture was then grown till it reached absorption of ~0.6 at OD_{560}.

2x1 ml where withdrawn from the inoculum and put on ice. The rest of the culture was supplemented with 1 mM IPTG and grown for 3 h more. Afterwards again two samples of 1 ml were withdrawn. All samples were centrifuged in a table top centrifuge at 6000 rpm for 10 min. The sedimented cells were dissolved in SDS-sample buffer and denatured at 85 °C for 10 min. Subsequently samples can be applied to SDS-PAGE, an overexpression results can be checked with Coomassie staining. Comparable amounts of material should be put onto the gel concerning uninduced and induced samples. As for the purification, cells were disrupted with a microfluidizer, centrifuged at 45.000 rpm for 2.5 hours to remove cellular debris. Lysate was then used for purification with Ni-NTA from QIAGEN after the instruction in the manual.

2.14. Preparative Methods

2.14.1 Plasmid isolation

Plasmid isolation from *E. coli* cells can be achieved with kits of the company Quiagen. According to the amount of plasmid that should be isolated or volume of cell culture used one can choose between the QIAprep Miniprep Kit or the Midi, Maxi or Giga Kits. The isolation method is based on the method of alkaline lysis established by Binboim and Doly (Birnboim 1983). After the cells are lysed the adsorption of DNA is achieved with silica gel columns (Mini-Prep) or with anion exchangers (Midi and Maxi Prep). The purification of DNA is carried out with extensive washing of the column. In the mini prep protocol a low salt concentration is used for elution (water or 10 mM Tris-HCl, pH 8.5). When using the anion exchangers for bigger preparations the elution is carried out under high salt conditions (Buffer QF). Since the volume is bigger with this Kit the eluted DNA has to be precipitated with isopropanol to concentrate the plasmid. The complete procedure can be found on the supplier's web-page and should be followed precisely (http://www1.qiagen.com/Plasmid/Handbooks.aspx). Differing from the companies suggestions RNase A is not added to the lysis buffer, to prevent RNase

contamination of the lab ware which might result in inactivation of ribosome and other RNA compounds of the *in vitro* systems. Generally 5 ml overnight culture is used for Mini-Preps, 50 ml LB-overnight cultures are used for Midi-Preps and 200 ml are used for overnight cultures.

2.14.2 Purification of DNA from Agarose Gels (Gel extraction)

The isolation and purification of DNA-fragments after separation on an agarose gel can be accomplished directly from the gel piece. Since DNA becomes visible by staining the gel with ethidium bromide or by directly adding it to the agarose prior to the run one can make the fragments visible under UV light and excise them precisely with a sterile blade. The isolated gel piece can then be treated with the gel extraction kit by the company Invisorb following the instructions in the manual.

2.14.3 Phenol chloroform extraction

To separate nucleic acids in aqueous solutions from proteins, namely after incubating them with dephosphorylase or restriction enzymes extraction with organic solvents is performed. The DNA/RNA solution is brought together with an equal volume of phenol (70 % v/v) vigorously mixed for at least one minute. To separate the phases of organic solvent and aqueous solution a centrifugation step at 10,000 rpm for 1 min is performed. The denatured protein then is separated in the organic and interphase and the nucleic acid can be removed in the aqueous phase and brought to a separate vial. Here it is mixed with one volume of chloroform/isoamylalcohol (24:1), phase separation is again achieved by centrifugation for 1 min at 10,000 rpm. The upper phase carries the DNA. If the volume is too big the concentration of the nucleic acid can be up scaled *via* precipitation with ethanol and salt, solving it in a smaller volume of buffer afterwards.

2.14.4 Ethanol precipitation

Nucleic acids can be precipitated in the presence of monovalent cations adding EtOH to an aqueous solution. 0.1 volumes of a 3 M sodium acetate solution and 2.5 – 3 volumes of ice cold 96 % (v/v) EtOH are added. The precipitation process is supported by centrifugation (30 min, 14,000 rpm, 4 °C). The supernatant is discarded and the pellet washed with 70 % EtOH (v/v). The centrifugation (15 min, 14,000 rpm, 4 °C) is stabilizing the pellet. The supernatant again is discarded and the pellet dried

at RT or if possible in a vacuum centrifuge. Subsequently it can be solved in an appropriate amount of water. When dealing with very small amounts of nucleic acid glycogen can be added or another suitable carrier, also precipitation time can be prolonged and incubation can be carried out at -20 °C for 2 h to ON or at -80 °C for 1 h.

2.14.5 Cold TCA precipitation (tRNA)

A 10 µl sample (normally two per assay) was delivered into a glass test-tube (8 x 10 cm) containing 20 µl of precipitation carrier solution (yeast $tRNA_{bulk}$ 5 mg/ ml in EDTA 0.5M pH 8.5). Two ml of ice cold TCA (10 % w/v) was immediately added and vortexed for 1-2 seconds. The aminoacylated tRNA was precipitated on ice for 30 min and the precipitation mix was then filtered through glass fiber filters. The filters were washed three times with 2 ml of cold TCA (10 %) and once with 2 ml diethylether/ethanol (1:1 v/v). The filter was submerged in 3 ml of Rotiszint and shaken ON in a counter vial. The radioactivity adsorbed on the filter was measured the next day in the Wallac 1409.

2.14.6 Cold TCA precipitation proteins

To precipitate proteins from dilute mixtures TCA is used. To a given sample volume 100 % TCA is added to bring the solution to a f.c. of 10 % TCA. Then sample is then kept on ice for 30 min or incubated overnight at 4 °C. This step follows centrifugation at 14,000 rpm for 45 min. The supernatant is carefully removed and the pellet washed with ice cold acetone. Another centrifugation step follow spinning the sample for 10 min at 14,000 rpm. The supernatant is removed completely and the pellet dried. Since residual TCA sometimes remains in the pellet it can result in acidification of the sample making SDS-PAGE runs troublesome. To avoid acidification a basic sample buffer should be prepared, just by adding 1 M Tris-base to a final concentration of 10 % to the SDS loading dye. This way left over TCA is neutralised and does not distort the run in SDS gels.

2.14.7 Preparative isolation of RNA from PAA gels

Via preparative gel electrophoresis separated RNA can be extracted with Phenol and extraction buffer from the gel. The gel piece with the RNA of choice is crushed with by pushing it through a syringe. It is then mixed with 10 ml phenol (70 % in extraction

buffer v/v) and 10 ml extraction buffer; for very small gel pieces the reaction can be carried out in an Eppendorf tube using instead of 10 ml 0,5 ml of each. The extraction is carried out ON for at least 12 h shaking vigorously on a Vortex mixer. The solution then is centrifuged for 60 min at 10,000 rpm or more to force phase separation. The aqueous phase is taken to another tube and the rest is re extracted with 10 ml of extraction buffer. The top layers of both extractions are then brought together and precipitated by adding 2.5 volumes of EtOH and centrifuging at 14,000 rpm for 2 h. The precipitated RNA is then washed with 70 % EtOH. The isolated RNA is solved in MQ water aliquotized, shock frozen and stored at -80 °C.

2.14.8. ^{32}P-labelling of tRNA, mRNA and short oligos

To label 5'ends of nucleic acids with ^{32}P the enzyme T4 polynucleotide kinase and [γ-[^{32}P]-Adenosine-5'-triphosphate are used. Beforehand the 5' phosphate group is removed with the enzyme Calf Intestinal Alkaline Phosphatase (CIP) from natural nucleic acids such as tRNAs and mRNA (for oligos this step is not needed). Here 500 pmol of material can be incubated with 2.5 U of enzyme in a volume of 50 µl of appropriate buffer supplied by the company. The reaction is put together on ice and incubated 45 min at 50 °C. To stop the reaction 5 µl of 3 M sodium acetate are added and the nucleic acid is separated from the protein *via* phenol/chloroform treatment. Afterwards the 5'-labeling can take place. Here 200 pmol of dephosphorilated nucleic acid 40 U of polynucleotide kinase and 20-50 µCi [γ-^{32}P]-ATP are incubated in the enzyme buffer supplied by the company. The reaction volume of 40 µl is put together on ice and then incubated for 1 hr at 37 °C. After the reaction is finished the samples are loaded on a G-25 quick spin column to remove most of the unincorporated nucleotides. The procedure should be carried out according to the protocol from Roche. Usually the eluate is about 50 µl or more. If additional gel purification is needed, it is advisable to reduce the sample volume in a speed vac before loading.

2.14.9 tmRNA preparation - Isolation of RNA from whole cells after Jünemann (Jünemann 1996)

1L culture of the strain BL21DE3/pGEMX2-ssrA was used for overexpression. Cells were pelleted by centrifugation at 6000 rpm for 10 min at 4 °C. Then the pellet was washed twice using 15-20 ml wash-buffer. The suspension is transferred to S600-tube and again centrifuged for 10 min at 6000 rpm. Then the cell pellet is balanced

and diluted with $H_{20}M_{10}$-buffer (0.25 g cells/ ml). All is transferred to a tightly closable lid and one volume of phenol is added and vortexed for 45 min at 4 °C. After the phenolisation the mixture is centrifuged at 7000 rpm for 1 h. The RNA is then going to the aqueous phase, proteins and cell debris are forming a thick pellet. Therefore after the centrifugation 3 phases should be clearly visible clearly visible, one containing phenol, one the aqueous phase containing buffer and RNA and in between a thick pellet of debris and protein. The aqueous phase should be taken to a fresh tube and 1 volume of chloroform added and 1 min vortexed to remove residual phenol. Then the sample is centrifuged again for 20 min and the aqueous layer is removed from the top. To remove the large RNAs 0.65 volumes of 5 M NaCl are added and everything kept on ice for 30 min. To pellet the unwanted large RNAs the sample is centrifuged for 1 h at 7000 rpm. The pellet is discarded and the supernatant is being precipitated with ethanol *via* addition of 2-3 volumes of 100 % EtOH. Precipitation can be carried out either ON at -20 °C or within 1-2 h at -80 °C. The sample is centrifuged at 10,000 rpm for 1 h and the pellet washed twice with 70 % EtOH to remove salts. The resulting pellet is then solved in a suitable amount of MQ aliquotized and shock frozen and stored at -80 °C. After the Jüneman protocol further purification with a denaturing PAA gel has to be carried out, since the tmRNA is still contaminated with tRNAs and 5S rRNA.

2.14.10 Purification of alanylated tmRNA

When charging tmRNA with alanine (2.14.11) we can detect efficiencies of around 30% meaning the rest of the molecules remain uncharged after the reaction is finished. To be able to separate charged from uncharged species HPLC is a method of choice, because the tmRNA is very large and alanine not very hydrophobic, it is impossible to estimate whether a purification works out properly. Using a C8 column (Figure **12.14.10-1 A**) we could observe an improvement from 30% to 50% of charged tmRNA. Yet we could not detect binding of tmRNA to the ribosome. To be able to keep tmRNA in a “native” state throughout the purification, we also tried to purify alanylated tmRNA with an immobilized EF-Tu column according to Ribeiro and Sprinzl (Ribeiro, Nock et al. 1995). To conduct this method we purified His-tagged EF-Tu from *Thermus thermophilus*, which is more stable than the *E. coli* pendant, and bound it to Ni-NTA raisin (Figure **12.14.10-1 B**). EF-Tu forms ternary complexes specifically in its GTP bound conformer with charged tRNAs. The trick of this method

is to incubate a tRNA mixture after charging with this Ni-NTA•EF-Tu slurry, where EF-TU•GTP then only fishes out the charged molecules. The slurry then is washed excessively losing all the non bound uncharged tmRNA molecules.

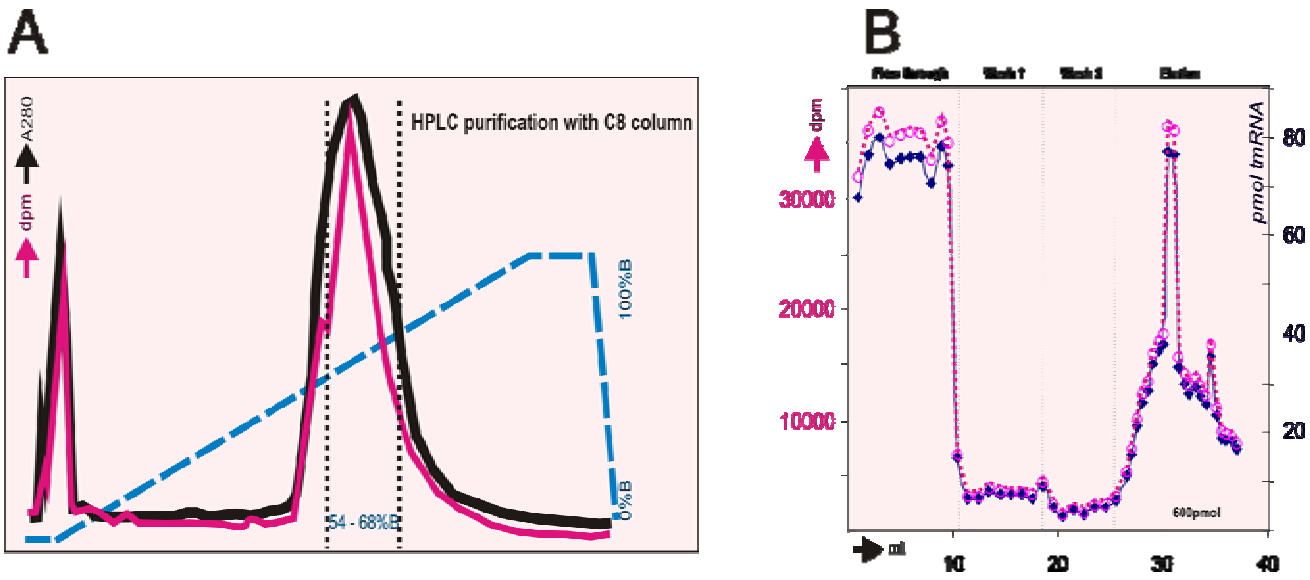


Figure 12.14.10-1: A. Typical profile of charged tmRNA applied to a HPLC C8 column; B. tmRNA elution profile of self build EF-Tu column (Immobilized EF-Tu column after Rebeiro et al. 1995 (Ribeiro, Nock et al. 1995)

Afterwards Ala-tmRNA molecules which are bound to the EF-Tu are eluted by administering GDP to the slurry. EF-Tu then looses its bound GTP which is replaced by GDP. When this happens EF-Tu undergoes large conformational rearrangements. Switching to the GDP conformer means that it can not bind to charged-tmRNA molecule any longer. This way we were able to enrich the charged fraction from 30% to approximately 50%.

2.14.11 Preparative tRNA aminoacylation

The preparative aminoacylation of specific tRNAs with the cognate amino acid was achieved by the synthetase activity present in the S-100 preparation or with purified synthetases in the case of tmRNA (AlaRS). The tRNA-free S-100 fractions were the enzyme source. Additionally the low RNase content of the tRNA-free S-100 fractions introduces less degradation risk during the incubation time and subsequent manipulation of the aminoacylated-tRNA.

In general, the reaction mix contained 10-50 A_{260} units of a pure specific tRNA (or 100-200 A_{260} units of $tRNA^{bulk}$) plus 2-7 fold molar excess of the cognate radioactive labelled, (^{3}H or ^{14}C) amino acid and an optimal amount of S-100 fraction enriched in the corresponding synthetase (usually ~200 µl tRNA-free S-100 per 10 A_{260} units of specific tRNA) for tmRNA a 8-10x excess of labelled alanine was used. The final ionic conditions fixed for the tRNA charging was $H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}ATP_3$, for tmRNA charging the condition were slightly modified here magnesium concentration was elevated to 6mM and the polyamines were omitted. Before the addition of the S-100 enzyme fraction, the pH was adjusted between 7.5-8 with 1 N KOH. The mixture was then incubated for 30 min at 37 °C. The reaction was stopped by addition of 1/10 volume of sodium acetate pH 5.5 and quickly put on ice. The aminoacylation reaction was stopped after 15 minutes incubation at 37 °C with the addition of 3 M sodium acetate, pH 5.0 (1/10 of the volume), and the mixture was put immediately on ice bath. Phenol-chlorofom, isoamylalcohol extraction was performed and aqueous phase containing the tRNA was precipitated by ethanol (2.5 volumes of cold ethanol 100 %). The precipitation was quantitative at -80 °C for 45 minutes or at - 20 °C for 2 hours. The aminoacyl-tRNA was pelleted by centrifugation (30 minutes at 12,000 x g) and washed with 70 % (v/v) ethanol, shortly lyophilized (~5 minutes) and re-dissolved in water. Small aliquots were prepared, shock-frozen in liquid nitrogen and stored at - 80 °C until further use.

The level of aminoacylation could be determined at this point by means of an analytical cold TCA precipitation and a direct measurement of the recovery radioactivity.

2.14.12 Isolation of tight coupled 70S from *E. coli*

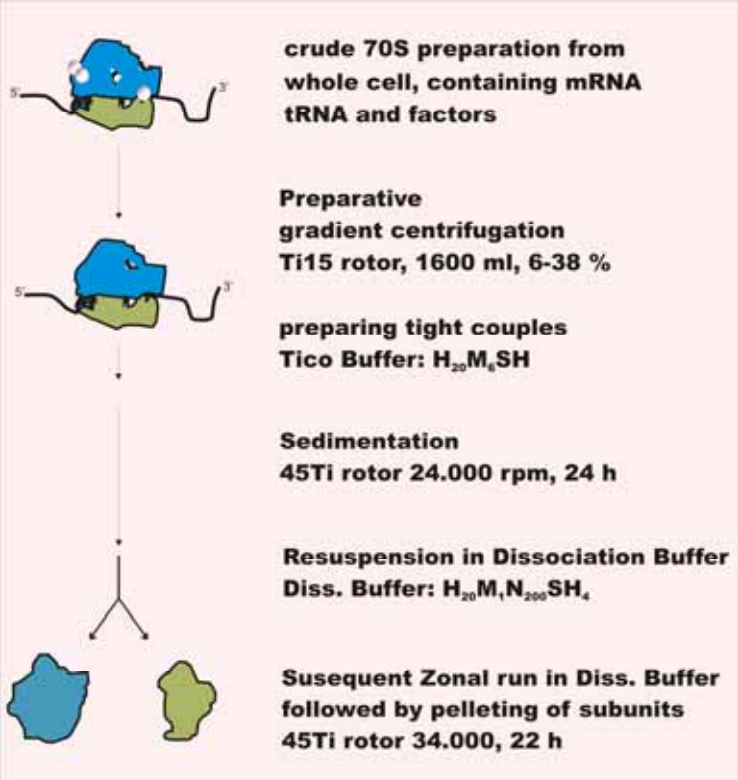


Figure 2.14.12-1: Experimental procedure to prepare ribosomal subunits

The isolation of ribosomes was carried out after the method describe in Bommer et al (Bommer, Burkhardt et al. 1996). The ion composition of the buffers used for isolation of 70S is kept close to *in vivo* conditions ($H_{20}M_6N_{30}SH_4$: Tico buffer) and makes isolation of intact ribosome possible (Hapke and Noll 1976). For one large scale preparation 200 g of frozen cells of the strain CAN/20-12E are dissolved in 400 ml of Tico buffer while being slowly stirred at room temperature. The bacterial suspension has to be homogenised to a degree that there are no clumps of non-dissolved cells or aggregate left, therefore pulling the solution twice through a 60 ml syringe is advisable. A micro fluidizer is then used to break the cells; the machine should be washed with Tico buffer beforehand and in this way equilibrated. The cell suspension is then filled into the loading chamber and under high pressure of 18 psi pushed through a small channel. During this procedure cells break due to friction. The procedure is repeated 3times and the collected suspension filled in sterilized and RNAse free centrifuge bottles. It is centrifuged at 16,000 rpm for 45 min in an SA-600 rotor. The pellet consisting mainly of cell debris is discarded and the supernatant holding ribosomes and soluble enzymes (also called as S-30) is centrifuged further at 22,000 rpm for 20 h in a 45Ti-Rotor to pellet the 70S-ribosomes. The pellet is then solved in Tico buffer and centrifuged again for 10 min to remove insoluble aggregates. The supernatant (S100) is shock frozen and kept for further purification processes. The “crude 70S” particles are aliquotized and also shock frozen in liquid nitrogen. Usually a yield of 400 A_{260} crude 70S per g cell can be expected.

2.14.13 Preparative isolation of 30S and 50S subunits

Ribosomal subunits can be obtained by dissociation of 70S ribosomes. To prepare subunits one has to first purify the crude 70S further by zonal centrifugation in a linear sucrose gradient (0-40 %) in Tico buffer. During this zonal run the crude 70S are separated from factor contaminations. Not more than 5,000 A_{260} should be loaded for one run. The centrifugation is carried out in a Beckmann zonal rotor Ti15 at 22,000 rpm for 17 h at 4 °C. Subsequently the gradient is pumped out by feeding 50 % sucrose solution into the rotor while running at 3,000 rpm. The gradient is then pushed out by the sucrose solution. The 70S peak containing fractions are pooled and centrifuged in a Ti45 rotor at 24,000 rpm for 24 h to pellet the tight coupled 70S free of factors. The pelleted 70S (tight couples) are dissolved in dissociation buffer and a second zonal run with the same conditions mentioned above is performed. After the zonal run now the 50S and 30S containing fractions are pooled and pelleted overnight at 34,000 rpm for 22 h in a Ti 45 rotor. Subunits are then dissolved either in Tico buffer or in re-association buffer to form re-associated 70S which are needed for the functional assays. Crude 70S or tight coupled 70S can not be used for this purpose since they still contain mRNA and tRNA. The concentration of the subunits is determined by measuring their optical density at A_{260}. Subunits are then aliquotized, shock frozen and stored at -80 °C. The typical yield is 600 A_{260} units of 30S and 800 A_{260} units of 50S per 3,000 A_{260} tight coupled 70S.

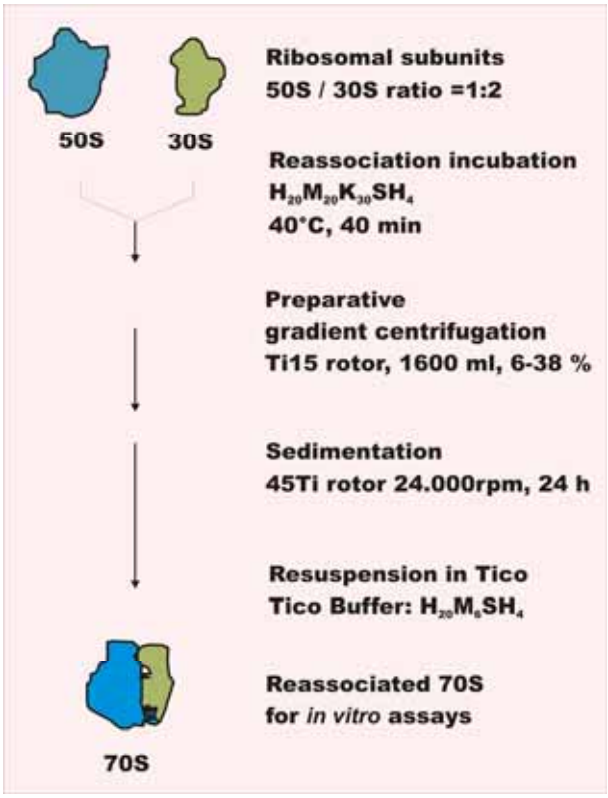


Figure 2.14.13 -1: Experimental steps to create reassociated 70S ribosomes

2.14.14 Preparation of re-associated 70S

Crude 70S and isolated tight couples are obtained from translating ribosomes in the cell (polysomes) and usually hold fragments of mRNAs and tRNAs. These are then split into their subunits with the method mentioned above. Afterwards follows the re-association step in high magnesium concentrations to recover 70S particles (Blaha, Burkhardt et al. 2002). Reassociated ribosomes are more efficient in the binding of tRNAs as well as in their activity in Poly(U) directed Poly(Phe) synthesis. Tight coupled 70S carry fragments of both tRNA and mRNA, per average 0.6 x tRNAs per 70S ribosome (Remme, Margus et al. 1989). After verifying the intactness of the subunit RNAs with agarose gel electrophoresis, they are mixed together in a ratio of 2:1 (6000 A_{260} of purified 30S with 3000 A_{260} of 50S) The mixture is diluted to a final concentration of 140 A_{260}/ ml reassociated and incubated for 60 min at 40 °C gently rocking in a water bath. After the incubation step is done the solution is checked for aggregates, if some have appeared the sample is quickly spun at 10,000 rpm for 15 min, since they might disturb the following zonal gradient. A high excess of 30S is used in this incubation step to minimize the amount of free 50S, thus improving the separation of re-associated 70S ribosomes from 50S subunits in the following centrifugation. The re-associated are then applied to the zonal gradient in re-association buffer. The zonal run is prepared in a linear sucrose gradient (0-40 %) for 17 h at 18,000 rpm and 4 °C. The gradient is fractionated, the 70S peak collected and the re-associated particles pelleted in a Ti 45 rotor for 24 h at 24,000 rpm. The pellets are finally dissolved in Tico buffer and their concentration measured at A_{260}. The ribosomes are then aliquotized in 50 µl aliquots shock frozen and stored at -80 °C.

2.15. Other methods in ribosomology

2.15.1 Analytical sucrose gradient centrifugation of ribosomes

Sucrose gradient centrifugation is a technique that allows the separation of complexes based on the sedimentation coefficient (S). This technique can be used to separate 70S, 50S and 30S ribosomal subunits.

A sucrose gradient (10-30 % (w/v) in binding buffer) was prepared in an Ultra-Clear or polyallomer tubes (14 x 95 mm Beckman). The reaction mix (0.5-1 A_{260}) was overlaid on the gradient and centrifugation was performed in SW 40 rotors

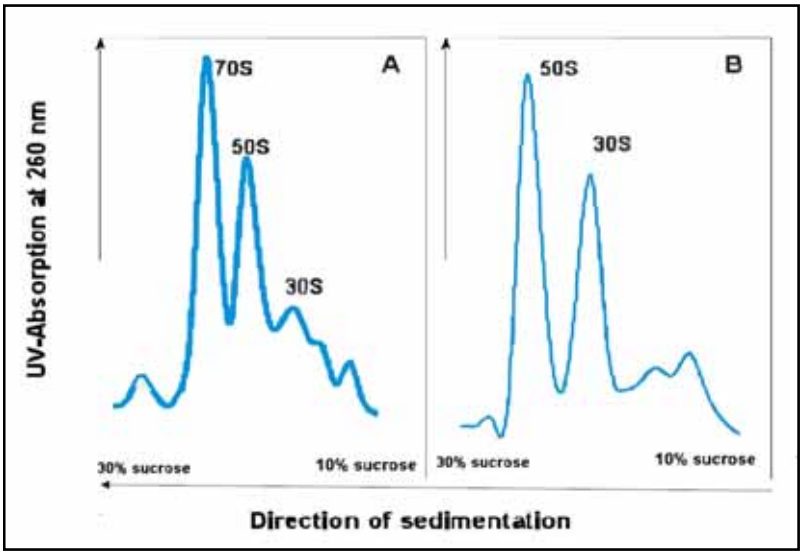


Figure 2.15.1-1: Typical sedimentation profile of ribosomes and subunits after sucrose gradient centrifugation

(Beckmann). In the SW 40 up to 10 A_{260} units of pure ribosomes or ribosomal subunits per tube can be loaded. The centrifugation was performed at 18,000 rpm for 18 h, 4 °C. After centrifugation the gradient was fractionated while monitoring the absorbance at 260 nm.

2.15.2 ^{14}C-labeling of ribosomal subunits with ^{14}C-Formaldehyde

Labelling of subunits is achieved *via* reductive methylation of N-terminal amino acid residue. The reaction involves the formation of a Schiff base between the N-terminal amino groups of proteins and formaldehyde which is subsequently reduced to a secondary amine. For the reaction 500 pmol 70S are incubated with 50 µCi of ^{14}C-formaldehyde and 25 mM dimethylamine borane complex in a total volume of 500 µl under Tico-Buffer conditions containing potassium acetate instead of ammonium acetate. Mix the solution gentl

y and incubate everything for 3 h at 4 °C or on ice. After three hours the concentration of dimethylamine borane complex was elevated to 50 mM and incubation was carried

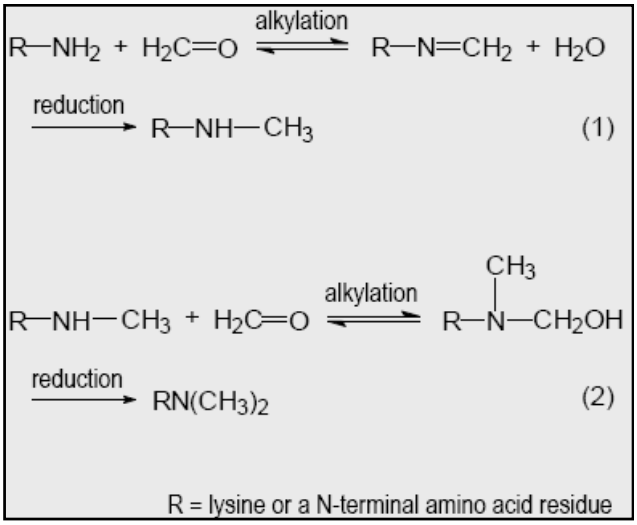


Figure 2.15.2-1: Reaction scheme for reductive methylation

out for 3 h more. Then the reaction was stopped by addition of 100 mM Tris, pH 7.5. The sample is then dialysed against 1000 volumes of dissociation buffer for 45 min at 4 °C. Then the samples are applied to SW28 sucrose gradients (10-30 %) in dissociation buffer and centrifuged for 23,000 rpm and 11 h (4 °C). The gradient is fractionated and 50S and 30S carrying fractions are pelleted at 44,000 rpm for 18 h in a TLA 100 rotor (according to sample size this pelleting step can be also carried out in Ti70 rotor or bigger). Pellets are dissolved in Tico, the A_{260} is measure and radioactivity quantified. Afterwards labelled subunits are stored at -80 °C.

2.15.3 Crosslinking ribosomes with DMS

The typical crosslinking reaction is carried out in a buffer free of substances carrying free alpha amino groups, in our case we used Tico-buffer supplemented with KAc instead of Ammonium acetate. The crosslinker DMS (Dimethyl Suberimidate) is added to a f.c. of 10 mM. The buffer should then be adjusted to a pH of 8.5 – 9.0, at lower pH the crosslinking reaction does not take place. Ribosomes are added to a f.c. of 0.3 µM. Lower concentrations are more optimal since they prevent a multimerization of 70S, to create “oligo” ribosomes. The reaction is carried out for 2 h at 30 °C. The reaction is stopped by the addition of 0.1 Vol 1 M Tris-HCl, pH 6.7. Subsequently the sample is kept on ice or at 4 °C. It is then dialysed against 100 V of VD-buffer for 45 min. Afterwards the crosslinked and thus dissociation resistant 70S fraction has to be separated from non-crosslinked ribosomes and multimers, which form because an inter rather than an inter-ribosomal crosslink has occurred. Depending on the the initial reaction volume, a 10-30 % sucrose gradient in VD(-) buffer is prepared. Sucrose gradient centrifugation is carried out and fractions with dissociation-resistant ribosomes are collected and pelleted in a Ti Rotor ON. Crosslinked ribosomes are dissolved in Tico buffer, the A_{260} is measured. They are aliquotized and stored at -80 °C. Subsequently functional tests such as Poly(Phe) synthesis or Pi-complex formation should be carried out to assure the functionality as well as an analytical sucrose gradient centrifugation in an SW 60 rotor (45,000 rpm , 2 h 15 min) under dissociating conditions were native and crosslinked ribosomes are finally compared in their dissociation behaviour.

2.15.4 Alkali ladder and T1 digestion as comparative ladders in RNA-PAA gels

To estimate length and sequence of certain mRNA fragments alkali ladders and T1 cleavage patterns can be prepared as comparative standards. A ladder of truncated RNAs is generated by alkaline hydrolysis.

100,000 dpm of 5' or 3'end labelled mRNA should be taken per reaction. The reaction should occur in alkaline hydrolysis buffer (AH buffer: 10 µl MQ/2.5 µl NaOH 1 M/ 37 µl Urea 9 M). Add 5 µg of *E. coli* $tRNA_{bulk}$ to the radiolabeled RNA and lyophilize quickly. Then solve the material in 4 µl AH buffer and incubate it at 95 °C for 15 s (time can be increased to 40 s if insufficient cleavage occurs) and put on ice. Before applying the sample to gel add urea loading dye, load different amount to choose the best amount for good resolution.

For T1 cleavage that produces fragments of the RNA cleaved at guanosines 100,000 to 200,000 dpm of 5' or 3' labelled RNA should be used. To the material 5 µg of *E. coli* $tRNA_{bulk}$ has to be added. Then the sample is lyophilized and 4 µl of TC buffer are added (28 µl MQ/ 4 µl citrate buffer, pH 3.5/160 µl Urea 9 M). Incubate the sample 5 min at 50 °C then adding 0.1 U of RNase T1 incubating it further for 15 min at 50 °C. Sample should then be put to dry ice to stop the reaction. On ice the reaction will continue, so if no dry ice is available sample should be either shock frozen or kept on ice for less than 30 min! Urea loading dye is added before loading the sample to the gel.

2.15.5 RelE activity assay

The RelE protein was a gift of Dr. Hiroshi Yamamoto who purified this factor according to the methods described in Cherny et al. (Cherny, Overgaard et al. 2007). For assaying RelE activity a Pi complex was formed with AcPhe-$tRNA^{Phe}$ and MFstop messenger on 70S ribosomes. After 15 min incubation RelE (5x over ribosome) was supplemented so that the cleavage reaction could occur. Subsequently the cleaved mRNA was recovered *via* phenol-chloroform purification and analysed on a denaturing 20% PAA gel. As standards an alkali-ladder as well as the T1 cleavage product of the ^{32}P labelled MF-stop mRNA was applied to the gel to indicate

successful cleavage (Figure.). When comparing Lanes –R and +R we can see that RelE has cut twice. One position is the proper position with the UAA codon in the A-

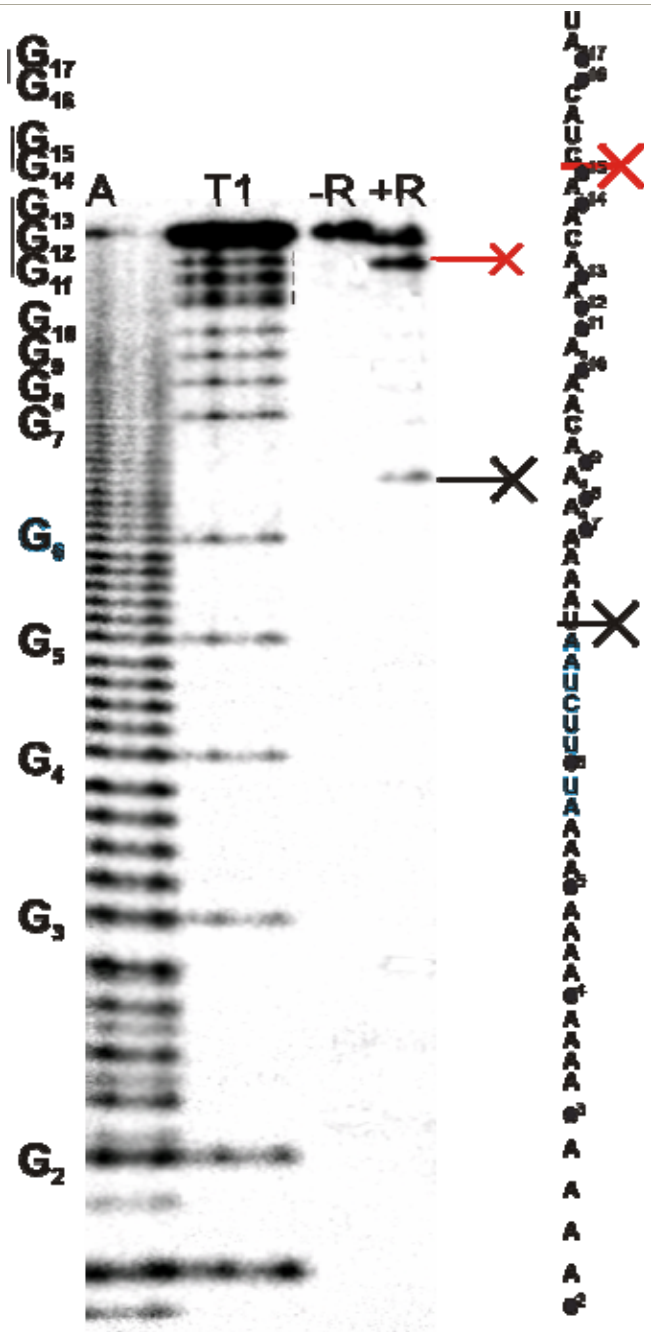


Figure 2.15.5-1: Analysis of RelE cleavage of ribosomal complexes with MFstop mRNA. A, alkali ladder; T1, T1 cleavage product of naked MFstop mRNA; -R and +R :MFstop mRNA from Pi-complex incubated without and with RelA, respectively. The left sequence depicts the T1 ladder, where after each G in the messenger sequence a cleavage occurs, together with the alkali ladder and the given sequence of the MFstop mRNA (sequence on the right) the cleavage sites was reconstructed

site, yet a second band could be observed, due to a SD sequence in the 3' terminus of the messenger which probably directed the ribosome close to the 3' end creating a second site for RelE cleavage.

It follows that the purified RelE protein is active and can be used for the formation of a 70S complex ready for tmRNA binding.

2.15.6 GTPase-assay to determine the activity of G-Proteins

Many in vitro systems are dependent on the activety of G-Proteins such as EF-G, EF4 or IF2. Before usage it is necessary to quantify their activety, this is possible due to their characteristic feature to cleave GTP in the presence of ribosomes or specific ribosomal states. The easiest method is the evaluation of uncoupled GTPase activety that can be measured for IF2 and EF-G (Figure **2.15.6-1**) . Here 5 pmol of ribosomes are incubated in the presence of γ^{32}P GTP (f.c. of 0.05 mM with 20 – 50 dpm/pmol) with a given amount of factor for 5 min at 37°C under binding buffer conditions. The reaction is stopped upon addition of 120 µl of stop solution A (0.5 M H_2SO_4, 1.5 mM NaH_2PO_4) and 30 µl of stop solution B (200 mM $MoNaO_4$) and mixed briefly. Then 800 µl of 2-Butanol are added and everything is vortexed for 1 min and subsequently centrifuged for 10 min at 16.000 xg.

After this step 400 µl of the upper phase are withdrawn, put together with 5 ml of

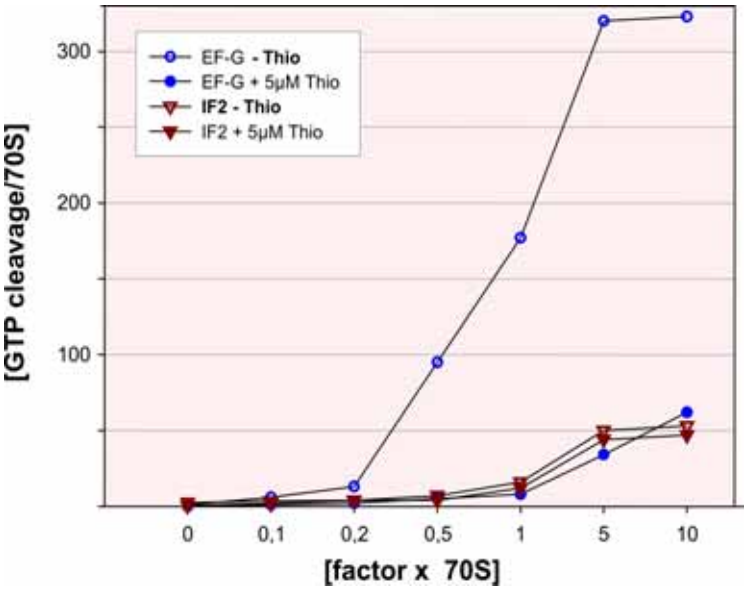


Figure 2.15.6-1: GTPase activety of IF2 and GTP in the presence and absence of the antibiotic thiostreptone which is known to inhibit the function of EF-G

Ready-Mix (scintillation mix) and counted in a scintillation counter. The amount of GTP hydrolysed per 70S gives a read-out of the factor activety.

2.15.7 Primer extension analysis (Toe-printing)

For toeprinting, synthetic MstopML mRNA annealed to ^{32}P-end-labeled primer as described in Hartz et al., 1988 (Hartz, McPheeters et al. 1988) was used to program ribosomes for post-termination complexes. Annealing reaction is carried out in 1x VD Buffer (-Mg^{2+}) for 3 min at 80°C, then snap frozen in dry ice/EtOH or liquid nitrogen and then slowly thawed on ice (lasts about 30 min). The magnesium level of the annealing product must now be adjusted to 6 mM. Then 10 pmol reassociated 70S are briefly, incubated with 0.25 pmol mRNA (annealing product) and 20 pmol of $tRNA^{Phe}$ in binding buffer for 5 min. Aliquots of this reaction mixture are used for single omission tests and further incubated with different sets of factors in binding buffer for 5min at 37°C in the presence and absence of $tRNA^{fMet}$ (1 x 70S).

Reactions are now used for toe printing, here the labelled-primer, which is complementary to the 3' region of the mRNA was extended using 100 units of MuMLV (Fermentas) reverse transcriptase in the presence of 135 µM each of the four dNTPs in VD (2.11.6 - MuLV-Mix; add 2.5 µl per reaction) at 37°C for 10 min. The reactions were stopped by addition of 7.5 µl formamide dye and heated to 95°C for 5 min. 2.5 µl toeprint reactions were analyzed on 8% denaturing polyacrylamide gels. The gels were quantified using a Molecular Dynamics Phosphor Imager.

As for sequencing ladders 0.5 pmol of the anneal mix were used. Four sequence ladder mixes are prepared according to 2.11.6 and incubated at 42°C for 15 min. To stop the reaction 10 µl of formamide dye are added. The reaction is then heated to 95°C for 5 min and quickly put to ice. For each lane 2.5 µl should be loaded.

2.16. In vitro systems

2.16.1. Estimation of the functional competence of ribosome preparations

The elongation activity of the ribosome preparations was routinely checked using a modification of the poly (U)-dependent poly(Phe) synthesis system described by Traub and Nomura (Traub and Nomura 1969), and/or the AcPhe-tRNA primed

poly(Phe) synthesis described by Bartetzko and Nierhaus (Bartetzko and Nierhaus 1988) modified according to Y. Teraoka and K. H. Nierhaus, (unpublished).

2.16.1.2. Poly(U)-dependent poly(Phe) synthesis

Under standard conditions (15 µl of reaction mix), the assay contained, $H_{20}M_{4.5}N_{150}SH_4Spd_2Sp_{0.05}$, i.e. 20 mM Hepes-KOH pH 7.6 (0 °C), 4.5 mM $Mg(Ac)_2$, 150 mM NH_4Ac, 4 mM ß-mercaptoethanol, 2 mM spermidine and 0.05 mM spermine. The binding reaction was carried out by incubating 25 µg of poly(U), 0.2 A_{260} of 70S re-associated ribosomes sometimes primed with a two molar excess of acetyl ([3H] or [^{14}C]Phe-tRNA^{Phe}; specific activity: 1,000 - 3,000 dpm/pmol).

The charging reaction contained 100 µM [^{14}C] or [^{3}H]Phe (10-100 dpm/pmol), 3 mM ATP, 1.5 mM GTP, 5 mM acetyl-phosphate, 1/3 A_{260} of tRNA^{bulk} (*E. coli*), and an optimal amount of S-100 preparation (3 µl) in a total volume of 10 µl under the same conditions as the binding mix. The binding reaction was incubated for 15 min at 37 °C, then mixed with the charging mixture previously incubated for 2 min at 37 °C.

Aliquots were withdrawn at indicated times and after addition of one drop of 1% BSA (as precipitation carrier) the synthesis was stopped by 2 ml of 10 % TCA. The mix was incubated at 90 °C for 15 min in order to hydrolyse the tRNA^{Phe} that otherwise would remain as aa-tRNA together with the [^{14}C]-or [^{3}H]poly(Phe). The samples were cooled to 0 °C and filtered through glass filters. These filters were washed 3x with 10 % TCA and twice with 5 ml of diethylether/ethanol (1:1) to remove the TCA and to dry the filters. The radioactivity adsorbed on the filters was measured as described before.

2.16.1.3. Minimal Poly(U)-System

The minimal system for Poly(U)-dependent poly(Phe) synthesis is essentially similar to the method described in 2.16.1.2 yet the S-100 fraction is replaced by the factors EF-G,-Tu and –Ts.

2.5 pmol 70S are incubated together with 25 µg of poly(U) and pre-charged [^{3}H]Phe-tRNA^{Phe} as well as 1.5 mM GTP and the three proteins in a ratio of 2 x aa-tRNA for EF-Tu, 0.8 x 70S in the case of EF-G and 1x EF-Tu for the factor EF-Ts. The incubation mixture is then kept at 37°C for 15 min. The synthesis is stopped by TCA. The following steps are comparable to the method described in 2.16.1.2. This test can be used to give a quick answer about the activety of EF-G,-Tu and-Ts in protein

synthesis and should be carried out for each new preparation. At the most optimal conditions a synthesis rate of 7-10 Phe/ ribosome can be observed and should be taken as comparison.

2.15.1.4. Determination of the AcPhe-tRNAPhe binding

The binding of N-acetyl[^{3}H] or [^{14}C]Phe-tRNA^{Phe} to ribosomes was determined by nitrocellulose filtration. Aliquots from the binding reaction were placed in glass tubes in an ice bath. The samples were then diluted with 2 ml of ice cold binding buffer ($H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$) and filtered immediately through a nitrocellulose filter previously equilibrated in the same buffer (Nirenberg and Leder 1964). The filter was then washed two times with 2 ml of binding buffer. The amount of AcPhe-tRNA^{Phe} bound on the ribosomes was calculated as function of the amount of radioactivity retained on the filters and, this value was normalised to pmol bound per pmol of ribosomes (ν).

2.16.2. Watanabe assay: site specific binding of tRNA to ribosomes, translocation and puromycin reaction

The functional states of the elongating ribosome (Pi, PRE and POST states) appearing during the elongation cycle were studied using the methodology described by Watanabe (Watanabe 1972) with some modifications. The Watanabe assay allows a controlled stepwise execution of the partial reactions of the ribosomal elongation cycle. In the first step a 70S-mRNA-tRNA complex is formed, in which the tRNA is located in the ribosomal P-site. If N-acetylated-tRNA (e.g., N-Acetyl-Phe-tRNA^{Phe}) or N-formylated-Met-tRNA is used in the first step, an initiation complex is simulated (Pi-complex, I for initiation). In a second step the A site can be filled with the corresponding cognate tRNA enzymatically (with EF-Tu) or non-enzymatically (without EF-Tu) forming a pre-translocational complexes (PRE complex). In the third step, PRE-complexes containing tRNAs in P and A sites are translocated to the E and P sites respectively (POST-complexes) upon addition of elongation factor G (EF-G) and GTP. The efficiency of the translocation reaction and /or the binding state of the tRNAs is determined in a fourth step taking advantage of the antibiotic puromycin (analogue of the 3' aminoacylated end of a tRNA). This antibiotic reacts specifically with the P-site bound acyl-tRNA, if the ribosomal A site is free, forming an acyl-puromycin derivative (Allen and Zamecnik 1962). The puromycin reaction defines the location of a charged tRNA on the ribosome, i.e., if the P-site binds a peptidyl-tRNA,

the puromycin reaction will be positive, whereas if the aminoacyl or peptidyl bound tRNA is present at the A site, the puromycin will not react (Traut and Monro 1964). In any case, after the addition of EF-G that does not affect the binding state, the puromycin reactivity of a P-site bound aminoacyl- or peptidyl-tRNA will be positive, while the A site bound species should show a translocation factor-dependent puromycin reaction.

The enzymatic binding (plus EF-Tu) of aminoacyl-tRNA at the A-site will be described separately in the section corresponding to the di-peptide formation The final ionic condition used in this experimental scheme were $H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$ pH 7.5, the same as the poly(Phe) synthesis. A typical experiment was conducted as follows:

2.16.2.1. First step: P site binding or Pi complex formation

Pi complexes were prepared with 5-10 pmol of re-associated ribosomes in a volume of 12.5 µl incubated with: 25 µg of homo-polymeric mRNA (e.g., poly(U)) or 6 fold excess of a natural or hetero-polymeric mRNA over ribosomes and 1.5-2 fold excess of labeled N-Acetyl-Phe-$tRNA^{Phe}$ or N-formyl-Met-$tRNA_f^{Met}$, respectively. The first step was incubated for 15 min at 37 °C in a volume of 12.5 µl.

For P-site blockage with deacylated tRNA, programmed ribosomes with specified messengers were primed with 1.5-2 fold of deacylated-tRNA (sometimes 5' labeled with γ-^{32}P) keeping constant the same size aliquots and the ionic binding conditions.

2.16.2.2. Second step: A site binding and/or PRE complex formation

Keeping constant the ionic conditions (binding buffer), the volume of reaction was increased to 25 µl per single determination. Non-enzymatic A site occupation was carried out (PRE complex formation) adding 0.8 to 1.5 molar excess of N-acetyl-aminoacyl-tRNA ([^{3}H] or [^{14}C] labeled) to ribosomes whose P site was pre-occupied with deacylated tRNA in the first step. The mix was incubated for 30 min at 37 °C. The binding was measured with double determination by nitrocellulose filtration as described before.

2.16.2.3. Third step: Translocation reaction

At this step, a GTP-mix (5 µl per aliquot) was added to Pi or PRE complexes maintaining constant the binding ionic conditions ($H_{20}M_{4.5}N_{150}SH_4Sd_2Spm_{0.05}$). Samples were split in 30 µl aliquots and 2.5 µl of EF-G was added to each (0.1-

0.4fold EF-G per ribosome). Control aliquots contained binding buffer instead of EF-G. After the addition of EF-G the aliquots were incubated for 10 min at 37°C.

2.16.2.4. Fourth step: puromycin reaction

Four aliquots from the binding assay containing EF-G and four without EF-G, were processed in the following way: 2.5 µl of puromycin stock solution in binding buffer (final concentration = 0.7 mM) were added to two aliquots from every group (± EF-G), while the other two received 2.5 µl of binding buffer. After these additions the samples were incubated at 37 °C for 5 min and the reaction was stopped adding 32.5 µl of 0.3 M sodium acetate, pH 5.5, saturated with $MgSO_4$. The amount acyl-puromycin formed was determined by extraction with 1 ml of ethyl acetate. After the addition of the organic solvent, the samples were strongly vortexed for 1 min, left 10 min on ice and centrifuged for 30 seconds at 15,000 x g in order to achieve complete phase separation. 800 µl of the organic phase was withdrawn and counted.

The radioactivity extracted in the controls (minus puromycin) was subtracted from that plus puromycin in order to calculate the amount of acyl-puromycin formed.

A successful puromycin reaction depends critically in the way in which the puromycin solution is prepared and handled. Two basic rules for the preparation of the puromycin stock solution with the maximal activity should be observed:

The pH of the solution must be neutral. Since the puromycin is obtained commercially as hydrochloride, the pH of the solution had to be neutralised adding 1 M KOH (1/100 of the reaction volume).

The puromycin stock solution must be maintained at room temperature (otherwise it precipitates lowering the effective concentration). Under these conditions the stock solution retained its maximum activity for about one hour. However, one can prepare a larger stock of puromycin solution that if aliquotized and shock-frozen in liquid nitrogen keeps its activity for months.

Binding assays without ribosomes were included in all the experiments as standard controls in order to determine the background of radioactivity adsorbed to the filters. This background was normally low (below 10 % of the binding signal) and directly proportional to the concentration of the radioactive component in the assay. Controls without mRNA plus ribosomes were also included when needed (e.g. the test a new heteropolymeric mRNAs).

2.16.3 RTS 100 High Yield *E. coli* Kit (Roche)

The RTS 100 system is a coupled transcription translation system for DNA-templates or linearised DNA fragments containing a T7-promoter. 5 separate lyophilisates are supplied with the system, which need to be reconstituted and combined: the *E. coli* lysate, energy components, amino acids, reaction buffer and control DNA.

To reconstitute the system one has to follow the guidelines of the supplying company which are listed in the following table:

Table 8: Composition details of the RTS Kit

Compound	Description	Reconstitution procedure
1 . *E. coli* lysate	contains all components for trancription and transation such as factors, ribosome	add 0.36 ml of reconstitution buffer, mix carefully; never vortex
2 . Reaction mix	substrate mix for reaction	add 0.3 ml of reconstitution buffer, mix carefully; never vortex
3 . Amino acid mix	mix of 19 amino acids	add 0.36 ml of reconstitution buffer, mix carefully; never vortex
4 . Methinonine	seperate methionine; if radiolabeling of the product is needed ^{35}S-Methionine can be added instead	add 0.33 ml of reconstitution buffer, mix carefully; never vortex
5 . Reconstitution buffer	Buffer to reconstitute solutions 1-4 from lyophilisate	1.6 ml ready solution
6 . DNA template	(with the Kit a control GFP vector is supplied)	vector is solved in 50 µl MQ

After reconstitution, all components should be aliquotized and shock frozen. It should be noted that the composition between the batches can slightly vary; therefore it is advisable to never completely use up the lysate of the recent kit to have a positive control at hand for following experiments.

For analytical usage (10 µl reaction), i.e. when measuring luciferase translation, the following mix of compounds mentioned above should be used:

Reconstitution buffer	*E.coli* lysate	Reaction mix	Met	amino acid mix	Template/ MQ
0,87 µl	2,4 µl	2 µl	0,4 µl	2,4 µl	2,13 µl
Σ 10 µl					

In contrast to GFP synthesis which is carried out with the RTS system for 6 hrs at 900 rpm with an additional incubation of 16 hrs at 4°C to allow proper folding of the protein, luciferase expression is carried out for only 2 hrs at 30°C.

2.16.4 PURE System

In contrast to the RTS system, the PURE system offers a more refined in vitro translation system. Though possible using this system with a plasmid template, experiments described in this thesis a solely carried out with mRNA transcripts (T7-RNA polymerase for instance is not added).

The protein expression is performed in two steps. The first step includes the initiation reaction. Here 70S ribosomes (8 pmol) or an equal amount of subunits are incubated together with a given mRNA (4x over ribosome) and initiator [^{3}H]-fMet-tRNA (2x over ribosome). This initiation complex formation is carried out for 10 min at 30°C. To be able to have a defined buffer composition we need to dilute 70S in 70S dilution buffer.

- 70S dilution-buffer: H^{20} M^{6} K^{180} $Spd^{4.2}$ $Spm^{0.1}$ SH^{4}
- dilution of reassociated ribosomes (33.6pmol/µl) 1:2.1 with 70S dilution-buffer resulting in 70S sample with 16 pmol/µl in H^{20} M^{6} K^{100} Spd^{2} $Spm^{0.05}$ SH^{4}

For a single reaction the following pipeting scheme should be followed:

70S ini. buffer	mRNA 4x 70S	H_2O	[^{3}H]-fMet-tRNA 2x 70S	70S 8 pmol
1 µl	Σ 6,5 µl			0.5 µl

The final buffer composition of this reaction step has H^{50} K^{100} $M^{4.5}$ Spd^{2} $Spm^{0.05}$ SH^{4}, the composition of the 70S initiation buffer mix is given below in table 9.

Table 9.: 70S initiation buffer mix - PS I

390 mM	Hepes-KOH pH 7.6
750 mM	K-Glutamate
33 mM	$Mg(OAc)_2$
15 mM	spermidine
0.375 mM	spermine
30 mM	β-mercaptoethanol

Next the second reaction step for elongation and thus protein synthesis is carried out at 30°C for 1 h. 12 µl of reaction mix are added to 8 µl of initiation mix.

Reaction buffer PS II (given in Table 10) is used to adjust the final reaction mix to H^{50} K^{100} $M^{8.4}$ Spd^{2} $Spm^{0.02}$ SH^{4}.

Table 10. : 5x reaction buffer mix - PS II

250 mM	Hepes-KOH pH 7.6
500 mM	K-Glutamate
55 mM	$Mg(OAc)_2$
10 mM	spermidine
5 mM	β-mercaptoethanol

To be table to determine the amount of protein synthesized (in our case GFP), we need to separate the reaction mixture using SDS-PAGE. Gels are then dried and an exposition on phosphoimager plates is carried out for 14-16 hrs to detect radiolabeled reaction-products. GFP synthesis is finally quantified using the Image Quant software. The defined composition of the second reaction step is mentioned in Table 11.

Table 11: Composition of elongation reaction in the PURE System

5x PS II	2.4 µl
19 amino acids (1 mM)	2.0 µl
Cys (10 mM)	0.2 µl
tRNA mixture (700 OD_{260}/ml)	1.6 µl
ATP-GTP mix (40 mM each)	1.0 µl
creatine phosphate (500 mM)	0.8 µl
formyl donor (1 µg/µl)	0.2 µl
EF+RF+IET-mix (w/o IF1, IF3)	0.585 µl
[^{35}S]-Met	0.2 µl
ARS-mix	0.216 µl
IF1	(0.5 µl)
IF3	(0.5 µl)
H_2O	Σ 3.2 µl
70S initiation-mix	8.0 µl
	Σ 20 µl

3 Results: Testing a new concept for translational initiation

3.1. Setting up a system to elucidate the function of initiation factors in translation of polycistrons

To investigate whether initiation factors can stimulate the 70S-type initiation in the translation of polycistronic mRNAs a minimal system has to be set up, which can mimic the *in vivo* near situation of translation of mRNAs that code for more than one protein, which is the case for about 65 % of *E. coli* mRNAs. In this thesis we test a hypothesis, according to which two modes of initiation exist: (i) The canonical 30S-binding type, where the small subunit binds to the Shine-Dalgarno sequence next to the initiation AUG, and (ii) the 70S-scanning type, which might exist at the second or further downstream cistrons, when some prerequisites are fulfilled. One example is a narrow intercistronic distance of <30 nt, where a terminating 70S and a downstream initiating 30S subunit would collide; another one an initiation site after a preceding cistron, which is hidden in a secondary structure. A minimal system for testing this hypothesis would be therefore a bicistronic mRNA with a 30S-binding mode at the first cistron, and after a short intercistronic distance of <30 nt a second cistron, which therefore would be initiated by the 70S-scanning mode. This is possible after the ribosome has terminated the translation of the first ORF moving in a scanning like motion to the next initiation start.

To be able to discern and trace back two initiation pathways we have designed both *in vitro* and *in vivo* approaches. *In vitro* approaches comprise the usage of several unique methods such as the RTS system, the PURE system, as well as toe-printing studies, site specific tRNA binding tests and poly(U)dependent-poly(Phe) synthesis. In the context of the RTS and PURE system activity ratios of two different luciferases both giving an individual chemoluminiscent read-out or the synthesis of GFP were taken as reporters to quantify synthesis and thus initiation events. *In vivo* approaches haven been discussed in the thesis of Romi Gupta. To give a reasonable insight into the complete spectre of this study, the results of this thesis will be mentioned and discussed throughout the text as well.

To allow optimal function of all assays, we need not only a defined mRNA construct; moreover all factors taking part in the process of translation are required.

Additionally compounds which make it possible to enforce either the scanning modus of 70S or the *de novo* 30S initiation have to be identified.

In the following paragraph the design and procedure of the experimental system as well as the purification of all accessory components will be discussed in detail.

3.1.1 Prerequisites –
Purification of accessory factors and their activity test

3.1.1.1 Initiation Factor 2 (IF2)

Initiation factor IF2 is with 890 amino acids the largest of the three proteins involved in the initiation step of protein synthesis of bacteria kingdom, whose main role is that of positioning the initiator fMet-tRNA$_f^{Met}$ in the ribosomal P site. To accomplish this function, IF2 interacts with both 30S and 50S ribosomal subunits and with the initiator tRNA. The latter is one of the most important interactions in the pathway of translation initiation, playing an essential role in the selection of mRNA initiation sites (Gualerzi and Pon 1990). The specificity of fMet-tRNA recognition by IF2 rests mainly on the presence of the blocked -NH_2 group of methionine, a typically bacterial feature.

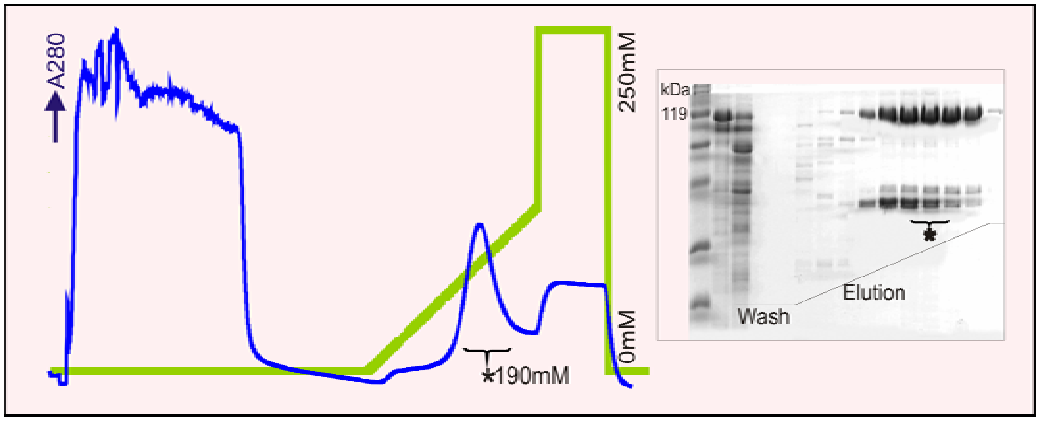


Figure 3.1.1.1-1: HisTrap assisted purification of IF2 from S30 extracts of an overexpression in *E. coli* strain XL-1; Protein eluted in fractions with 190 mM imidazole, peak-fractions were pooled and used for further purification with an anion-exchange column. Insert: elution fractions analyzed on a 15% SDS gel; *asterisk* indicates peak fractions containing IF2, which were pooled and further purified (blue – elution profile; green – elution gradient)

The factor carrying a His-tag was purified from an overexpression strain with the help of a HisTrap column (Figure **3.1.1.1-1**) and further with a MonoQ anion-exchange column (Figure **3.1.1.1-2**) to remove residual contaminating proteins.

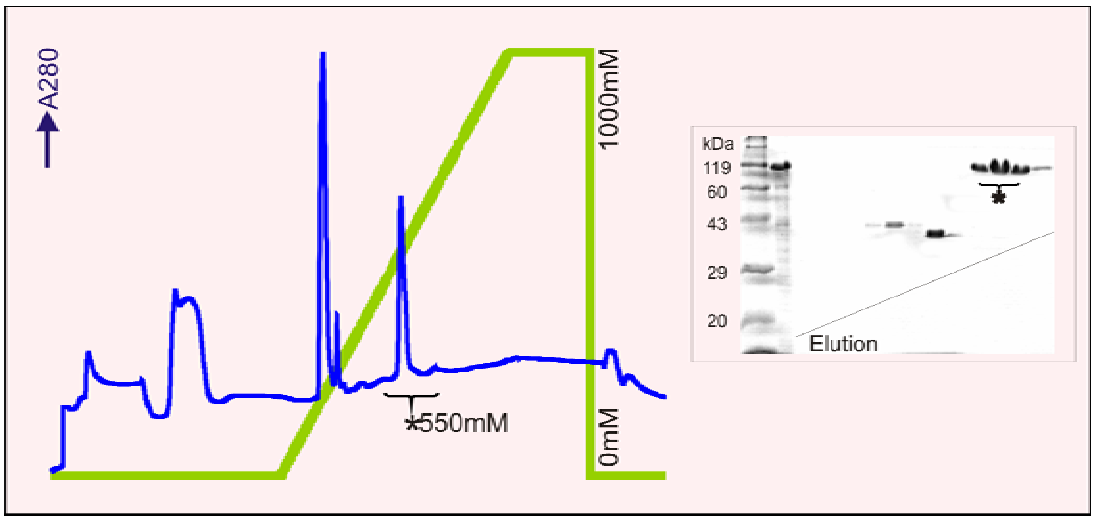


Figure 3.1.1.1-2: MonoQ assisted purification of IF2 from peak fractions of HisTrap; Protein eluted in fractions with 550 mM ammonium acetate, peak-fractions were pooled and extensively dialysed against IF2-buffer. Insert: elution fractions analyzed on a 15% SDS gel; *asterisk* indicates peak fractions containing IF2

After purification of the factor a protocol had to be identified that assures its functionality in our *in vitro* systems. Thus the protection conferred by the initiation factor on the initiator tRNA with respect to spontaneous hydrolysis occurring at alkaline pH was measured (Spurio, Brandi et al. 2000). Reaction mixtures (15 µl) in Tico buffer (pH 8.0) contained 10 pmol [^{3}H]fMet-tRNA$_f^{Met}$ and 10 pmol of the factor. Samples were withdrawn at various times from 0 to 120 min, choosing an incubation temperature of 37 °C. Samples were spotted on glass filter discs for determination of the cold trichloroacetic acid-insoluble radioactivity.

We were able to detect a subtle protection activity of IF2 hinting that the isolated protein bears a certain active fraction (**3.1.1.1-3**). Yet to be absolutely sure of the functionality a second activity test was conducted. Here the function of IF2 in the formation of a 30S initiation complex (namely 30S•mRNA•fMet-tRNA$_f^{Met}$•IF2) was examined.

For this experiment either 5 pmol of 30S or 70S were incubated with 1.5x initiator tRNA, 1.5x factor and different kinds of mRNA under binding buffer (BB) condition.

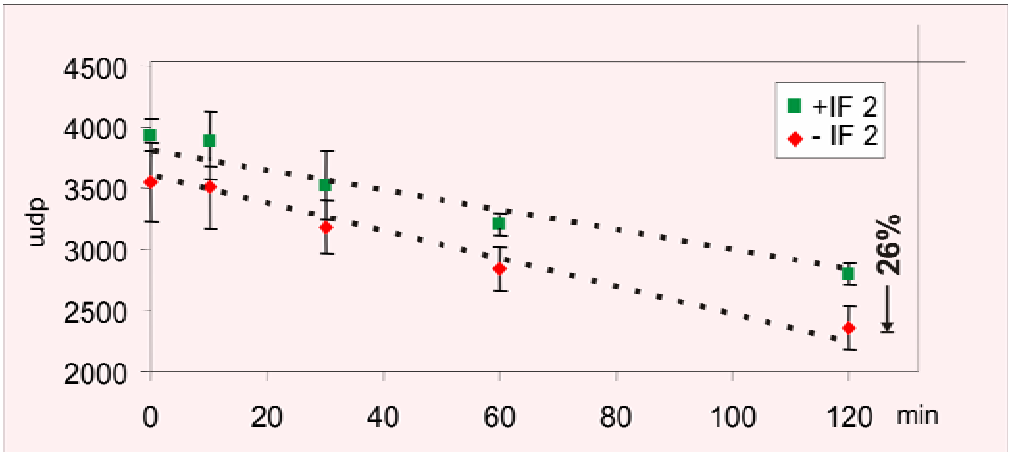


Figure 3.1.1.1-3: Protection from spontaneous hydrolysis of initiator fMet-tRNA$_f^{Met}$ by IF2; arrow indicates a cleavage-protection of 26 % after an incubation for 120 min.

Two mRNAs were tested, one (MS2-Fluc-fragment mRNA ~62 nucleotides) carrying a Shine Dalgarno (SD-region) the other mRNA (MF-mRNA) lacking it. Since a SD region is a prerequisite of contact formation of the mRNA to the 30S subunit and thus of fMet-tRNA to 30S, MF-mRNA was taken as a negative control (there is no contact between mRNAs without SD and small subunits). Thus the addition of IF2 should not

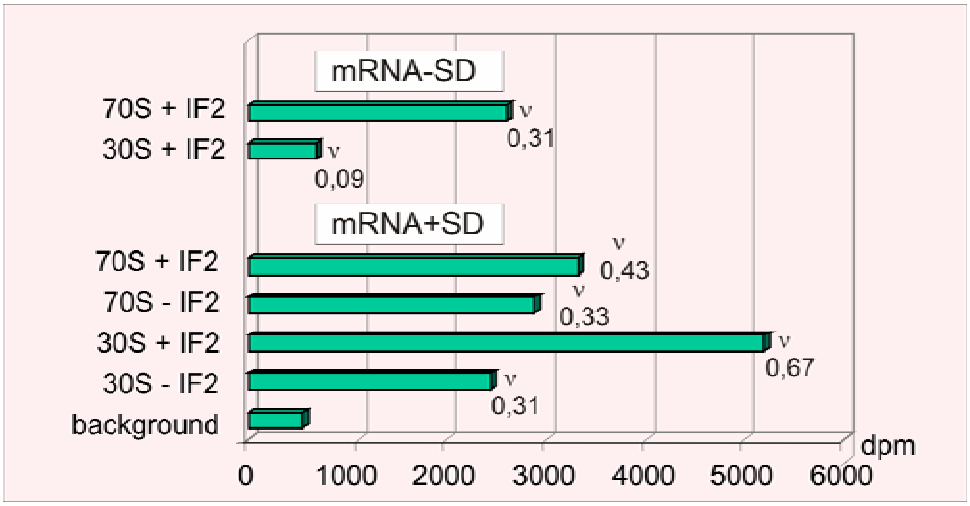


Figure 3.1.1.1-4: Binding of fMet-tRNA to the 30S•IC formed with mRNA with or without an SD sequence (mRNA+SD/ mRNA-SD); ν describes the amount of fMet-tRNA$_f^{Met}$ bound per ribosome; binding to 70S was taken as an internal control of the system.

stimulate binding of the initiator tRNA to the subunit in this case whereas the binding be enhanced in the presence of the factor when MS2-Fluc-fragment mRNA is used.

Experiments showed that IF2 can stimulate binding of fMet-tRNA$_f^{Met}$ to 30S (Figure **3.1.1.1-4**), this is one of the essential functions of this factor; therefore we concluded that our protein is active and can be used.

3.1.1.2 Initiation Factor 1 (IF 1)

IF1 is the smallest of the three initiation factors (72 amino acids in *E. coli* K-12). According to general wisdom it is essential for the growth of *Escherichia coli* (Cummings and Hershey 1994) and seems to play an important role in cold shock responses of the bacteria (Giangrossi, Brandi et al. 2007). It accelerates dissociation of empty 70S ribosomes into subunits (Grunberg-Manago, Dessen et al. 1975) and

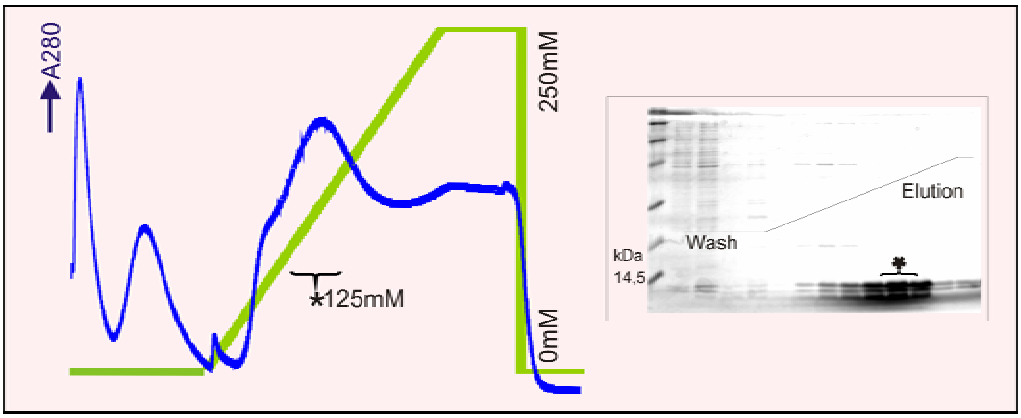


Figure 3.1.1.2-1: HisTrap assisted purification of IF1 from S30 extracts of an over-expression in the *E. coli* strain XL-1; Protein eluted in fractions with 125 mM Imidazole, peak-fractions were pooled and used for further purification with cation-exchanger; Insert: elution fractions analyzed on a 17% SDS gel; *asterisk* indicates peak fractions containing IF1 that were pooled and further purified with MonoS.

stimulates formation of the 30S pre-initiation complex (Hartz, McPheeters et al. 1989).

The hypothesis of IF1 - being of particular importance for the mechanism of 70S scanning - made it utterly important to purify active IF1 for the usage in our *in vitro* system. The factor carrying a His-tag was purified from an over-expression strain with the help of a HisTrap column (Figure **3.1.1.2-1**) and further with a MonoS cation-exchange column (Figure **3.1.1.2-2**) to remove residual contaminating proteins. A

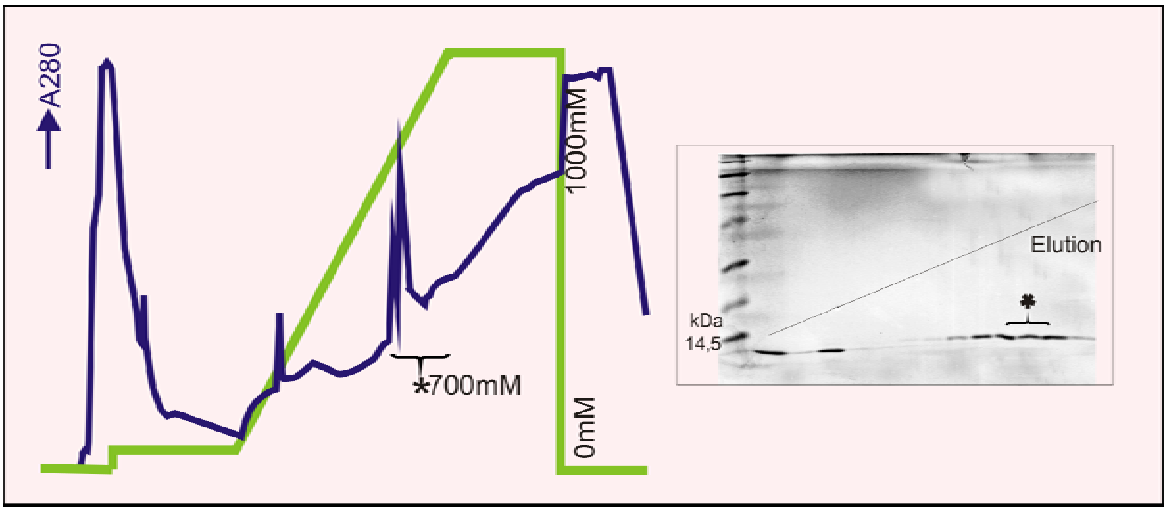


Figure 3.1.1.2-2: MonoS assisted purification of IF1 from peak fractions of HisTrap; Protein eluted in fractions with 700 mM ammonium acetate, peak-fractions (*asterisk)* were pooled and extensively dialysed against IF1-Buffe

test for IF1 activity is rather tricky. Its function is yet poorly understood, which is one of the main reasons for our extensive analysis of this protein raising the hypothesis for a new initiation modus. From the Cryo-EM analysis of Simonetti et. al. (Simonetti, Marzi et al. 2008) as well as the crystal structure analysis of Carter et. al. (Carter,

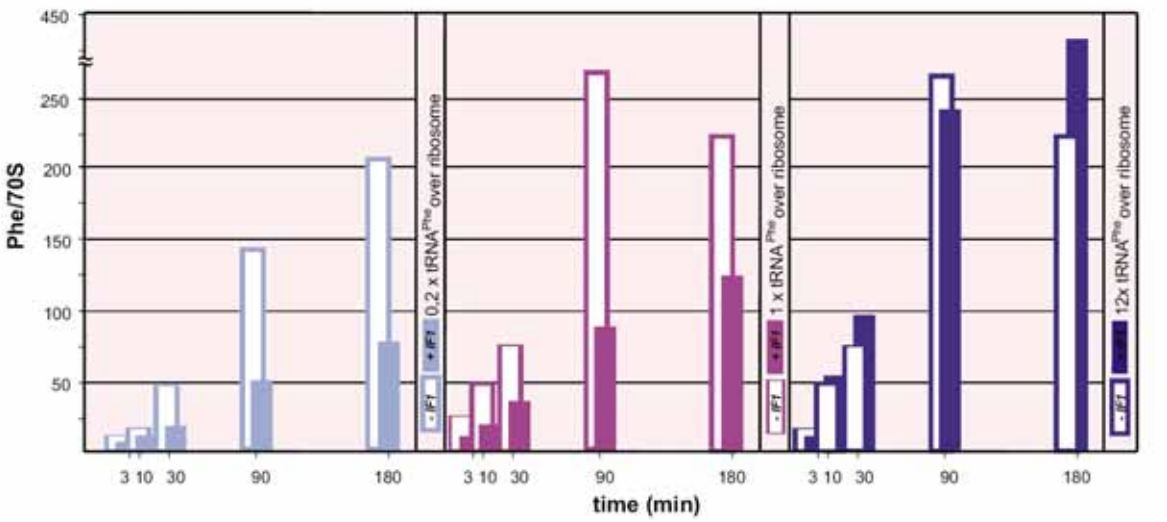


Figure 3.1.1.2-3: IF1 effects in poly(U) dependent poly(Phe); 5 pmol of 70S were incubated with 1.5x Ac-Phe and 5x IF1 over ribosome as well as 20 µg poly(U) mRNA for 15 min at 37 °C; after priming poly(Phe) synthesis was started by addition of the charging mixture containing the indicated amounts of $tRNA^{Phe}$ and was stopped after different time periods.

Clemons et al. 2001) we have a precise idea of the binding site of IF1 on the 30S subunit. The factor is interacting with the decoding centre occluding the A-site, it changes the conformation of helix 44 and triggers the flipping out of conserved bases 1492 and 1493. Thinking of the function of IF1 in 70S-scanning, the idea of the factor blocking the A-site for ternary complexes, which might otherwise interfere with the scanning process to the next AUG, seems reasonable.

To observe such a function we programmed 70S ribosomes with Ac-Phe-tRNA^{Phe} and incubated such a Pi complex with IF1, then we allowed for poly(U) directed poly(Phe) synthesis for up to 180 min. The surprising finding was that in the presence of limiting amounts of tRNA^{Phe} concentrations, 5x IF1 were enough to sufficiently block poly(Phe) synthesis. The effect was most prominent when creating a system with a short supply of tRNA^{Phe} of 0.2x tRNA^{Phe} over ribosome. Here the turnover rate of tRNAs is highest, making the blockage effect of IF1 most visible. Using higher concentrations of tRNA erased the IF1 effect probably due to the effective IF1 chasing *via* the excess of ternary complexes. This observation was not the only interesting finding concerning the function of this protein. Moreover we were able to observe an effect of IF1 when performing ternary complex binding to the ribosome in the presence of deacyl-tRNA^{Phe}. We could measure ~ 40 % decrease in ternary complex binding when IF1 was present. tRNA binding in the absence of EF-Tu - so to say non-enzymatic binding - was not inhibited.

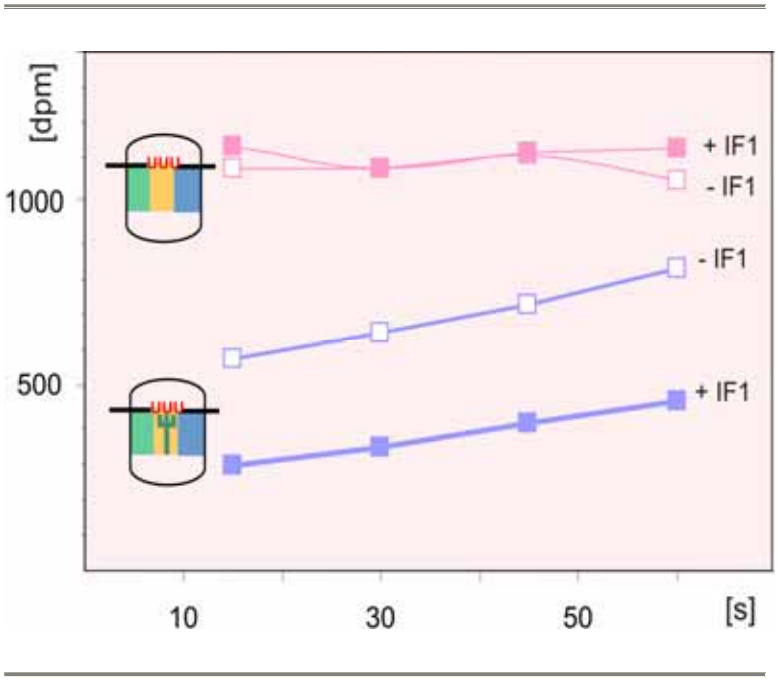


Figure 3.1.1.2-4: IF1 effects in ternary complex binding; 5 pmol of 70S were incubated in the presence and absence of 2x tRNA^{Phe} and 10 x IF1 over ribosome as well as 20 µg poly(U) mRNA for 5 min at 37 °C; [14C]Phe-tRNA^{Phe} (0.5 x 70S) as ternary complex with EF-Tu (1.5 x tRNA) and binding was allowed for up to 60 s at 25 °C.

The Inhibition itself can be described only as kinetical hampering because it ceases after 5 min and is then no longer detectable. [ternary complex formation was carried out for 5min at 37°C in the presence of PK (1.5 µg), PEP (5 mM) and GTP (2mM)].

3.1.1.3 Initiation Factor 3 (IF 3)

Same as IF1 and IF2 the initiation factor IF3 is essential for cell viability. It has been described as important for the 30S type of initiation functioning both as a dissociation factor to split the 70S ribosome into subunits after translation has been terminated, and as an anti-association factor to prevent premature 50S binding without the preceding event of 30S IC formation.

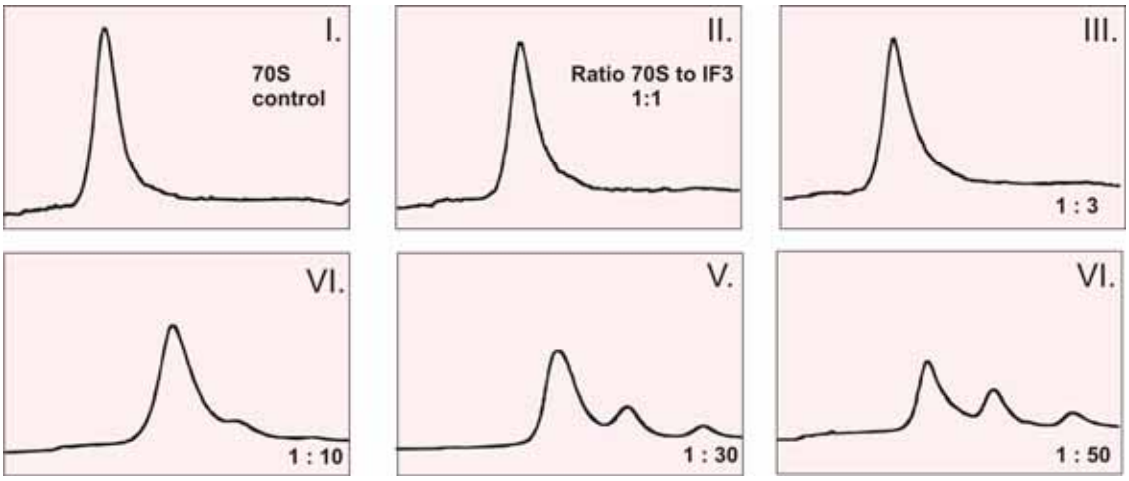


Figure 3.1.1.3-1: IF3 dissociation activity was tested by incubating increasing amounts of the factor with 70S ribosomes at 37 °C for 15 min in the presence of 1x binding buffer. The reaction was then put on ice and later loaded on a 10-30 % sucrose gradient which was then run in a SW60 rotor for 3 hours at 38,000 rpm. I. presents the control while profiles II-VI show different set ups where increasing amount of IF3 were added to 70S.

Similar to other two initiation factors carrying a His-tag, IF3 was purified from an over-expression strain with the help of a Ni-NTA agarose and further with a MonoQ anion-exchanger to remove residual contaminating factors. Detailed description of this procedure can be found in the thesis of Romi Gupta.

IF3 dissociation activity was used as a quick check of the factors activity. This can be observed particularly well in Tico-buffer without polyamines. Different amount of IF3 were incubated with an adequate amount of ribosomes at 37 °C for 15 min. Samples were put on a sucrose gradient and centrifuged with SW60 rotor. Patterns

were collected and dissociation activity assessed by the 70S particles that had dissociated into subunits (see Figure **3.1.1.3-1**).

3.1.1.4 Release Factor 1 (RF1)

Termination of translation is signalled by any one of the stop codons UAA, UAG, and UGA present at the ribosomal A-site. Two release factors, RF1 and RF2, recognize

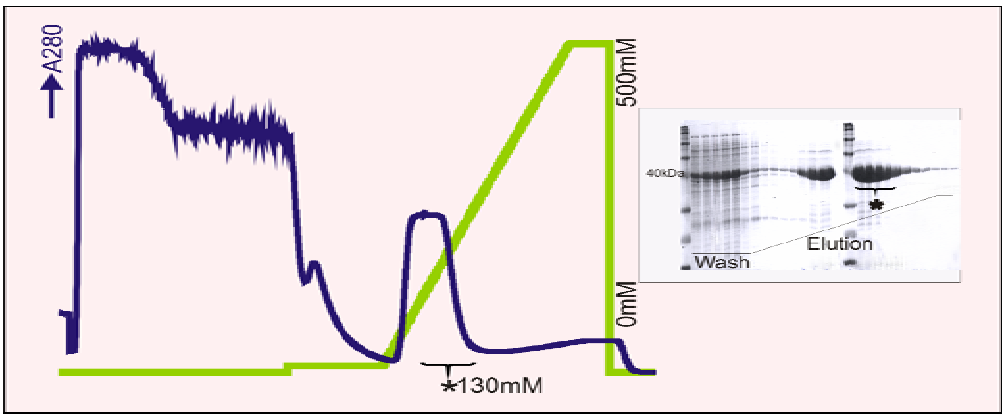


Figure **3.1.1.4-1: :** HisTrap assisted purification of RF1 from S30 extracts of an over-expression in the *E. coli* strain BL21(DE3); protein eluted in fractions with 130 mM imidazole, peak-fractions were pooled and used for further purification with anion-exchange column. Insert: elution fractions analyzed on a 15 % SDS gel; *asterisk* indicates peak fractions containing RF1 that were pooled and further purified with a MonoQ column

UAA and UAG or UAA and UGA and trigger the hydrolysis of the ester bond that links the polypeptide with the P-site tRNA, thus leading to a loss of the polypeptide and leaving the ribosome with a deacylated tRNA in the P-site.

RF1 is a medium size protein (360 amino acids in *E. coli* MG1655). To form termination complexes, the starting point for testing the 70S-scanning activity to the second ORF in our bicistronic mRNAs, we need active release factors. Therefore we had to overexpress and isolate this protein (see Figure **3.1.1.4-1** and **3.1.1.4-2**)

Assaying for RF1 activity is carried out in an *in vitro* system testing the individual reactions of the elongation cycle. 5 pmol of 70S are primed through incubation with deacylated $tRNA_f^{Met}$ (1.5x) and MFstop-mRNA (8x over 70S). A-site filling is achieved by incubating the Pi-State with two fold Ac-Phe-$tRNA^{Phe}$ over 70S. EF-G is added (0.2x) for translocation. Finally RF1 is supplemented (2x) and release is monitored by filtrating the sample trough a nitrocellulose filter counting the retained

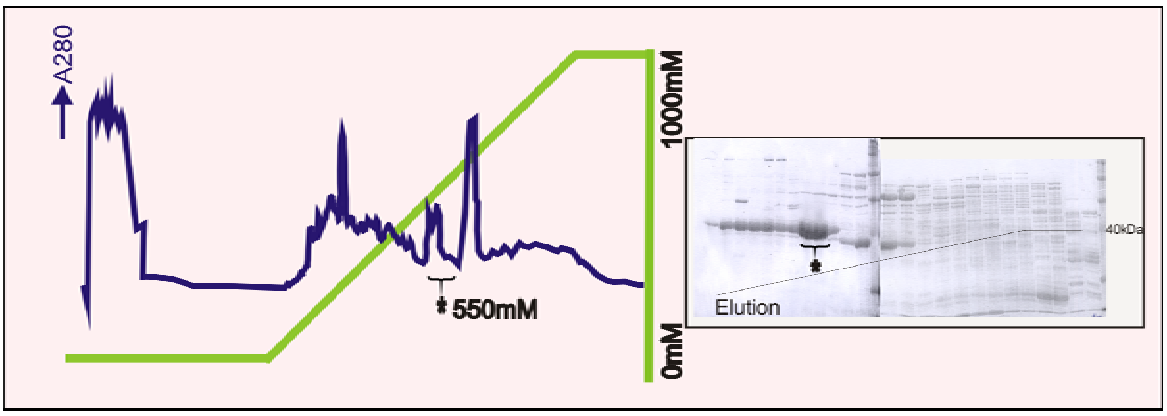


Figure 3.1.1.4-2: Purification of RF1 (MonoQ) from peak fractions of the HisTrap column. Protein eluted in fractions with 550 mM ammonium acetate, peak-fractions were pooled. Insert: elution fractions analyzed on a 17 % SDS gel; *asterisk* indicates peak fractions containing RF1 and dialysed against binding buffer.

radioactivity. The test demonstrates that the isolated protein is active. After a 5 min incubation of the POST-complex with RF1 53 % of P-site bound AcPhe was released. (Figure **3.1.1.4-3**).

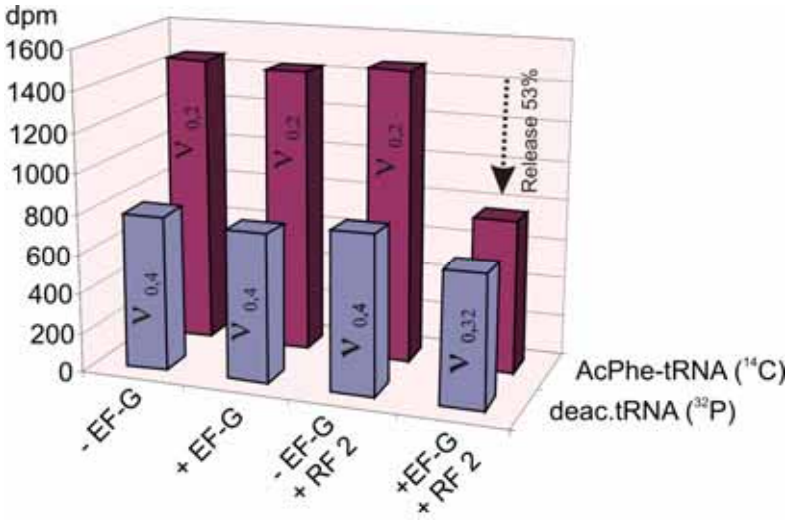


Figure 3.1.1.4-3: Assaying RF1 activity by measuring factor-dependent release of AcPhe from 70S ribosomes programmed with MFstop-mRNA.

3.1.1.5 CCA-adding Enzyme (CCA)

tRNA nucleotidyltransferase is responsible for both the addition and repair of the universally conserved 3′ terminal CCA sequence of tRNAs. This is due to the fact that this enzyme also catalyzes the reverse pyrophosphorylysis reaction: It can both degrade and reform the CCA sequence and thus maintains the 3′ end of all tRNAs in organisms such as *Escherichia coli* which do encode the CCA sequence. Since some commercially available tRNAs have relatively low acceptance for radiolabeled amino acids in charging reactions, sometimes a degraded CCA-end is the reason. In a trial to make our system more efficient we set out to purify CCA-adding enzyme to be able to improve tRNA charging with this enzyme. CCA-adding enzyme is also interesting for the exchange reaction that can be achieved with [α-^{32}P]ATP exchange labelling of tRNA (Ledoux and Uhlenbeck 2008). The purification was conducted with Ni-NTA agarose (Figure **3.1.1.5-1;** method see chapter 2.13.6).

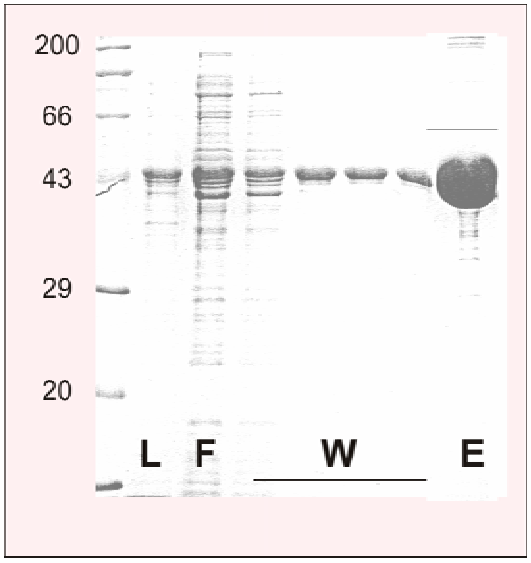


Figure 3.1.1.5-1: Purification of CCA-Adding enzyme in batch from Ni-NTA agarose (L = lysate; F= flow-through of unbound proteins; W= fractions of Ni-NTA wash steps; E= elution fraction, with 250 mM imidazole)

Analysis of our tRNA batches showed that CCA could not increase charging efficiencies; therefore the work with this enzyme was discontinued.

3.1.1.6 SmpB, small binding protein B

To trigger trans-translation, a process that deliberates ribosomes stalled on truncated mRNAs, SmpB is needed. This protein binds tmRNA and helps to accommodate it in the A-site.

The complex formation of tmRNA and the ribosome which is described in the appendix of this work is dependent on the presence of SmpB, which binds together with the large RNA to the 70S and maybe even co-migrates with the molecule through the ribosome.

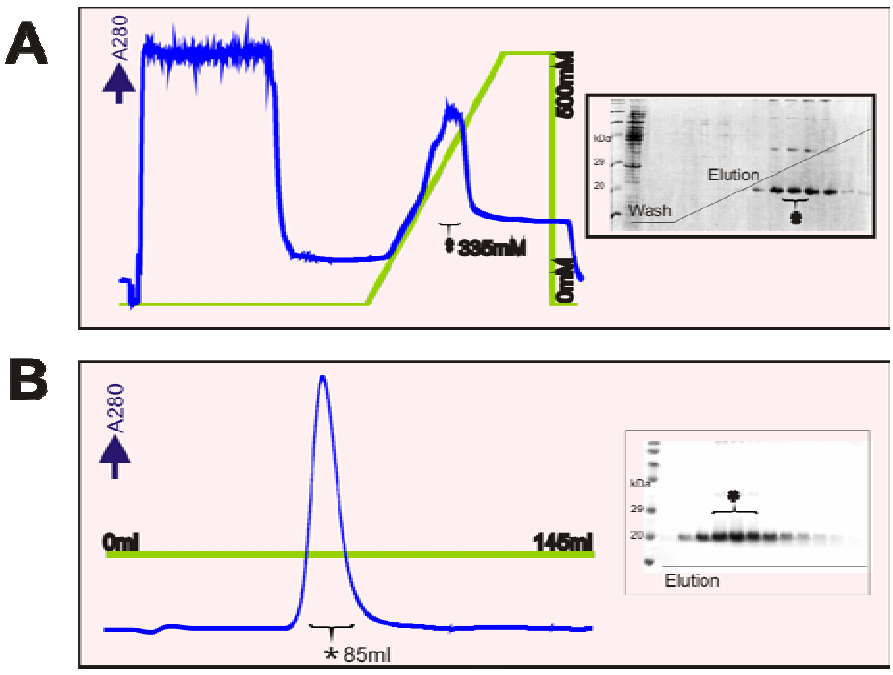


Figure 3.1.1.6-1: A. HisTrap assisted purification of SmpB from S30 extracts of an over-expression in XL-1; Protein eluted in fractions with 335 mM imidazole (see indent; SmpB peak fractions (*) were pooled and used for further purification with Superose 12), B. Superose 12 run get rid of impurities within the SmpB fractions of after HisTrap purification; Protein eluted after 85 ml in SmpB buffer, peak-fractions were pooled and concentrated using an Amicon with a cut-off of 10 kDa.

Thus to purify SmpB is very important for the success of this study. Purification procedures were conducted using a HisTrap and Superose column (Figure **3.1.1.6-1**)

To test for SmpB activity different concentrations of the protein were incubated with 35 pmol of uncharged tmRNA which can be easily detected in a native 8 % PAA gel after staining with toluidine blue (Figure **3.1.1.6-2**) showing a band shift and proving information that our SmpB is active in binding tmRNA.

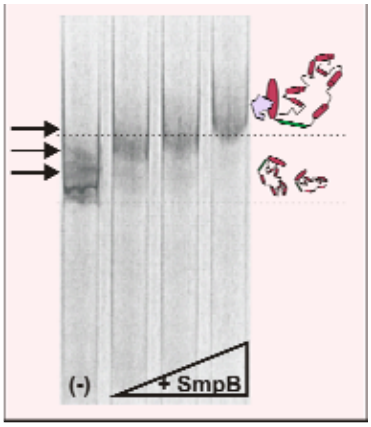


Figure 3.1.1.6-2: Band-shift experiment tmRNA. From left to right 0x, 0.5x, 1x and 2x SmpB were added to uncharged tmRNA and incubated under BB conditions for 15 min at 37 °C. Arrows indicate the shift of tmRNA due to the binding of the protein

3.1.2 Tools to dissect the initiation modes in a bicistronic mRNA – How to distinguish 70S-scanning from 30S-binding initiation

To analyze the mode of initiation is an important prerequisite for our study. To do so, the scanning region between two cistrons should be lockable either with a protein or a DNA/RNA oligo forming a stable double strand thus preventing a possible 70S-scanning between the cistrons allowing exclusively the de-novo 30S-type initiation.

We have tested MS2-protein, which is a viral coat protein being able to specifically interact with a stable stem loop structured termed as MS2-binding site. This is an example of a protein, which could also be used for a twin-cistron in an *in vivo* experiment being co-expressed and shutting-off scanning. An alternative is an LNA oligo, a special RNA-molecule which binds stably to an RNA *in vitro*. The LNA has been chosen because of its selectivity and special stability forming double helical structures.

A central problem studying initiation is to specifically distinguish the initiation *via* the 30S subunit from that one started with an associated 70S ribosome, which was up to now only attributed to special scenarios of translation in bacteria (see Figure **1.3.3-1**). Since 70S scanning would call for an intact ribosome moving between two cistrons without dissociation into subunits, we needed a possibility to rule out a dissociation event under such circumstances.

Therefore we constructed a) 70S particles which are cross linked and therefore can not fall apart into subunits anymore, and b) labelled 50S subunits which would show up in 70S under conditions, where we start with unlabeled 70S and have dissociation and re-association events during the experiment. Such labelled 70S particles could then be identified *via* radioactive measurements after sucrose density centrifugation and separation from the administered labelled 50S subunits.

3.1.2.1 Blocking Scanning with MS2

The interaction of MS2 coat protein and its translational operator hairpin is a very well-characterized RNA–protein complex. Only 10 min after infection of *E. coli* by the bacteriophage MS2, the product of the phage coat protein reaches a concentration to bind specifically to the MS2-mRNA. Since the discovery of this interaction this RNA-protein complex has been used in numerous systems to purify and identify proteins and their interaction partners (Ji, Fraser et al. 2004; Youngman and Green 2005).

The MS2 protein is relatively small with 129 amino acids. Generally it is used as a fusion product, since the MS2-binding protein itself tends to form oligomeric conglomerates, which make it hard to keep it in solution. In this study the MS2-MBP (maltose binding protein) was used. The massive MBP-tag makes it possible to keep the protein in solution and active. The protein was purified by my college Romi Gupta, the purification essentially followed the method described in Ji et al. (Ji, Fraser et al. 2004).

For our study we wanted to block 70S scanning specifically. When inserting the MS2 RNA-tag in between the two ORFs, scanning can be stopped by the binding of the MS2 protein.

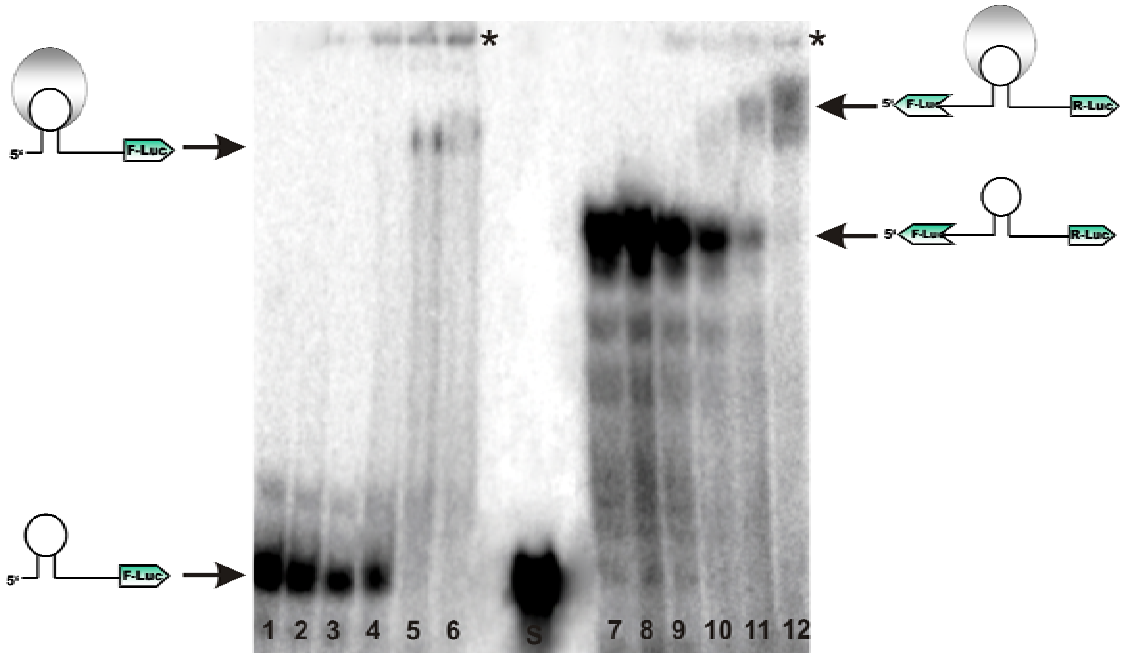


Figure 3.1.2.1-1: (MS2)F-Luc and F-Luc(MS2)R-Luc mRNA fragments were incubated with raising amounts of MS2-protein (lanes 1-6 and 7-12: 0x, 1x, 3x, 5x, 10x or 20x of MS2 proteins over RNA); arrows indicate the migration of bound and unbound mRNAs; * , complex that was unable to migrate into the gel.

Beforehand we had to test the binding activity of our isolated MBP-MS2. We used mRNA fragments, which were controls for the constructs we wanted to use *in vivo* and *in vitro*. First we determined the ratio for binding of MS2 to mRNA *via* a gel shift analysis. ^{32}P-endlabeled mRNA carrying the MS2 region was titrated with different concentrations of MS2 until a 100 % shift of the mRNA was visible in a native 8 %

PAA gel (**3.1.2.1-1**). Various amounts of protein were incubated with 1 pmol of mRNA at 37 °C for 15 min (Binding buffer condition). The samples were then quickly put on ice and analysed by electrophoresis.

One can clearly see that 10x MS2 over mRNA generates a shift in the gel suggesting that this is an optimal ratio for binding. This shift occurs with long as well as with shorter messengers, no matter whether the MS2 region is in front or between the two cistrons.

Since the MS2 protein binds RNA, we also wanted to control, if it also binds RNA unspecifically. Unspecific binding would block translation of any cistron and thus result in the collapse of any experimental setup. The same binding reaction was carried out in the presence of raising amounts of poly(U) mRNA, which has no specific RNA elements and is completely unstructured (Figure **3.1.2.1-2**) Unfortunately, that poly(U) was able to compete for MS2 binding: The signal of the band-shift slowly disappeared with higher concentrations of poly(U). This and further data suggested a significant unspecific RNA binding activity, an observation that was also reported already in the literature (Shtatland, Gill et al. 2000), which rendered this protein useless for our purpose. Therefore the MS2 project was discontinued.

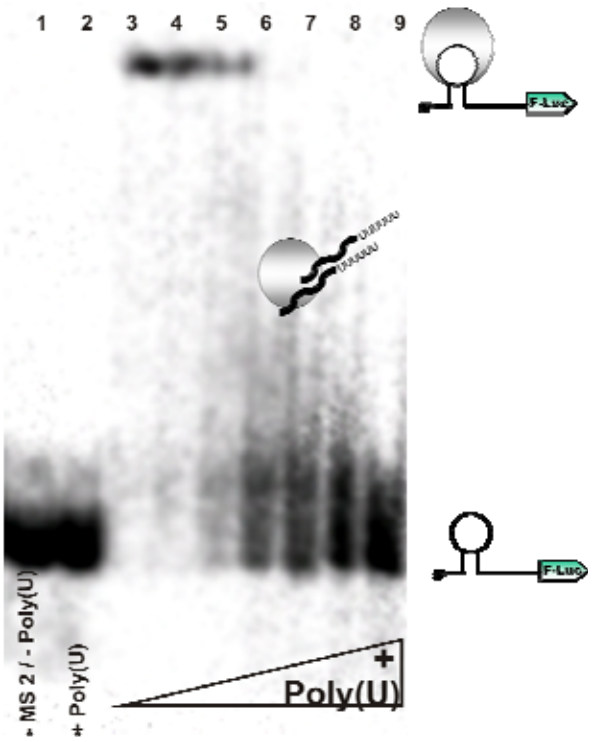


Figure 3.1.2.1-2: The (MS2)F-Luc mRNA fragment was incubated with MS2 protein and raising amounts of poly(U) mRNA. Lane 1 and 2 are controls; lanes 3-9 are in the presence of 10x molar excess of MS2-protein over (MS2)F-Luc mRNA and raising amounts of poly(U): lanes 3(0 µg), 4 (0.5 µg), 5 (1 µg), 6 (3 µg), 7 (5 µg), 8 (10 µg), 9 (20 µg).

3.1.2.2 Blockage of Scanning with LNA

A locked nucleic acid (LNA), which is an inaccessible RNA for RNases, is a modified RNA nucleotide: The ribose moiety of an LNA nucleotide contains an extra O-bridge connecting the 2' and 4' carbons (Kaur, Arora et al. 2006). The bridge "locks" the ribose in the 3'-endo structural conformation, which is often found in the A-form of DNA or RNA. LNA nucleotides can be mixed with DNA or RNA bases in the oligonucleotide whenever desired. The locked ribose conformation enhances base stacking. These features significantly increase the thermal stability and make the LNA molecule attractive for our study to block a possible 70S-scanning between two ORFs. We ordered an LNA of 22 nucleotides complementary against the MS2-element in our mRNA constructs. First of all the binding ability of the LNA to our mRNA had to be tested. Increasing amounts of LNA over mRNA were shortly incubated at 85 °C for 5 min for removing any secondary structure and then left for cooling down to room temperature at 30 °C. If double strand formation occurred due to the binding of the LNA this can be detected in a native PAA gel due to a band shift (Figure **3.1.2.2-1**).

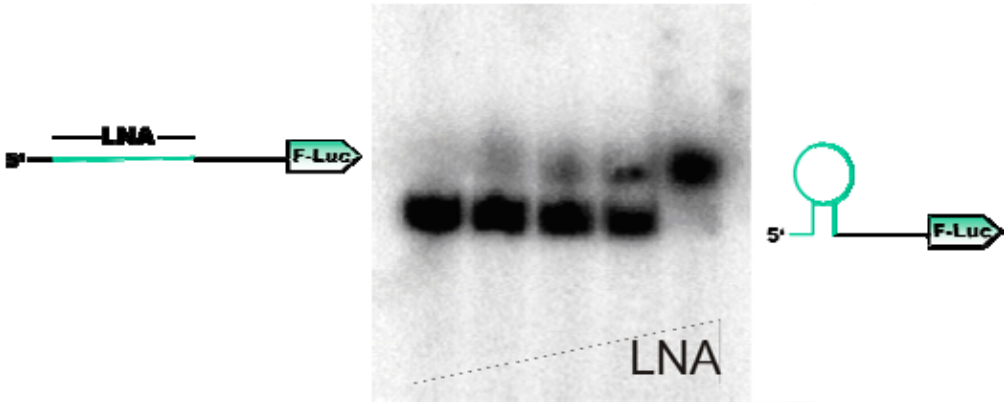


Figure 3.1.2.2-1: The (MS2)F-Luc mRNA fragment was incubated with raising amounts of LNA directed against the MS2-binding site (green). From left to right rising amounts so 0x, 1x, 5x and 10x and 100x of LNA over mRNA were used

Surprisingly we observed that a rather high concentration of LNA is needed to give a 100 % shift. When we were testing for temperature optimum that would still allow annealing of the LNA with our mRNA, we found that the LNA does not need a high temperature to form a double strand with the mRNA: it already does so at room temperature (Figure **3.1.2.2-2**) which makes the use of LNA for our studies very

attractive even if we need to use very high concentrations. Though the LNA was used in preliminary experiments concerning the luciferase constructs, we were

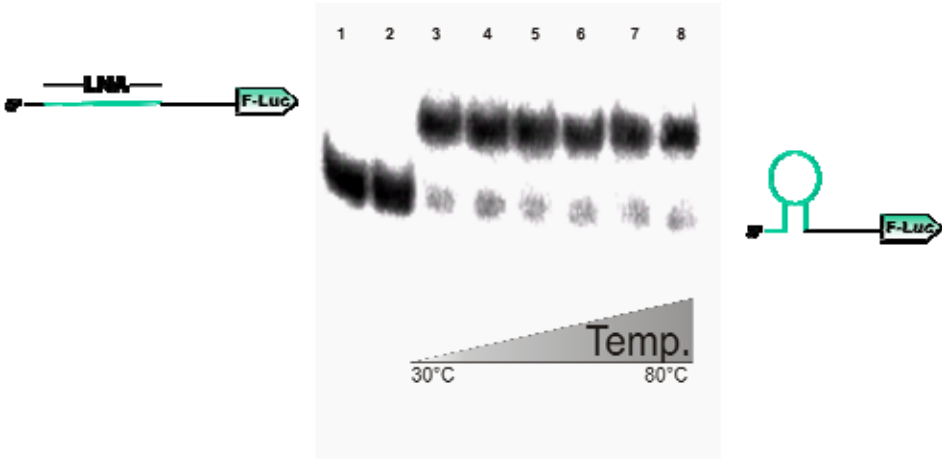


Figure 3.1.2.2-2: The (MS2)F-Luc mRNA fragment was incubated with 100x excess of LNA directed against the MS2-binding site at different temperatures. Both were incubated at 30 °C, 50 °C, 60 °C, 70 °C and 80 °C. Lanes 1 and 2 are controls showing mRNA only.

unable to use them throughout the complete study because they were rather expensive and oligos proved to be as effective in RTS experiments. Thus final experiments presented later in this work were solely carried out with antisense DNA-oligos.

3.1.2.3 Labelling of 30S and 50S subunits

[^{14}C]-Labeling of ribosomal subunits was carried out according to the method described in section 2.14.16. The labeled particles were isolated *via* sucrose gradient centrifugation and their ability to re-associate was measured in a simple experiment. Labeled 50S were added to unlabeled 30S (and *vice versa)* and their ability to perform poly(U) directed poly(Phe) synthesis was measured and compared to untreated re-associated 70S particles (Figure **3.1.2.3-2**).

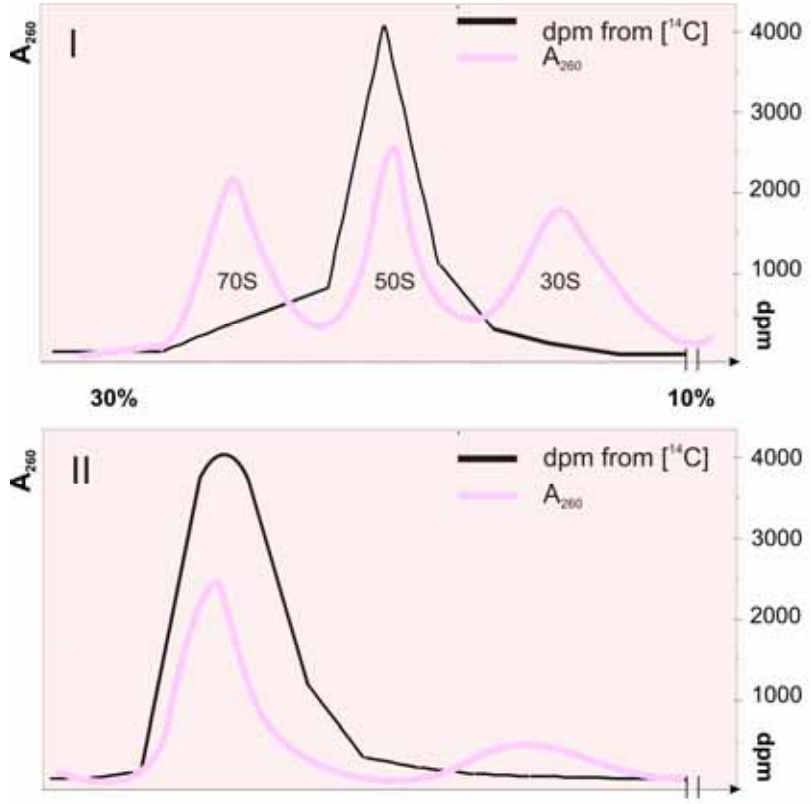


Figure 3.1.2.3-1: Checking purity, integrity and re-association activity of labelled 50S subunits I. [^{14}C]-50S and native 70S / 30S under Tico buffer conditions. ; II. Formation of re-associated 70S from labelled [^{14}C]-50S and native 30S (see materials and methods, chapter 2.14.14) can be followed by dpm of [^{14}C]-50S,

The integrity of the labelled large subunits was also tested with sucrose gradient centrifugation and compared to the profile obtained with unlabeled 50S (Figure **3.1.2.3-1**). Under Tico-buffer conditions the labelled large subunits clearly migrated in the region of 50S and showed no shoulder or smear that would hint to a degradation problem rendering these 50S useless for further experiments. Under reassociation conditions labelled subunits were able to form 70S particles. This could be quantified by measuring [^{14}C] signals in the fractionated gradient. Only when radiolabeled 50S

were able to associate together with native 30S subunits, radioactivity can be detected within the 70S peak. Both experiments showed that the subunits have 100 % activity compared to wild type.

3.1.3.2 Production of crosslinked 70S

The use of crosslinked ribosomes has been proven an elegant way in experimental setups that call for 70S particles, since they are unable to pass the cycle of dissociation and re-association events during protein synthesis (Moll, Hirokawa et al. 2004; Takahashi, Akita et al. 2008). The characteristics of these dissociation resistant particles are essential to examine the modes of translation described in our study.

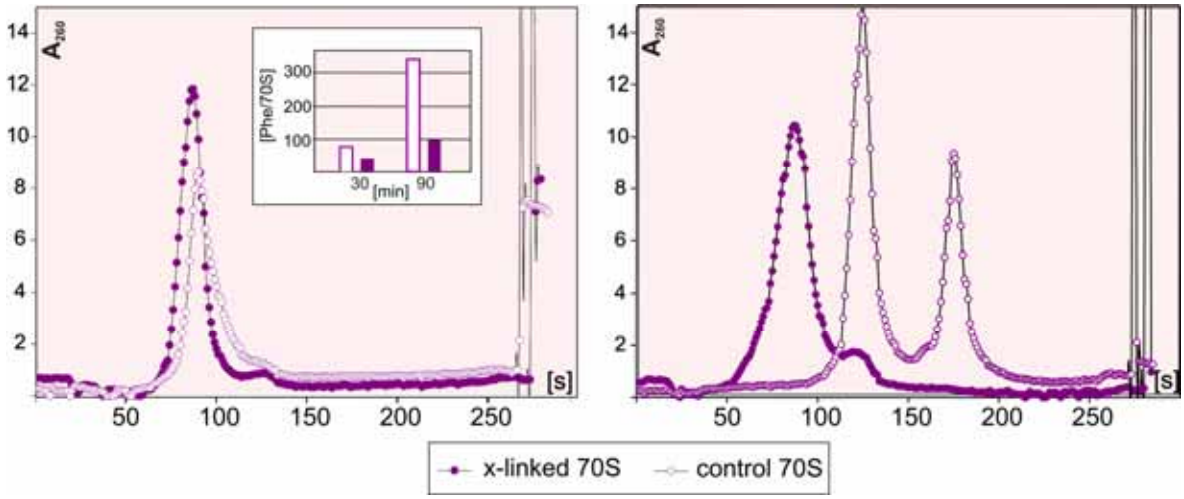


Figure 3.1.3.2-1: Sucrose gradient profiling of crosslinked (closed circle) and native ribosomes (open circle) under 4.5 mM Mg^{2+} (A) and 1 mM Mg^{2+} conditions; indent shows the activity in poly(Phe) synthesis.

Ribosomes were crosslinked according to section 2.14.16 and subsequently tested for their behaviour in different buffer systems. Sucrose gradient analysis revealed (Figure **3.1.3.2-1**) that our isolates did not dissociate in low magnesium buffer. Results regarding their activity in protein synthesis pointed out that the initial activity was 50 % lower of that observed in native ribosomes. After 90 min of continuous synthesis an even higher reduction of 70 % was observed. Due to the crosslinks a rapid recycling of ribosomes from homopolymeric mRNA is not possible observed as

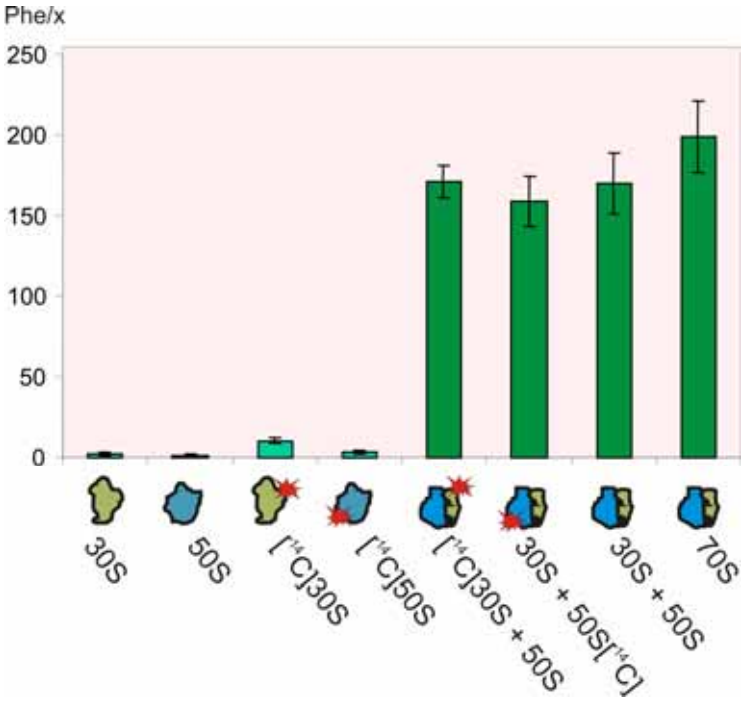


Figure 3.1.2.3-2: Checking the poly(Phe) synthesis activity of subunits and 70S ribosomes.

with native 70S ribosomes (Szaflarski, Vesper et al. 2008). Synthesis is massively inhibited and ribosomes remain on longer poly(U) chains, thus slowing down the overall rate of synthesis. During the synthesis of dipeptides from heteropolymeric constructs this effect is negligible; a recycling capability is not needed.

Crosslinked ribosomes show little poly(Phe) synthesis; most of them get inactivated during this process of chemical modification, because of disruption of factor binding sites and other spots that are necessary for the fidelity of protein synthesis. ~70 % are inactive; the amount of crosslinked ribosomes therefore should be tripled to be in the range of the active fraction of our native, reassociated 70S preparation.

3.1.3 Working with the PURE System

The PURE system is a special *in vitro* system to synthesise proteins with a defined set of factors. It was developed in the Ueda laboratory (Shimizu Y 2005) and is commercially available. The system is reconstituted out of highly pure *E. coli* proteins needed for translation, as well as other translational components such as ribosomes, tRNA, energy sources etc. which can be added to the reaction or not, according to the experimentator's desire. Transcripts as well as DNA can be used as a template to starts synthesis. Besides, all proteinacious compounds can be removed from system *via* metal affinity resin after the reaction is completed.

Given that single components can be excluded "one by one" we were particularly interested in using this setup to get answers about the importance of initiation factors for different initiation modes.

A number of mRNAs were designed to serve as template for protein synthesis in 30S and 70S initiation. They were designed as bicistrons; a short, leaderless ORF preceding a long ORF coding for GFP. In all constructs the SD of GFP is hidden by a weak secondary structure. 70S can start this way only on the short ORF and 30S initiation should be inhibited in two ways: 1. it can not initiate at the first ORF because this one is leaderless and 30S bindin mode does not work on leaderless mRNAs and 2. it can not initiate at the second ORF because the start is blocked by a secondary structure. After 70S initiation has happened, the ribosome will proceed until termination at the stop codon of the short ORF and if scanning happens it will proceed without dissociation, until it finds the next translation start. Since some ribosomal proteins possess helicase activity the 70S ribosome can easily unwind secondary structures in a given mRNA with a stability of up to -20 kcal. This way it should be able to reach the hidden translation start of GFP and translate the second cistron. GFP synthesis can thus be taken as readout for 70S-scanning type of initiation. We were unable to say how long the scanning window would be, so several constructs were designed, which harboured a scanning widow of -1 (start and stop codon are overlapping) and one of +9, +19, +24 or +39 (distance between start and stop codon had an increasing distance of the mentioned nt seize). To begin with we tested the longest and shortest window to get an overview how to proceed.

The experiment allowed us to answer several questions about 70S scanning. First of all we found GFP synthesis when the complete set of factors was present (Figure **3.1.3-1 B;** +IF1/IF3). We were also able to see that the scanning window between

the two cistrons is quite large; so that both the -1 and the +39 constructs yielded very similar results. The second remarkable observation to be made is the fact that IF3 is absolutely essential for the translation of the second cistron. To gain 100 % activity of GFP IF1 seems to be required, too. These facts argue for a dependence on IF3 and IF1 for the 70S-scanning type of initiation.

The antibiotic kasugamycin is known for disturbing the binding of initiator tRNA to the small subunit rather than to act on the 70S ribosome, up to now there is no described effect on the translating ribosome (Schluenzen, Takemoto et al. 2006), therefore it is considered to be an initiation inhibitor.

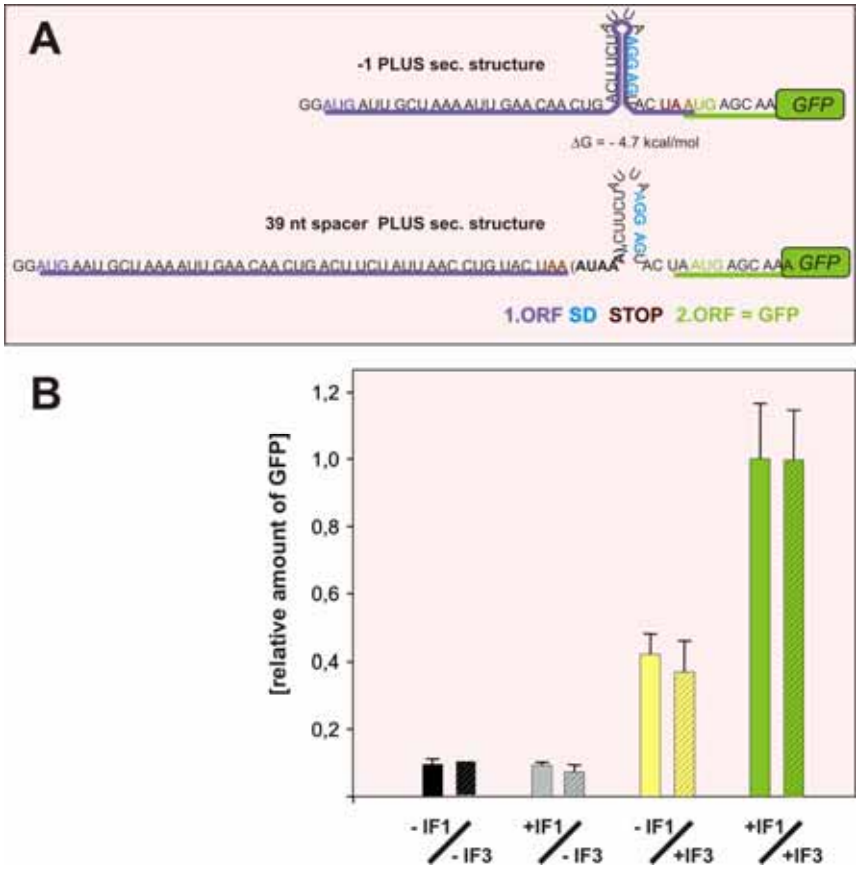


Figure 3.1.3-1: A. Details of the mRNA used in the experiment shown in B. GFP synthesis from two mRNA constructs in the presence of different sets of initiation factors is shown; solid bars describe results of the -1 construct; hedged bars show results with the 39 nt spacer construct

Besides it has been reported that Kasugamycin has no effect upon translation initiation of 70S on leaderless mRNAs (Chin, Shean et al. 1993). Because Ksg has such an impact on 30S but none on 70S leaderless initiation, we wanted to test

whether there is a consequence on the 70S-scanning type of initiation *in vitro*. For this experiment we choose the -1Plus mRNA as well as the -1Minus (here the start of the second cistron is not shielded by a secondary structure element; Figure **3.1.3-2 A**). In the case of the -1Plus construct we are able to reproduce the results seen in Fig. **3.1.3-1 B**, the synthesis of the second cistron is IF3 dependent, the presence of IF1 is highly stimulatory. Looking at the -1Minus construct we see a slightly different picture; the background without initiation factors seems slightly higher, besides the stimulation through IF1 is less than what we see for the -1Plus construct. The start of the second ORF is not shielded in this mRNA, the difference might account for some 30S-binding events happening.

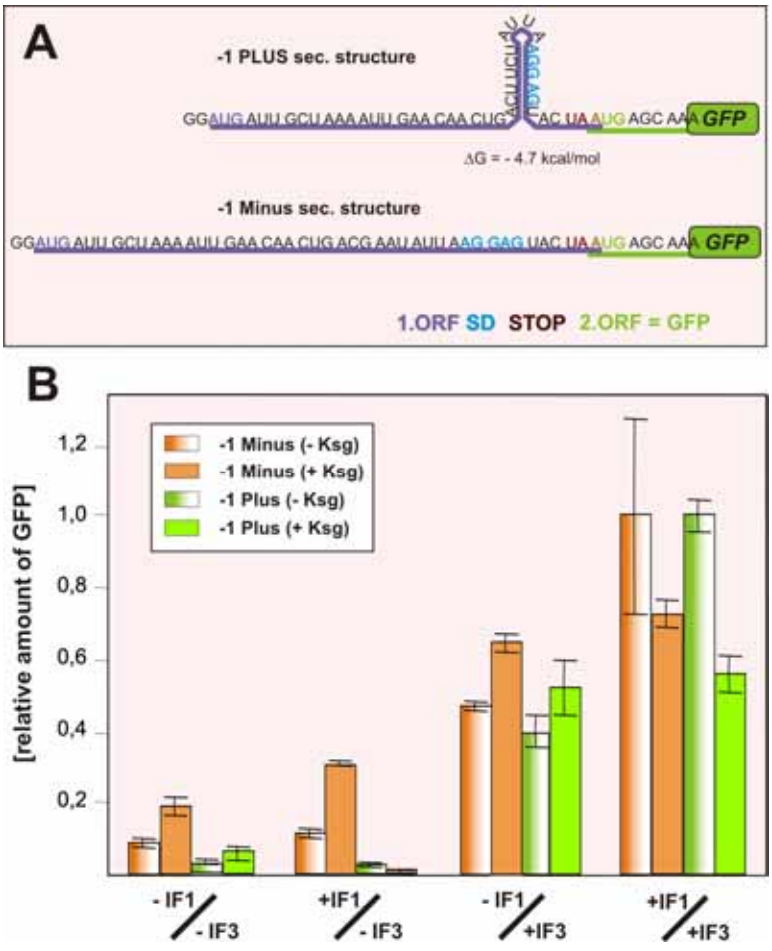


Figure 3.1.3-2: A. Details of the mRNA used in the experiment shown in B. Here GFP synthesis from two mRNA constructs in the presence of different sets of initiation factors as well as in the presence and absence of the antibiotic Kasugamycin (Ksg) are shown.

Yet one observation is striking similar; the stimulatory effect of IF1 seems completely abolished upon addition of 250 µM Kasugamycin (Ksg), whereas there is no effect on

the IF3 function during 70S initiation. Maybe Ksg can interfere with IF1 in the case of the 70S-scanning mode.

As for 30S initiation on the aforementioned constructs, there was absolutely no GFP synthesis detectable when using the -1PLUS/39nt spacer PLUS construct upon addition of subunits as we had already expected. This is not surprising, the first ORF is leaderless and the start of the second is hidden by a secondary structure. Leaderless mRNAs do not lead to the formation of 30S IC whereas 70S ribosomes are well known to initiate on such messengers. Ksg addition to 30S initiated -1MINUS constructs lead to an overall decrease in GFP synthesis. In contrast to the results obtained with 70S initiation, both IF1 and IF3 alone posses the ability to promote GFP synthesis together with IF2, which is present in all reactions discussed in this chapter. When both are added it results in an additive effect of GFP synthesis. Ksg addition on the other hand results in a loss of this additive effect (Figure **3.1.3-3**).

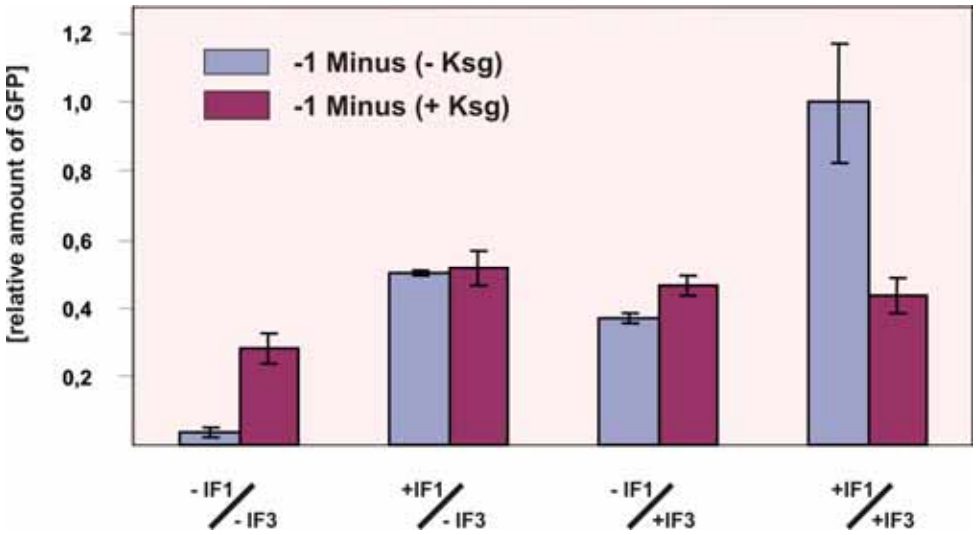


Figure 3.1.3-3: GFP expression measured when using the -1 Minus construct in the presence of variant sets of IF1 and IF3; here initiation was started with 30S instead of reassociated 70S

3.1.4 Two luciferases as a tool to discriminate one initiation mode from the other

Results of the PURE system had given us good implications for our model, though to find more accurate answers, we should be able to measure the ratio of two translation products in a bicistronic construct and to quantify both of them. The need of two readouts lead us to the usage of luciferases as reporter proteins. Various luciferase kits are commercially available which make quantification easy, the assay highly reproducible and precise data assessment possible. The kits make use of the fact that a different set of reaction partners for Fluc (Firefly luciferase) and Rluc (Renilla luciferase) exist. Fluc needs ATP and Mg^{2+} to oxidize luciferin resulting in chemoluminescence, whereas Rluc only needs the presence of oxygen to oxidize coelenterazine, resulting in emission too. Thus it is possible to measure both concomitantly and without overlap within one sample that can be either derived from cell-extracts or samples from an *in vitro* translation system. Two reagents are supplied in the kit. Mix I has all the components that Fluc needs, so after mixing, activity can be directly measured with a luminometer. Afterwards Mix II is added to the same sample containing chelators such as EDTA to remove the Mg^{2+} needed for the Fluc reaction. This way Rluc activity can be assessed giving feedback about the relative amount of the two proteins and thus their translation ratio.

The first set of messengers to use this system was made up out of the dicistron Fluc-MS2-Rluc. The MS2 region was added to be able to control scanning by the addition of the MS2 protein that interacts with this RNA element resulting in the block of 70S moving between the cistrons. Due to the uncontrollable behaviour of the MS2 protein we were not able to get interpretable read-outs, so the region was changed from MS2 to an IR (intercistronic region) that would bind either LNA or DNA oligos also leading to a block. Antisense-oligos not only for the IR but also specifically for both luciferases were designed to have a large set of controls at hand. The design of the specific antisens sequences was carried out with the help of Szyma M. Kielbasa (MPI Bioinformatics' Department) and the program "Unique Probe Selector". The selectivity of inhibition of the different anti-oligos was first tested in RTS before further experiments were conducted.

If not described otherwise plasmid (~250 ng) along with the inhibitory antisense or LNA was added to RTS reactions of 10 µl with a total amount of 14 pmol ribosomes. The reaction was incubated for 20 min at 30 °C. Longer incubation times proved to

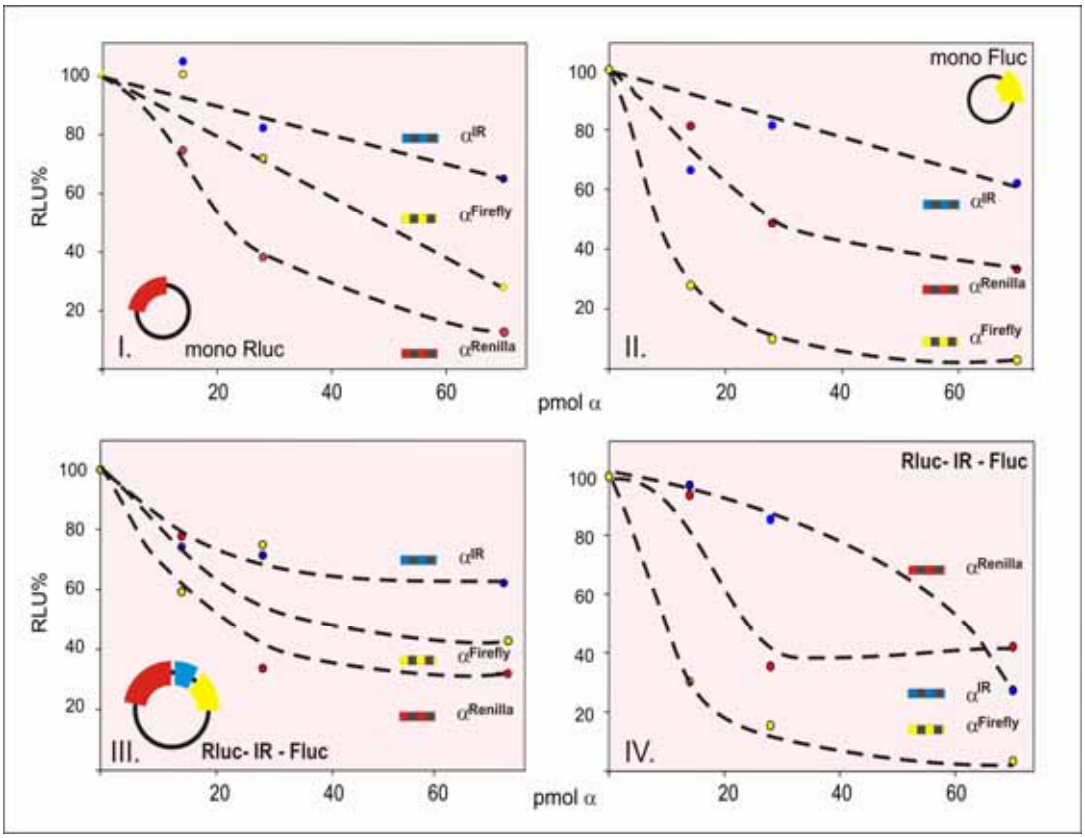


Figure 3.1.4-1: Inhibition profiles of different reporter protein constructs for three antisense sequences to specifically block Renilla luciferase (Renilla), Firefly luciferase (Firefly) and to block scanning in between the two ORFs of a bicistronic construct (IR – intercistronic region); results for di-Fluc and di-Rluc were both carried out with the same bicistronic construct, yet the result for each of the luciferases was depicted separately to

be deleterious to the inhibition effects of the oligos probably because they were degraded during this long incubation period. After incubation 1 µl of the total reaction was mixed with 10 µl of Mix I. According to the manufacturer's note 10 min incubation at RT followed and Fluc activity was measured in a luminometer, then Mix II was added and after another 10 min incubation Renilla activity was quantified. Regarding these preliminary experiment we could clearly determine the amount of antisense DNA which would allows us to specifically inhibit each of the luciferases in the various constructs without causing artefacts due to unspecific binding out of an excess of antisense (Figure **3.1.4-1**). To precisely block Renilla we decided for an amount of 1.4 µM (corresponding to 14 pmol per aliquot), to achieve the same for Firefly 2.8 µM (corresponding to 28 pmol) seemed sufficient, whereas to make an efficient block for scanning we chose the highest concentration of antisense possible

(7 µM = 70 pmol/aliquot) that would otherwise not interfere with the expression of the reporter proteins from the monocistronic constructs.

With the defined set of controls our scanning hypothesis could now be tested easily. Figure **3.1.4-2** summarizes the experimental results for mono and bicistronic reporter constructs. In A. we see the design of mono and bicistronic mRNA. In the bicistron an IR is placed between the stop codon of Renilla and the Shine-Dalgarno sequence of Firefly luciferase. The length of the IR is 67 nt. It is composed of two stretches which are with AC_4 repeats unstructured and span 25 nt left and right from the antisense binding region which itself is composed of 17 nt. 25 nt spacers are used to make sure that the oligo biding here will not interfere with the binding of neither 30S at the Shine-Dalgarno region and 70S terminating at the stop codon of Renilla.

The inhibition profile which is depicted in Figure **3.1.4-2 B** shows the results in solid bars for the monocistronic construct and in hedged bars for the bicistronic mRNA

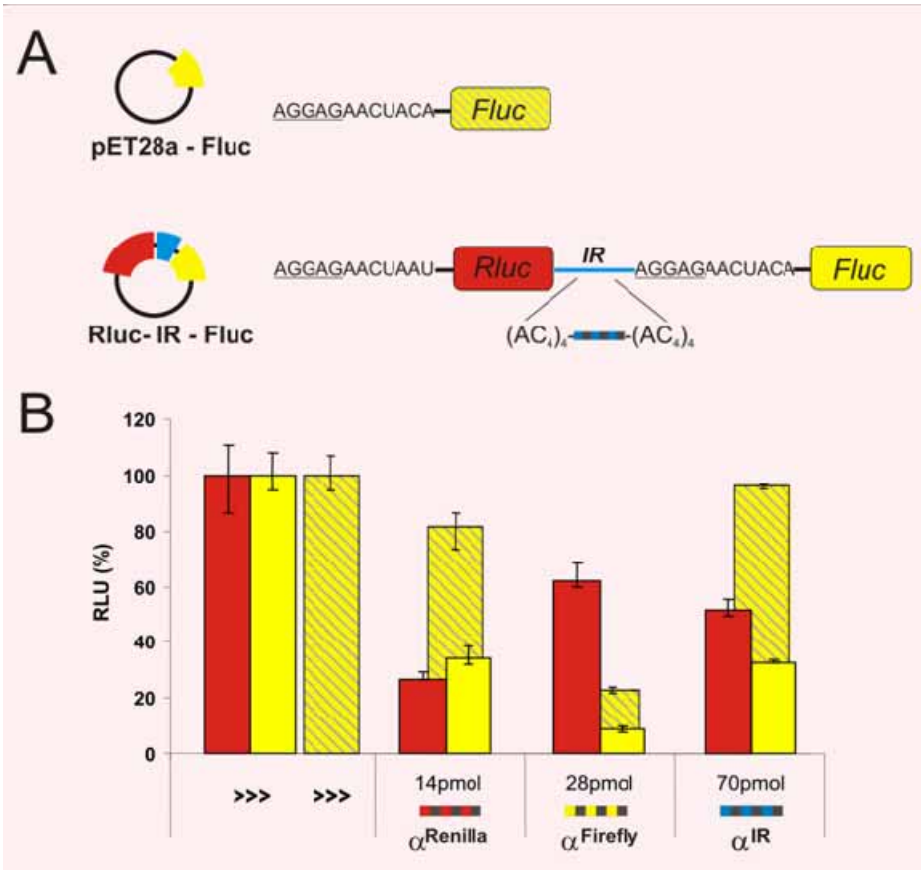


Figure 3.1.4-2: Inhibition profiles of different reporter protein constructs for three antisense sequences to specifically block Renilla luciferase (Renilla), Firefly luciferase (Firefly) and to block scanning in between the two ORFs of a bicistronic construct (IR – intercistronic region).

The translation of Firefly in the monocistronic environment is not inhibited in the presence of antisense oligos directed against IR or Renilla. Upon addition of anti Firefly the translation of the protein decreases by 70 % because translation is blocked upon oligo binding within the ORF of the protein. In the bicistronic construct we can observe a strong inhibition effect on Renilla in the presence of the Renilla antisense, as well. A mild repression can be observed in the presence of anti Firefly or IR, due to unspecific binding of these sequences within the ORF of Renilla. Looking to Firefly we get a strong inhibition of translation in all three cases. The presence of anti Renilla pulls the expression down to 65 %. We expected here that 70S scanning is inhibited, yet the translation of anti Firefly can still continue with the 30S binding type of initiation because the Shine Dalgarno region of the Firefly ORF is still available. The same is probably happening in the presence of the anti IR. Upon hybridisation of the oligo in the intecistronic region scanning of 70S from the Renilla ORF towards the Firefly ORF is blocked but 30S binding can still continue.

3.1.5 Profiling the minimal factor composition for the novel 70S scanning type of initiation

Upon finishing the experiments that have been described in the preceding chapters, we had collected evidence for the existence of an initiation pathway that can function with intact 70S ribosomes. Yet the array of factors that promotes protein synthesis might go beyond the three initiation factors, so we chose a primer extension approach to gain further insight on the exact factor group that is needed to facilitate scanning of a 70S ribosome from one cistron to the next.

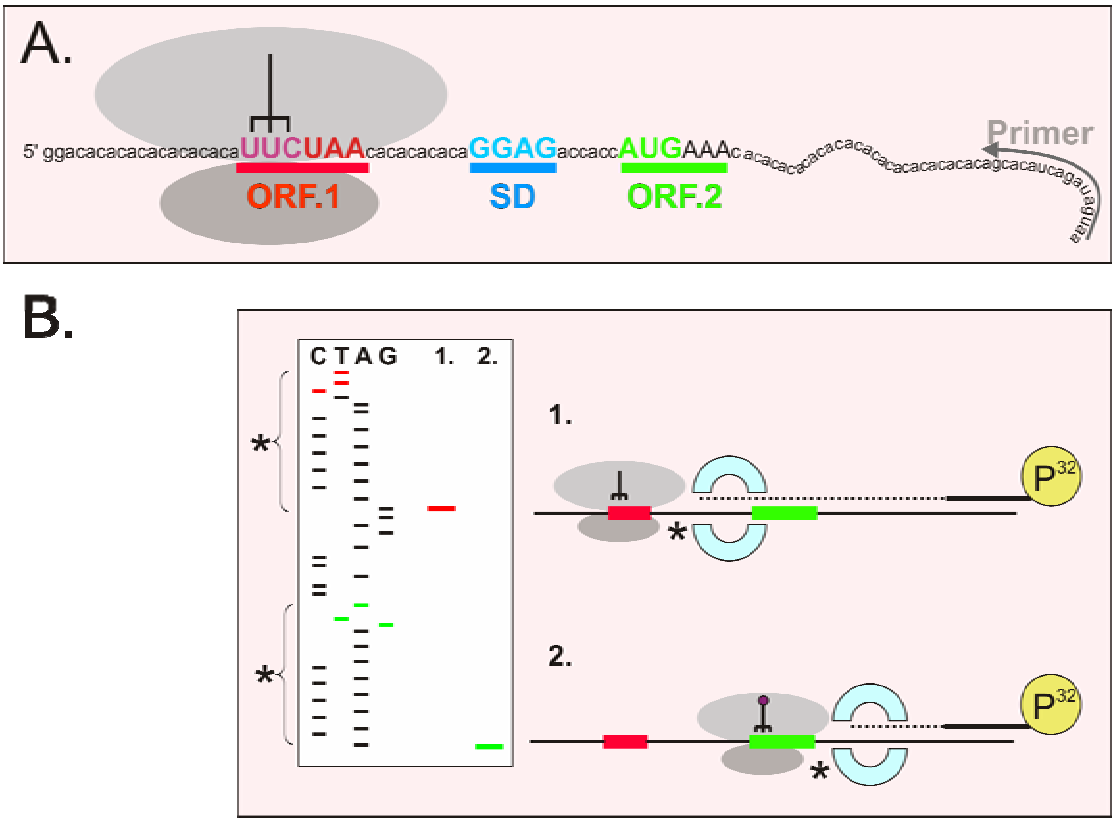


Figure 3.1.5.1: **A.** Toe-printing prerequisites: mRNA primed with deacylated $tRNA^{Phe}$ mimicking a post-termination complex at the first cistron. **B.** Explanation of the expected toeprint results. In **1.** we see a primer extension signal when the ribosome stays at the first ORF. In **2.** we see the signal shift, when the ribosome scans and reaches the second ORF; there is a 16-17 nt gap (*asterisk*) between the signal and the actual position of the ribosome on the mRNA, since the ribosome shields this region and does not allow the reverse transcriptase to move further on; light blue: reverse transcriptase

Primer extension (also called as toe-printing) is most commonly used to be able to determine the position of the ribosome on a given messenger with a precise resolution of 1 nt (Figure **3.1.5.1 B**).

For our study we designed an mRNA which as the other constructs should mimic a bicistronic environment. In the following case the first one made up out of two codons, one encoding phenylalanine and the other one a stop-codon, giving us the possibility to set up a post-termination complex when priming ribosomes with deacylated tRNA (Figure **3.1.5.1 A**). The second cistron on the other hand would comprise a start codon and a lysine codon.

Essential to our new initiation understanding is the fact that the ribosome does not dissociate to make a new initiation, so first of all we had to prove that x-linked ribosomes work in our assay system and behave similar to their non-modified counterparts, their usage underlines the behaviour of initiation without dissociation.

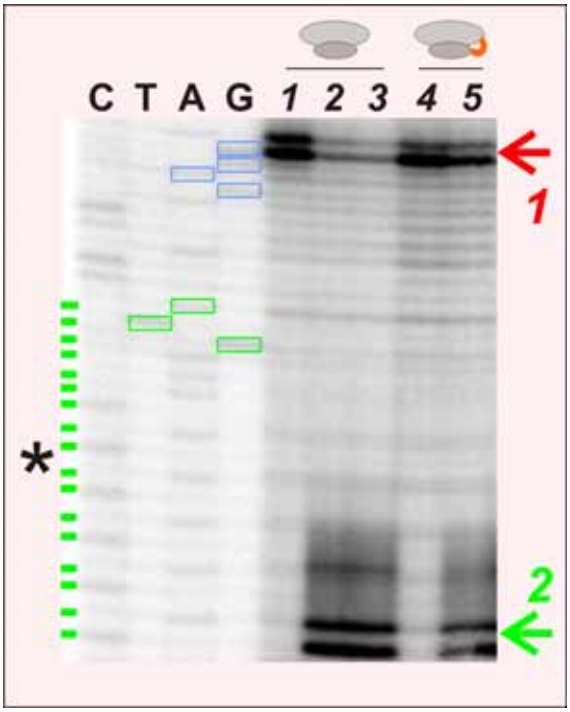


Figure 3.1.5.2: Primer extension study of the behaviour of native (lane 1-3) and crosslinked (lane 4-5) ribosomes primed with $tRNA^{Phe}$ (used in 5x excess over 70S) and subsequently incubated in the presence of $fMet\text{-}tRNA_f^{Met}$ (lane 3,5 - used 1x over 70S) or $tRNA_f^{Met}$ (lane 2); *asterisk/marker* again indicate the gap of 16-17 nt that occurs in toe-printing between the position of the ribosome and

Upon priming of non-modified and x-linked ribosomes we performed primer extension analysis and were able to show that both types allow complex formation at the first cistron (Figure **3.1.5.2**; 1 and 4). In the second round we added $fMet\text{-}tRNA_f^{Met}$ to these complexes and saw a surprising result (Figure **3.1.5.2**; 3 and 5). Both types of ribosome performed a shift to the second cistron, in other words were moving out of the post-termination state to the initiation site of the upstream cistron. Since both

types of ribosomes performed this action we must assume that the motion upstream is not connected to destabilization or “breathing” of the subunits. The ribosome might posses the inert ability to accommodate to the next start site when fMet-tRNA is present.

Concomitantly we wanted to test the contribution of the complete range of factors that allows protein synthesis in vivo. In a single omission test we removed one by one single proteins or groups of factors (elongation, termination, initiation) and observed the behavior of the ribosome.

The first group encompassed the three elongation factors EF-Tu, EF-G and EF-Ts which were administered in a ratio of 1.5x 70S, 0.8x 70S and 1x EF-Tu. The second group included release factors RF1 and RF3 that were added in a 2:1 and 1:1 stoichiometry to ribosomes. The third group consisted out of the three initiation factors IF1, IF2 and IF3 all added in 1.5x excess over ribosome. The fourth group was made up out of RRF only, the ribosomal release factor (1x 70S). The last protein group consisted out of two factors EF4 and EF-P that are more exotic to the translation mechanism yet under certain physiological conditions essential to translation, and have been extensively study in the Nierhaus group (Qin, Polacek et al. 2006; Connell, Topf et al. 2008). These factors were given in separate sixth group (ratio both 1:1 to 70S).

In the experiment described in Figure **3.1.5.2** we saw that fMet-tRNA$_f^{Met}$ by itself already has an extreme power to shift the ribosome to the second cistron thus we wanted to do our single omission test once in the presence and in the absence of the initiator tRNA, to be able to get a glimpse on the function the factors are doing.

Figure **3.1.5.3** lanes 1 – 7 show the set up in the absence of fMet-tRNA$_f^{Met}$. After priming the ribosome to produce a post-termination complex at the first cistron (lane 1) we added the protein mixes described in the table of 3.1.5.3 and allowed 10 min further incubation time at 37 °C. The toe-print results of lane 2 -7 show that in the absence of group 1 (elongation factors – lane 3), 3 (initiation factors – lane 5) and 4 (RRF – lane 6) the signal of the ribosome produced at cistron 1 does not vanish, whereas the omission of group 2 (release factors – lane 4) and group 5 (EF-P and EF4) leads to a disappearance of the signal. Since we did not add initiator tRNA here we can not expect a signal at position 2 to occur, rather the vanishing of the signal will be the signal for the ribosome having shifted and left the location that leads to the signal at ORF1.

In the second part of the experiment initiator tRNA was added. In lane 1 we can reproduce the results seen in Figure **3.1.5.2**; fMet-tRNA$_f^{Met}$ alone is capable to shift the majority of ribosomes to the second cistron yet the omission of the factor groups 1, 3 and 4 (Figure 3.1.5.3; lane 10, 12, 13) results in a partial elimination of this shift resulting in a signal at position one. Therefore we can conclude that groups 1 and 4 next to initiation factors are involved in the mechanism of the 70S-scanning type of initiation.

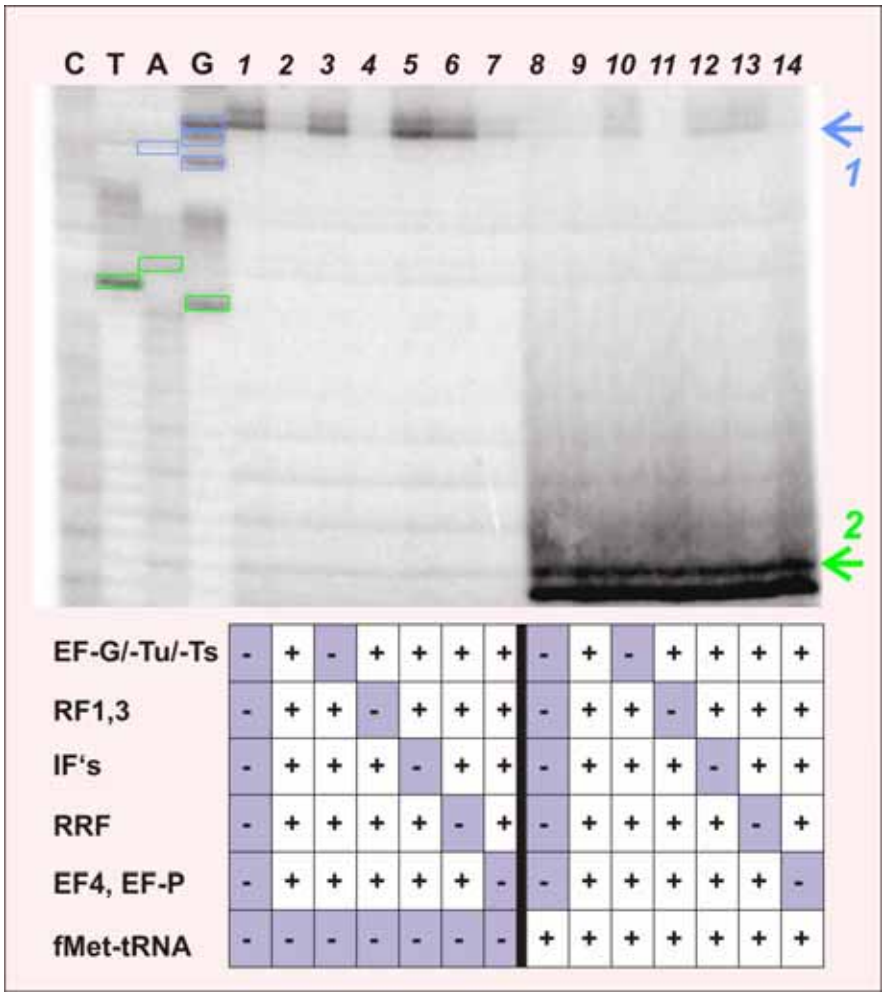


Figure 3.1.5.3: Primer extension study to understand the factor contribution to the scanning mechanism in the background of our model mRNA shown in Figure 3.1.5.1 A. Ribosomes primed with tRNAPhe (used in 5x excess over 70S) where incubated with the composition of proteins and tRNAs described in the table. Stoichiometry of single factors to ribosomes are given in the text. **1 – 7** show reactions in the absence of fMet-tRNA$_f^{Met}$ whereas **8 – 14** show reactions in the presence of fMet-tRNA$_f^{Met}$. Highlighted in red and green are the signals derived from the ribosome in the position of the first or the second cistron.

4 Discussion

The bacterial initiation mechanism has been analysed in detail over the past decades. Work that has been driven essentially by the groups of Gualerzi (Gualerzi and Pon 1990, Simonetti, 2008 #15377; Fabbretti, Pon et al. 2007) , Ehrenberg (Antoun, Pavlov et al. 2006, Antoun, 2003 #14165, Allen, 2005 #14828) and Noller (Dallas and Noller 2001) have put forward the general idea of an initiation pathway - the 30S-binding mode that needs free 30S subunits as well as three initiation factors to be carried out. IF3 for anti-association and masking the E-site, IF2 to deliver the initiator tRNA and IF1 to stimulate the functions of the two and to mask the A-site to make P-site occupation of the fMet-tRNA•IF2•GTP complex unequivocal.

In this thesis we set out to underline certain facts that this idea is not wrong but outdated since the majority of initiation events must be started with an intact

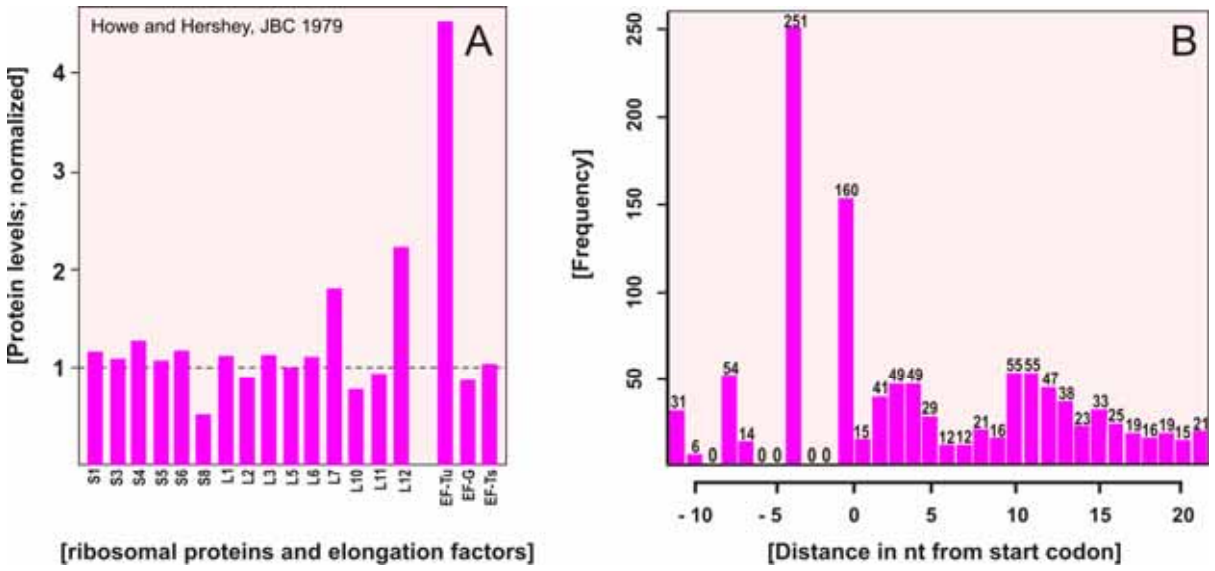


Figure 4-1: A. Representative study for the stoichiometry of ribosomal proteins and their ratios in *E. coli* B. Bioinformatics study shows that most coded cistrons, have their start codon located between -10 to +20 nucleotide.

ribosome. We were able to find hints throughout the literature that the un-dissociated ribosome must rule the majority of initiation events.

The first notion that a 70S-scanning mode of initiation must be predominant in the cell comes from the finding that ribosomal proteins are translated in a defined 1:1 ratio (Figure **4-1 A**). These ribosomal proteins are organized in polycistronic mRNAs;

starting with the canonical 30S-binding mode of initiation we have no possibility to explain why the stoichiometry of ribosomal proteins exists. Of course we could think of regulatory feedback-loops for each of the 51 proteins yet this seems unlikely, not only because such a feedback loop would have to regulate the synthesis of the protein itself but as well that of 50 others at the same time. Moreover some of the start codons of the r-proteins are hidden in secondary structural elements; here it has been proposed by Nomura that translational coupling is utilized to assure a 1:1 protein ratio as well as making it possible for subunits to reach hidden start codons. A 70S ribosome would melt such a secondary structural element while translating an upstream ORF giving way for 30S subunits to reach for the AUG codons of the downstream ORF. Such a model we can now rule out by means of bioinformatics analysis. We were able to show that the majority of proteins in *E. coli* are organized in policistronic mRNAs and that the majority of these polycistrons have a start and stop codon in close distance -10 upstream and +20 upstream of a given AUG (Figure **4-1 B**). The ribosome itself can cover about 40 nt on an mRNA, therefore the idea of regulation *via* translational coupling can be completely ruled out (Figure **4-2A**).

The second point of discussion is the essential character of IF1 for the bacterial cell. The idea that E-site and A-site must be shielded as a prerequisite for a proper binding of the ternary complex of IF2 and fMet-tRNA to the P-site cannot be taken in account as an explanation for the function of IF1. The small subunit itself has only one binding site, the P-site (Gnirke and Nierhaus 1986), thus there is no other chance for IF2 to deliver the tRNA but to this site only and rules out this fact to explain the essential nature of IF1. Hartz and Gold on the other hand were able to show that IF1 is necessary to select initiator tRNA on 70S (Hartz, McPheeters et al. 1989), with a detailed toe-print study they highlighted the fact that neither IF2 nor IF3 can select for the initiator tRNA unless IF1 is present. If we take the 70S-scanning type as a major initiation pathway in the cell, the fact that IF1 is necessary to select initiator tRNAs would explain the vital nature of this factor.

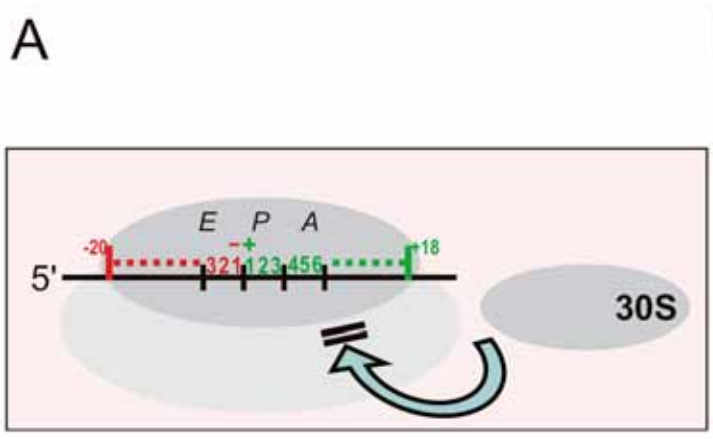
Another interesting point is the importance for formylated methionine in the initiation pathway of bacteria. Gruneberg-Manago and colleges put forward studies proving that the formation of the 30S-tRNA complex (with non-formylated initiator tRNA) is stimulated by all three initiation factors and is messenger dependent. However, the complex formation involving the 70S ribosomes is strongly inhibited by initiation factors when the non-formylated species is used (Petersen, Danchin et al. 1976 ; Petersen, Danchin et al. 1976). The fact that bacteria only use fMet as a starter and

not eukaryotes seems to be intriguing for only prokaryotes harbour polycistrons that would make 70S type of initiation necessary. It is puzzling to think of the initiator necessarily to be formylated but the 30S subunit having no chance to discriminate between the different species of initiator Met-tRNAs.

These facts lead to the hypothesis that he 70S scanning mode must be ruling initiation and that this mode must be dependent on initiation factor 1.

To be able to prove our new hypothesis we set up two experimental streamlines one that would follow a strict *in vivo* approach and another that would employ *in vitro* techniques to answer the question whether 70S or 30S predominantly facilitate initiation and in which way initiation factor 1 is involved in these mechanisms.

The *in vivo* experiments were conducted by my college Romi Gupta. Her studies were carried out with a special strain that had initiation factor one under the control of the arabinose promoter allowing for specific expression regulation by the addition of either glucose (downregulation) or arabinose (up regulation). Upon glucose addition and knockdown of IF1 in the cell she collected polysome profile and could make a unique observation namely that not only polysomes were extremely decreased (a common observation after down-regulation of initiation factors) but as well she found



B

-7 -4	-1 +1	+4 +7
NNN NNN	UAA	NNN NNN

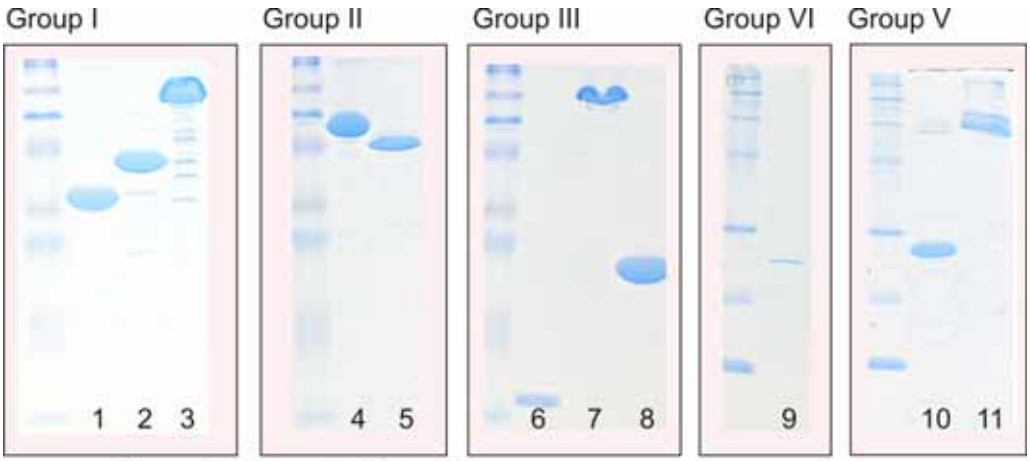
Cistron position	Ribosomal genes
-4	L23
-1	L29, S17
3	L1, L30, L15
4	S18
9	L18
10	L24, L4
12	L6, L16
14	L5, S14, L22, S5
15	S9
16	S19, S11
17	L2, S3
20	L27, L33
31	L36
32	L3
33	S8, S4
40	L17

Figure 4-2: A. Schematic figure of 70S preventing 30S to start initiation because of steric clashes on cistron borders B. Cistron position of ribosomal proteins on polycistrons counting the nucleotides before and after a stop codon; highlighted in red are proteins of the large ribosomal subunit both essential for assembly and dependent on the 70S scanning type

a scrambled 50S peak in the profile, hinting to a massive assembly defect. As already discussed we find ribosomal proteins organized in poly-cistronic mRNAs, bioinformatics could show that especially proteins that are essential for early assembly of the 50S subunits are organized with start and stop codons overlapping or in close proximity to each other (Figure **4-2 B)**, arguing for the fact that when 70S initiation is inhibited those proteins will not be translated at all or not in a correct ratio leading to assembly deffects and to cell death. Keeping in mind that this effect is observed when IF1 is depleted this idea would prove our hypothesis of IF1 being necessary for scanning. The next point of interest was the localisation of the initiation factors. *Via* a complex Western blot analysis we could show that in polysomal profiles initiation factor 1 could be detected exclusively on 70S monosomes but not on 30S subunits. This observation was in the light of present understanding of the IF1 role unique and new, besides it strongly supported our idea of IF1 function in a 70S initiation pathway. In a further analysis we were also able to detect significant amounts of IF3 on 70S and and on disomes. This was unexpected, for literature described IF3 as an anti-association factor. The finding that IF3 was partially also found on disomes promted us to believe that it might be engaged in scanning intercistronic distances. Cumulative these observations show that the function of IF1 and IF3 as well as the understanding of the 30S initiation pathway has to be revised.

To gain further insight on how this revision should be brought forward we constructed a second streamline of *in vitro* experiments that I have described in my thesis. With a broad spectrum of methods we looked for further evidence that the 70S pathway is predominant.

For this study certain prerequisites had to be fulfilled. First of all we had to purify all translational components and ribosomes to be able to set up an assay test that would give us the chance to look one by one at the function of each protein in the 70S scanning type of initiation. A summary of proteins that have been isolated for this study is given in Figure **4-3** along with their purity grade as well as their functional tests that had to be conducted to gain knowledge about the activity of each factor, something that is necessary to correctly interpret our results. During this course of preliminary experiments we were able to come up with a test to check the activity of IF1, as it binds to the A-site region of 30S we concluded that it might posses the ability to interfere with ternary complexes. Our guess proved to be right as a kinetical hampering of ternary complex binding in the presence of IF1 as well as a reduction of total poly(U) dependent poly(Phe) synthesis could be observed.



	No.	Purity	Activety-Test
EF-G	1	100 %	minimal system for poly(U) assisted poly(Phe) synthesis (chapter 2.16.1.3)
EF-Tu	2	76 %	GTPase activety (chapter 2.15.6); minimal poly(Phe) system
EF-Ts	3	70 %	minimal Poly(Phe) system
RF1	4	99 %	tRNA release from post-complex; protein is a kind gift of Dr. Oliver Vesper for purification and activety check refer to the thesis of Oliver Vesper
RF3	5	76 %	peptide release; see chapter 3.1.1.4
IF1	6	100 %	Inhibition of ternary complex binding to 70S programmed with $tRNA^{Phe}$ (Figure 3.1.1.2-4) and of poly(Phe) synthesis (Figure 3.1.1.2-3)
IF2	7	80 %	GTPase activity; fMet-tRNAfMet binding to 30S (Figure 3.1.1.1-4)
IF3	8	100 %	Dissociation activity (Figure 3.1.1.3-1)
RRF	9	100 %	Polysomal breakdown assay (Hirashima and Kaji, 1972; protein was a kind gift of Dr. Oliver Vesper
EF-P	10	69 %	no test described so far - protein was a kind gift of Dr. Markus Pech
EF4	11	72 %	GTPase activity (chapter 2.15.6); back-translocation, protein was a kind gift of Dr. Markus

Figure 4-3: Single components necessary for *an in vitro* analysis of the 70S-scanning type of initiation. 1-3 are routinely purified in the Nierhaus group – therefore there is no close up on purification, it is carried out after the general method of chapter 2.13.6

Furthermore we had to prepare an array of tools that would make it possible to dissect initiation modi. First of all we wanted to be able to rule out either 30S binding or 70S-scanning. 30S binding at the second cistron was impeded by the addition of a secondary structure that shielded the Shine-Dalgarno as well as the start codon (see Figure **3.1.3-1**) whereas 70S scanning was blocked by the presence of antisense DNA-oligos (Figure **3.1.4-2**)**.** Other methods were also taken into (MS2, LNA) account but were discontinued due to incompatibility problems. An overviewof this methodical approach is shown in Figure **4-4**.

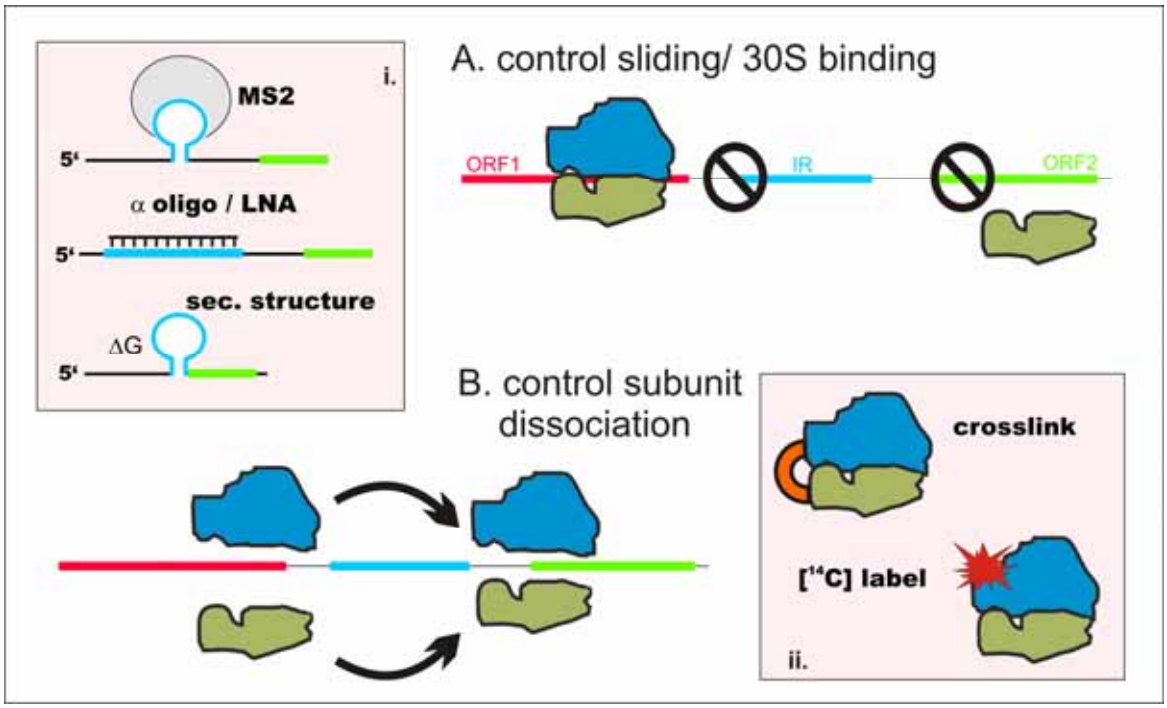


Figure 4-4: Prerequisites to dissect the initiation mode. **A** to control initiation modes the tools in i. were used B. to be able to rule out dissociation crosslinked ribosomes were produces, to monitor dissociation and re-association radiolabeled subunits were used

With these methods at hand we worked with different *in vitro* systems and reporter constructs to be able to answer the question about a *in vitro* prove for the 70S scanning type of initiation. Using the PURE system(Shimizu Y 2005), the RTS system and primer extension analysis we could clearly show that 70S ribosomes initiate protein synthesis and scan from cistron to cistron. Results from the PURE-system where a construct was used that had 30S RBS recognition blocked via secondary structure highlighted the fact that IF3 is essential and IF1 is still important for GFP-expression due to scanning.

The experiments with the bicistronic luciferase construct (Renilla and Firefly) underlined the fact that the second cistron is primarily reached by scanning of the undissociated 70S ribosome that triggers translation. Blocking this scanning reduces the translation of Firefly significantly; pointing out that 30S initiation is a minor initiation pathway in the translation of polycistrons.

Finally with the help of primer extension we could show that a certain set of factors is needed to move from one ORF to the other. The minimal bicistron consisting out of two codons each allowed us to see that next to the initiation factors, elongation factors as well as RRF are essential to the 70S scanning type of initiation.

Appendix A:

1.1 Initiating trans-translation

Translation proceeds through three steps: initiation, elongation and termination. Special circumstances can result in translational blockade so that termination can't be reached and the ribosome is stuck with an unfinished polypeptide and thus can not take part in protein synthesis anymore. To release such stalled ribosomes the bacterial cell has come up with a unique mechanism that is able to rescue a situation like this which is called trans-translation. It can release stalled ribosomes. Here a giant RNA molecule called tmRNA (transfer-messenger RNA) is able to march into a ribosome with an empty A-site offering no anticodon for ternary complexes or release factors. The tmRNA pushes the defective RNA out of the ribosome (Ivanova, Pavlov et al. 2005), takes over the incomplete peptide chain and adds a degradation code to its end (Keiler, Waller et al. 1996). The sequence of the RNA is unique; it has a CCA-end, which is charged by AlaRS (Muto, Sato et al. 1996) with alanine, and a tRNA like domain, which resembles a normal tRNA lacking only the ASL.

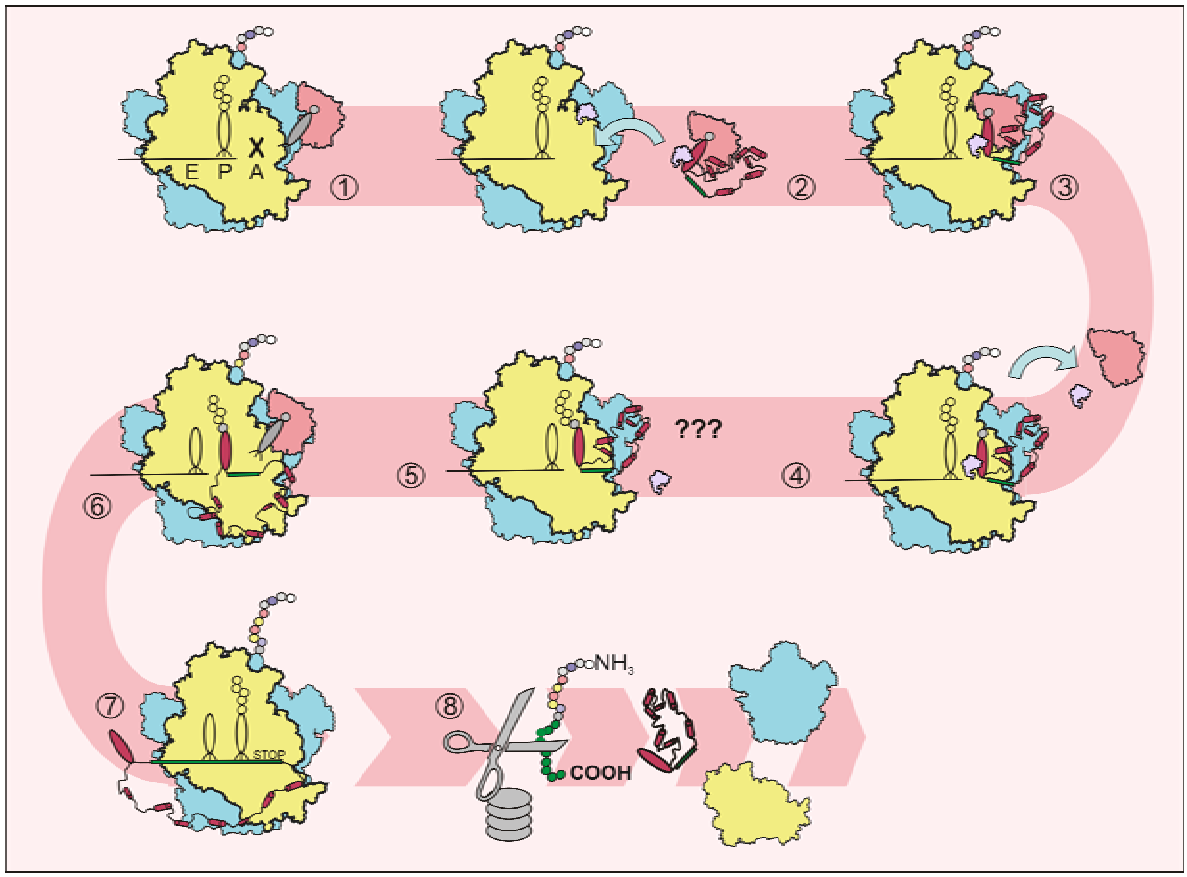


Figure A-1: Following tmRNA step by step through trans-translation; detailed explanation given in the text **(blue – 50S/ yellow – 30S/ tmRNA - red/ EF-Tu – pink/ light purple – SmpB)**

The second domain encompasses a large bundle of pseudoknots and a short ORF, encoding the degradation peptide that is added to the protein hanging unfinished on the ribosome (Burks, Zwieb et al. 2005).

tmRNA, like normal tRNAs,is brought to the ribosome in a ternary complex with EF-Tu (Fig. A-1.2). As mentioned before an anticodon is lacking (A-1.1), the ribosome cannot decode and accommodate the tmRNA like it does with tRNAs on a normal basis. Therefore a helper protein SmpB needs to give a hand here (Karzai, Susskind et al. 1999). SmpB is able to move together with the TLD of tmRNA into the A-site (Kaur, Gillet et al. 2006), contacting the major decoding players A1492, 1493 (Nonin-Lecomte, Germain-Amiot et al. 2009) perhaps triggers their movement to flip out and thus mimicking a decoding event (A-1.3). Subsequently the TLD of tmRNA must accommodate in the A-site and PTC reaction can happen transferring the incomplete peptide to the tmRNA molecule (A-1.5). Further steps of the mechanism include translocation of the TLD into the P-Site placing the mRNA like domain of tmRNA in the A-site allowing translation to occur again, until the stop codon of the internal mRNA segment of tmRNA is reached (A-1.7). The ribosome can then dissociate and the protein is targeted for degradation (Keiler 2008). Since the tmRNA molecule is rather large and of complex structure it is of general interest how this bulky object migrates through the ribosome. Several studies have yielded the hypothesis that while the TLD and MLD part migrate through the ribosome the pseudo-knot domains remain outside and somewhat unfold (Ivanov, Zvereva et al. 2002; Wower, Zwieb et al. 2005). Complexes of tmRNA with the ribosome have been resolved, only showing tmRNA at very early steps of trans-translation (see Fig. A-1.3 and A-1.4 (Valle, Gillet et al. 2003; Kaur, Gillet et al. 2006). To be able to gain a complete understanding of tmRNA interaction with the ribosome we set out to produce a complex of tmRNA with the ribosome at later stages of trans-translation (Fig. A-1.6 –7) to be visualized by cryo-EM. Encompassing this complicated task will be explained as a supplementary to the major work of this thesis dealing with a new initiation model.

1.2 Solving preparation problems for tmRNA•70S complexes

During my diploma thesis we were setting out to produce a 70S-tmRNA complex that would allow us with the help of cryo-EM to analyse the pass of this large RNA molecule through the ribosome. Yet the work remained unfinished so that we

continued to establishing proper binding conditions for tmRNA, yielding a complex of this monstrous RNA with the ribosome during the course of the doctoral thesis.

Our first choice was the MK10V system. Here a heteropolymeric mRNA that has a short 5'-end, followed by a sequence coding for Met (1x), Lys (10x) and Val (1x) was used. The experimental idea was to first prime the ribosome with fMet-tRNA, next allowing synthesis of the following 10 codons for lysine. tRNA decoding the Val codon would not be offered, so that the ribosome would get stuck, harbouring an empty A-site which would then be competent in accepting the incoming tmRNA molecule. In the first study MF10V also was a choice, but rendered a complex that was impaired in tmRNA binding. The reason for such a result could be due to the fact that poly(Phe)-chains have difficulties to find the tunnel entrance and thus can accumulate between the subunits rather than taking the tunnel (Picking, Picking et al. 1991).

A fast method to determine whether tmRNA can bind to ribosomes prepared with the MK10V messenger is looking for [^{14}C] counts in the TCA precipitate of a translation reaction. tmRNA is charged with radiolabled [^{14}C]-alanine that would be incorporated into the oligopeptide chain when the RNA successfully binds to ribosomes. Hot TCA precipitation leads to flocculation of oligopeptides longer than 3 aa, which can be filtered out of the reaction and tested for radioactivity.

In preliminary experiments we were able to detect [^{14}C] counts, prompting us to believe that tmRNA had indeed bound to ribosomes. This proved to be a wrong interpretation. SmpB, the small binding protein that brings tmRNA to stalled ribosomes was found to be able to form a complex with tmRNA protecting the ester bond between [^{14}C]Ala and the RNA, so that we could find positive TCA results without tmRNA binding to ribosomes.

Yet we were determined to modify the reaction conditions of the MK10V system more to finally be able to achieve tmRNA binding. When continuing the work on the MK10V system the first observation was that the MK10V messenger allowed a fast ribosomal recycling (Figure A 1.2-1). Elevated temperatures yielded a distorted ratio of fMet to lysine. If ribosomes would get stuck on an mRNA a ratio of 1:10 should be measured. Under elevated temperatures of 30 °C or higher the ratio shifted to over 1:20. Ribosomes therefore must recycle and continue with an initiation through ternary complexes, so that more and more lysine was incorporated in short oligo peptides. Therefore a temperature and time optimisation was conducted with the aim to repress recycling by slowing down the overall reaction (Figure A 1.2-1). Shifting

the temperature down to 20 °C made it possible to inhibit recycling completely. fMet to Lys ratios never rose above 1:10, results for 25 °C also showed promising results; a temperature which seemed to be more optimal to allow good charging of tmRNA too. Repression of recycling is an important prerequisite for tmRNA binding. When recycling occurs too fast the ribosome will not form a tmRNA●70S complex. Further experiments were performed at 25 °C for ~30 min.

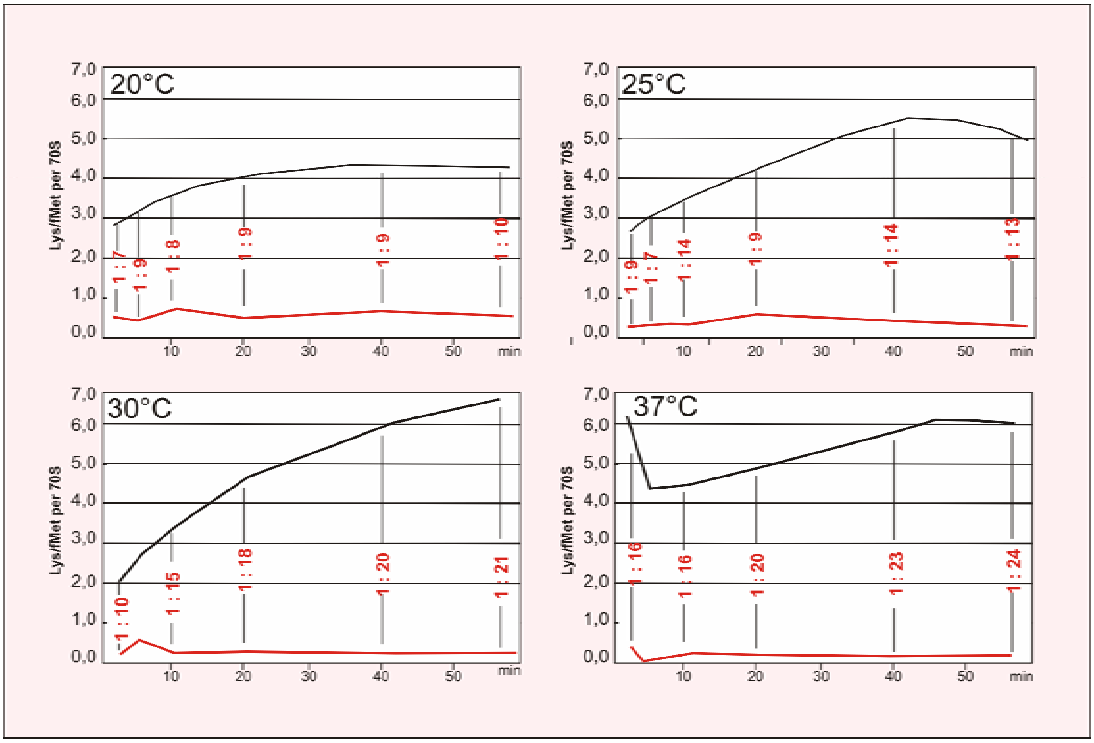


Figure 1.2-1: Kinetic measurements of MK10V synthesis under different temperature conditions; black lines mark the synthesis of [^{14}C]Lys per ribosome, red lines follow the incorporation of [^{3}H]fMet per ribosome; numbers in red mark the ratio of fMet to Lys per ribosome

Pre-charging tmRNA would also offer a possibility to reduce the time window for recycling. Unfortunately, the charging activity of tmRNA usually is 20-40 %. When using such samples one gives a large amount of uncharged tmRNA to the system. This is also problematic; SmpB binds to uncharged tmRNA, too causing a depletion of SmpB that can take part in complex formation. We tried two methods for enriching the alanylated fraction in tmRNA samples by separating uncharged from charged

molecules, one with HPLC and another using a self-made EF-Tu column (see 2.14.10).

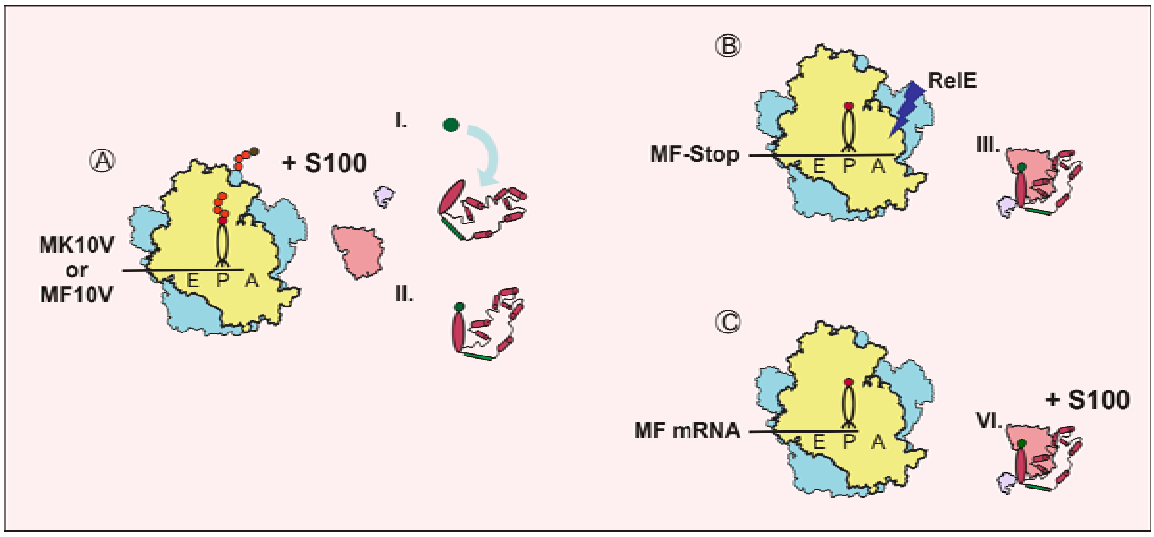


Figure 1.2-2: Strategies for the formation of a 70S●tmRNA complex; **A** I. describes the initial procedure with MK10V or MF10V mRNA, after priming ribosomes elongation mixture was added containing uncharged tmRNA as well as EF-Tu, SmpB, AlaRS and S100 fraction, to manage peptide synthesis as well as tmRNA charging and binding in one module. (II) Another approach used precharged tmRNA, reaction was carried out like in A I, AlaRS as well as free radiolabeled alanine were not added here **B.** RelE cleaves mRNA bound to ribosomes, producing a complex that accepts tmRNA (Ivanova, Pavlov et al. 2004) *in vitro*; the reaction was carried out in two steps, one encompassing ribosome priming and RelA cleavage, the second - a separate module - tmRNA charging - that contains SmpB, AlaRS and EF-Tu; after separate incubations both the RelE ribosome complex and the charged tmRNA-Tu-SmpB ternary complex were added together and after short incubation alanine incorporation was tested *via* puromycin reaction ***C***. The final approach also used a modular system, one part of the assay was used to prime ribosomes with Ac-Phe, the other one to produce a charged tmRNA that forms a ternary complex with SmpB and EF-Tu (like B III), to this reaction S100 fraction was added; both modules were after separate incubation combined and complex formation allowed for 5 min at 37 °C; whether tmRNA had bound to the ribosome was assessed by puromycin reaction measuring whether radioactivity of [^{14}C]-Alanine could be detected

The enrichment itself worked, but did not lead to enhanced binding of tmRNA to the ribosome.

Since we could not achieve binding of tmRNA to a complex formed with the MK10V messenger we changed the strategy. According to Ehrenberg, tmRNA can bind efficiently to ribosomal complexes which have been subjected to RelE cleavage. RelE is a sequence specific RNAse that cleaves mRNAs bound to the ribosome exposing the codon UAA in the A-site. Such a ribosomal complex carrying a peptidiyl tRNA in the P-site and an empty A-site due to RelE cleavage is a natural acceptor for tmRNA binding. We decided to use the MFstop mRNA for this purpose (Method described in 2.14.18). Though we could detect tmRNA binding using this method, the occupancy of 10-20 % was too low for cryo-EM.

Finally we used assay conditions as described in Figure **1.2-2 C**. To our surprise the addition of S100 to the ternary complex formation of SmpB●EF-Tu●Ala-tmRNA along with EF-G to drive translocation of the TLD immediately after it has accommodated into the A-site, pushed the occupancy to above 40 %. This sample is now being analyzed by cryo-EM.

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Curriculum Vitae

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