CHAPTER 2: Materials and Methods

2.1 Chemicals and enzymes-suppliers

Amersham Biosciences, England

γ ^{32}P-Adenosine-5'-triphosphate

γ ^{33}P-Adenosine-5'-triphosphate

FluorImager 595

ImageQuant™ TL (software)

L-^{35}S-Methionine

L-^{14}C-phenylalanine

L-^{14}C-Valine

L-^{3}H-Lysine

L-^{3}H-phenylalanine

L-^{3}H-aspartic acid

Personal Densitometer™ SI

Storage Phosphor Screens

Storm™ imaging system

Poly(A), (27-4110-01)

Poly(U), (27-4440-02)

PVDF western blot membrane

ECL western blot detection regents

Beckmann, München

Ready Value (scintillation liquid)

Ultracenrifuge tubes Ultra-Clear

Biolabs, New England (U.S.A)

Restriction endonucleases with Reaction buffers

T4 DNA ligase, 6 U/µl

2x Laemmli Sample Buffer

2x Native Sample Buffer

Sodium-dodecyl-sulfate (SDS)

Biomed, Maracay-Venezuela

tRNAs: Tyr, Phe, Asp (enriched fraction), $tRNA^{bulk}$ minus $tRNA^{Tyr}$

Biorad, Richmond (U.S.A)

Ammoniumperoxidisulphate

SDS (sodium-dodecyl-sulfate)

10x Tris/Glycine/SDS buffer

10x Tris/Glycine buffer

Ni-NTA HRP conjugate

Boehringer, Mannheim (now Roche Pharmaceuticals)

Adenosine-5'-triphosphate

Alkaline phosphatase (CIP, calf intestine phosphatase) 20 U/µl

Ampicillin

dNTPs

Guanosine-5'-triphosphate

Lysozyme

T4 Polynucleotide kinase

Phospho*enol*pyruvate

Puryvate kinase 10 mg/ml

T4 DNA polymerase, 1U/µl

$tRNA^{bulk}$ (from *E. coli*)

Calbiochem, Frankfurt

HEPES

Difco, Detroit (U.S.A)

Bacto agar

Bacto tryptone

Yeast extract

Fermentas:

2x Loading Dye Solution for RNA electrophoresis

6x Loading Dye Solution

GeneRuler™ 100bp DNA Ladder RNA ladder

GeneRuler™ 1kb DNA Ladder

RNA Ladder, Low Range

T4 DNA Ligase

MuMLV reverse transcriptase

Fluka, Neu-Ulm

Spermidine

Spermine

Fuji, Tokio (Japan)

Medical X-ray films

IBA:

Strep-tag® Starter Kit (2-1101-000)

Invitrogen:

Agarose (Ultrapure)

Urea (Ultrapure)

Sucrose (Ultrapure)

TEMED

Jackson ImmunoResearch, USA

Rabbit Anti-Sheep HRP conjugate

Kodak Eastman, Rochester (U.S.A)

X-ray films XAR-5

Xylencyanol

Merck, Darmstadt

All chemicals used in the laboratory and not extra listed here, and all essential amino acids

Bromophenol blue

Ethidium bromide (1%)

Urea

Tris

Xylene cyanol

Packard, Frankfurt

Filter Count (scintillation liquid)

Pharmacia, Uppsala (Sweden)

BSA (DNase and RNase free, 2.6 mg/ml)

Long polyuridine [poly(U)]

Sephadex

Sephacryl S-300 Spun Column

Sephacryl S-400 HR

Pineda Antikörper Service, Berlin

Rabbit polyclonal serum anti LepA (*E. coli*)

Promega Biotech, Madison WI (U.S.A.)

RNAsin

TNT Quick Coupled Translation/Transcription system

Qiagen, Düsseldorf

Qiagen Maxi Prep Tip 500

Qiagen Midi Prep Tip 100

Qiagen Mini Prep

Qiaquick PCR Purification kit

Quiquick Nucleotide Removal kit

Ni-NTA agar

RIKEN, Japan

Rabbit polyclonal serum anti EF-P *(E. coli*)

Roche, Germany

Adenosine-5'-triphosphate

Alkaline phosphatase (CIP, calf intestine phosphatase) 20 U/µl

Ampicillin

dNTPs

Guanosine-5'-triphosphate

Lysozyme

T4 Polynucleotide kinase

Poly-uridine [poly(U)]

Phospho*enol*pyruvic acid

Pyruvate kinase 10 mg/ml

RTS 100 E. coli HY Kit

RTS 500 E. coli HY Kit

RTS ProteoMaster Instrument

T4 DNA polymerase, 1U/µl

$tRNA^{bulk}$ (from *E. coli*)

Roth, Karlsruhe

1,4-Dithioerythritol (DTE)
Ampicillin

IPTG 25g

Phenol

Roti-Mark STANDARD 1ml

Rotiphorese® Gel 30 (37,5:1)

Rotiphorese® Gel 40 (19:1)

Trichloroacetic acid (TCA)

Santa Cruz Biotechnology, USA

Goat anti rabbit HRP conjugate

Sartorius, Göttingen

Nitrocellulose filters (Nr. 11306)

Schleicher and Schüll, Dassel

Selecta glass filter (Nr. 6)

Serva, Heidelberg

Acrylamide

Alcoa A-305 Aluminium oxide

Bis-acrylamide

Sigma-Aldrich, USA

NTPs-Tris

$tRNA_f^{Met}$

Ponceau S

5-Bromo-4-chloro-3-indolyl phosphate (BCIP)

Nitroblue tetrazolium (NBT)

Spectrum, Los Angeles (U.S.A)

Spectrapor dialysis membrane (MW 3500)

Whatman Ltd., England

Paper Filters

Non-typical laboratory machines

Wallac 1409 Liquid Scintillation Counter

RTS ProteoMaster Roche

Bacterial strains of *E. coli*

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, which is deficient in RNases BN, II, D (Zaniewski et al., 1984)

K12: F´proA+B+ lacIq Δ(lacZ)M15 zzf::Tn10(TetR)/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5

Plasmids

Rapid Translation System RTS pIVEX His-tag, 2nd Generation Vector Set (Roche, 3 269 019)

Luciferase T7 Control DNA(pProme-Lucy)

2.2 Buffers

2.2.1 Buffers and Electrophoresis solutions

Acrylamide and staining solutions have to be filtered before use.

44.4% AA/BAA (150:1)	Acrylamide	177.6 g
(stored at 4°C in dark bottles)	Bis-acrylamide	1.184 g
	Milli-Q-water (MQ-H_2O)	233.7 ml
44.4% AA/BAA (49:1)	Acrylamide	177.6 g
(stored at 4°C in dark bottles)	Bis-acrylamide	3.624 g
	MQ-H_2O	233.7 ml
40% AA/BAA (19/1) gel solution	Acrylamide	38% w/v
stock solution for RNA gel	Bisacrylamide	2% w/v
Agarose gel solution	10X TBE	5 ml
	Agarose	0.8-2% w/v
	Ethidium Bromide (1%)	1.5 µl
	MQ-H_2O	ad 50 ml

10% Sequencing gel	40% Acrylamide	20 ml
	Urea	36 g
	10X TBE buffer	8 ml
	MQ-H_2O	ad 80 ml
APS solution 10%	Ammonium*peroyx*disul-phate	10% w/v
Coomassie blue staining solution	Coomassie blue R-250	0.25% w/v
	Methanol	50% w/v
	Glacial Acetic acid	10% w/v
Destaining solution for Coomassiegels	Methanol	50% v/v
	Glacial acetic acid	10% v/v
Agarose gel sample buffer (5X) (for DNA)	EDTA	50 mM
	Ficoll 400	10%
	Bromophenol blue	0.25% w/v
	Xylene cyanol	0.25% w/v
Ethidium bromide staining solution	Ethidium bromide 1%	30 µl
	MQ-H_2O	300 ml
Agarose gel sample buffer (5X) (for RNA)	EDTA	10 mM
	Sucrose	60% w/v
	Bromophenol blue	0.1% w/v
	Xylene cyanol	0.1% w/v
RNA denaturing sample buffer (for AA gel electrophoresis with Urea)	Tris-HCl (pH 8)	10 mM
	EDTA	1 mM
	Urea	7.5 mM
	Bromophenolblue	0.05% w/v
	Xylene cyanol	0.05% w/v
SDS-Page protein sample buffer (for protein)	SDS	2% w/v
	Tris-HCl (pH 6.8)	90 mM
	Glycine	10% w/v
	ß-Mercaptoethanol	29 mM
	Bromophenolblue	0.1% w/v
TAE (10X)	Tris	48.4 g
	Glacial acetic acid	11.4 ml

	EDTA	3.7 g
	MQ-H_2O	ad 1000 ml
TBE (10X)	Tris	108 g
	Boric acid	55 g
	EDTA	7.4 g
	MQ-H_2O	ad 1000 ml
SDS-PAGE separation buffer	Tris	90.86 g
(pH 8.8)	SDS	1 g
	MQ-H_2O	ad 250 ml
SDS-PAGE stacking buffer	Tris	6.1 g
(pH 6.8)	SDS	0. 4 g
	MQ-H_2O	ad 100 ml
Transfer Buffer pH 8.3	Tris	25 mM
(Western blot)	Glycerol	190 mM
	Methanol	20% w/v
Tris-Glycine buffer 4x Running Buffer	Tris	100 mM
(Protein SDS-Page) pH 8.3	Glycerol	760 mM
	SDS	0.8% w/v
SDS-Separating gel for proteins	1.5 M Tris-HCl, pH	7.5 ml
(15% acrylamide)	8.844.4% Acrylamide 150:1	10.5 ml
	10% SDS	0.3 ml
	10% ammonium persulfate	0.2 ml
	TEMED	20 µl
	MQ-H_2O	11.18 ml
Stacking gel (10% acrylamide)	1.5 M Tris-HCl, pH 8.8	1.25 ml
	44.4% Acrylamide 49:1	2.25 ml
	10% SDS	0.1ml
	10% ammonium persulfate	0.1 ml
	TEMED	10 µl
	MQ-H_2O	6.29 ml
Toluidine blue staining solution	Glacial acetic acid	10% w/v
	Toluidine blue	0.1% w/v
Ponceau S stain	Ponceau S	0.01% w/v

	Glacial acetic acid	5% w/v
NBT solution	NBT	0.5 g
	70% dimethylformamide	10 ml
BCIP solution	BCIP	0.5 g
	100% dimethylformamide	10 ml
Alkaline phosphatase buffer, pH 9.5	Tris-HCl	100 mM
	NaCl	100 mM
	$MgCl_2$	5 mM

2.2.2 Buffers for microbiological and molecular methods

P1 (resuspension buffer for plasmid preparation)	Tris-HCl pH 8	50 mM
	EDTA	10 mM
P2 (Cell-lysis buffer for plasmid preparation)	NaOH	200 mM
	SDS	1% w/v
P3 (Neutralisation buffer for plasmid preparation)	Potassium acetate, pH 5.5	3 M
QBT (Equilibration buffer for Qiagen column)	MOPS-KOH pH 7.0	50 mM
	NaCl	1000 mM
	Ethanol	15% v/v
QC (Washing buffer for Qiagen column)	MOPS-KOH pH 7.0	50 mM
	NaCl	1250 mM
	Ethanol	15% v/v
QF (Elution buffer for Qiagen column)	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
10 x Klenow buffer	Tris-HCl, pH 7.6	500 mM
	$MgCl_2$	100 mM
	DTT	10 mM
	BSA (DNase free)	500 µg/ml
10 x Ligation buffer	Tris-HCl, pH 7.6	660 mM

	$MgCl_2$	50 mM
	DTT	50 mM
	Spermidine	5 mM
	BSA (DNase free)	500 µg/ml
10X Dephosphorylation buffer (for dephosphorylation of 5'phosphorylated ends of DNA or RNA)	Tris-HCl pH 7.6	500 mM
	$MgCl_2$	10 mM
	$ZnCl_2$	10 mM
	EDTA	5 mM
10X Phosphorylation buffer (for labeling of 5'-hydroxyl termini of DNA and RNA with [γ-^{32}P]-ATP)	Tris-HCl	500 mM
	$MgCl_2$	100 mM
	β-Mercaptoethanol	60 mM
	EDTA	10 mM
10X Transcription 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
	DTE	1 mM
	SDS	1% w/v
	NaCl	100 mM
Carrier solution for RNA precipitation	$tRNA^{bulk}$	1 mg/ml
	EDTA	50 mM
	$Na_2P_2O_7$	50 mM
LB Medium (Supplemented with 100 µg/ml ofampicillin)	Bacto-Tryptone	10 g
	Yeast-Extract	5 g
	NaCl	10 g
	NaOH (1 M)	1 ml
	dest. water	ad 1 L
LB-Agar plates (Supplemented with 100 µg/ml of ampicillin)	Bacto-Tryptone	10 g
	Yeast-Extract	5 g
	NaCl	10 g
	NaOH (1 M)	1 ml
	Agar	15% w/v

	destil. water	ad 1 L
Glycerol storage solution	Tris-HCl, pH 8.0	25 mM
	$MgSO_4$	100 mM
	Glycerol	66% v/v

2.2.3 Buffers for the functional studies and ribosome preparation

Dissociation buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_1N_{200}SH_4$)	MgAc	1 mM
	NH_4Ac	200 mM
	ß-Mercaptoethanol	4 mM
Elongation factor buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_6K_{150}DTE_1GDP_{0.01}$ $Gly_{10\%}$)	MgAc	6 mM
	KCl	150 mM
	Dithioerythriol	1 mM
	GDP	0.01 mM
	Glycerol	10% v/v
High Salt Wash Buffer	Hepes-KOH, pH 7.5	10 mM
($H_{10}N_{1000}SH_4$)	NH_4Ac	1000 mM
For the crude initiation factors preparation	ß-Mercaptoethanol	4 mM
Crude Initiation Factor Buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_6N_{150}SH_4Gly_{10}$)	MgAc	6 mM
Storage buffer for tRNA-free crude initiation factors	NH_4Ac	150 mM
	ß-Mercaptoethanol	4 mM
	Glycerol	10%
Binding buffer (standard buffer condition: $H_{20}M_{4.5}N_{150}SH_4Spd_2Spm_{0.05}$)	Hepes-KOH, pH 7.5	20 mM
	MgAc	4.5 mM
	NH_4Ac	150 mM
	ß-Mercaptoethanol	4 mM
	Spermidine	2 mM
	Spermine	0.05 mM

Tico-Buffer ($H_{20}M_6N_{30}SH_4$)	Hepes-KOH, pH 7.5	20 mM
	MgAc	6 mM
	NH_4Ac	30 mM
	ß-Mercaptoethanol	4 mM
HM_6K Buffer	Hepes-KOH, pH 7.5	20 mM
	MgAc	6 mM
	KCl	150 mM
	ß-Mercaptoethanol	4 mM
Mix I $H_{60}M_{10.5}N_{690}SH_{12}Spd_{10}Spm_{0.25}$ (Watanabe Ion mix I for tRNA enzymatic and non-enzymatic A site binding)	Hepes-KOH, pH 7.5	60 mM
	MgAc	10.5 mM
	NH_4Ac	690 mM
	ß-Mercaptoethanol	12 mM
	Spermidine	10 mM
	Spermine	0.25 mM
Mix II $H_{100}M_{22.5}N_{750}SH_{20}Spd_{10}Spm_{0.25}$ (Watanabe Ion mix II for tRNA non-enzymatic A site binding)	Hepes-KOH, pH 7.5	100 mM
	MgAc	22.5 mM
	NH_4Ac	750 mM
	ß-Mercaptoethanol	20 mM
	Spermidine	10 mM
	Spermine	0.25 mM
Mix IIe $H_{40}M_{8.3}N_{300}SH_8Spd_5Spm_{0.125}$ (Watanabe ion mix II for enzymatic A site binding)	Hepes-KOH, pH 7.5	40 mM
	MgAc	8.3 mM
	NH_4Ac	300 mM
	ß-Mercaptoethanol	8 mM
	Spermidine	5 mM
	Spermine	0.125 mM
Mix III $H_{66.7}M_{12.6}N_{500}SH_{13.4}Spd_{9.96}Spm_{0.26}$ (Watanabe ion mix III for tRNA A site binding, enzymatic and non-enzymatic)	Hepes-KOH, pH 7.5	66.7 mM
	MgAc	12.6 mM
	NH_4Ac	500 mM
	ß-Mercaptoethanol	13.4 mM
	Spermidine	9.96 mM
	Spermine	0.26 mM

Mix I $H_{100}M_{21}N_{870}SH_{20}Spd_{12}Spm_{0,3}$ (For poly(U) dependent poly(Phe) synthesis assay)	Hepes-KOH pH 7,5 MgAc NH_4Ac ß-Mercaptoethanol Spermidine Spermine	100 mM 21 mM 870 mM 20 mM 12 mM 0,3 mM
Mix II/Charging Mix $H_{80}M_{15}N_{840}SH_{16}Spd_{12}Spm_{0.3}$ (For poly(U) dependent poly(Phe) synthesis assay)	Hepes-KOH pH 7.5 MgAc NH_4Ac ß-Mercaptoethanol Spermidine Spermine	80 mM 15 mM 840 mM 16 mM 12 mM 0.3 mM
Mix E $ATP_{45}GTP_{22.5}(AcPO_4)_{75}$ Energetic mix/charging mix for poly(U) dependent poly(Phe) synthesis	ATP GTP Acetyl phosphate KOH	45 mM 22.5 mM 75 mM 360 mM
10X Buffer for RNase assay (Binding Buffer 10X)	Hepes-KOH pH 7.5 MgAc NH_4Ac ß-Mercaptoethanol Spermidine Spermine	200 mM 45 mM 1500 mM 40 mM 20 mM 0.5 mM
Re-association Buffer $H_{20}M_{20}K_{30}SH_4$ (For the 70S re-associated ribosomes preparation)	Hepes-KOH pH 7.5 MgAc KCl ß-Mercaptoethanol	20 mM 20 mM 30 mM 4 mM
X% Sucrose in different buffer	Sucrose binding buffer	X g till 100 ml
H-Mix (10% final reaction) for GTPase assay	Hepes-KOH pH 7.5 MgAc NH_4Ac ß-Mercaptoethanol	800 mM 9 mM 132 mM 1.6 mM

	Spermidine	20 mM
	Spermine	0.05 mM
Stop solution for GTPase assay	2-Butanol (H_2O sat.)	75%
	H_2SO_4	0.5M
	NaH_2PO_4	1.5mM
	$MoNa_aO_4 \bullet 2H_2O$	200mM

2.3 Analytical methods

2.3.1 Determination of ribosome and nucleic acid concentrations

The concentration of 70S ribosome, 30S and 50S ribosomal subunits was determined measuring the absorption at 260 nm. The following molar extinction coefficients were used:

70S	4.2×10^7 $M^{-1} \times cm^{-1}$	1 A_{260} unit = 24 pmol
50S	2.8×10^7 $M^{-1} \times cm^{-1}$	1 A_{260} unit = 36 pmol
30S	1.4×10^7 $M^{-1} \times cm^{-1}$	1 A_{260} unit = 72 pmol

The molar extinction coefficient of the synthetic RNA obtained via *in vitro* transcription was estimated as function of the base composition of the products. This estimation was compared with the values obtained by the Gene-Runner software. In both cases the results were in good agreement.

Base	**Molar Extinction Coefficient**
A	1.5×10^4 $M^{-1} \times cm^{-1}$
G	1.2×10^4 $M^{-1} \times cm^{-1}$
U	1.0×10^4 $M^{-1} \times cm^{-1}$
C	0.8×10^4 $M^{-1} \times cm^{-1}$

The extinction coefficient was the result of the sum of all coefficients corresponding to each base (Wallace and Miyada, 1987) minus 10 % of the total in order to account for the hypochromicity effect.

2.3.2 Conversion factors for the quantification of DNA and RNA

1 bp in double stranded DNA	660 g/mol

1 A_{260} unit of double stranded DNA	50 µg
1 A_{260} unit single stranded DNA or RNA (more than 100 bases)	40 µg
1 A_{260} unit of single stranded DNA (less than 25 bases)	20 µg
1 A_{260} unit of single stranded DNA (30-80 bases)	30 µg
A_{260}/A_{280} ratio for pure DNA	1.8
A_{260}/A_{280} ratio for pure RNA	2.0
1 triplet (codon) of RNA	~1000 g/mole

2.3.3 Radioactivity measurements

The radioactivity measurements were performed using a liquid scintillation counter Wallac model from LKB.

The samples were treated before measurement according to their physical nature and isotope content.

Liquid samples (1 ml or less) were delivered to scintillation vials (plastic, 20 ml maximal volume), and then 5 ml of Ready Value (Beckman) was added. The vials were tightly closed and their content mixed by quick and vigorous shaking (10 sec); after that the samples were immediately counted.

Nitrocellulose filters (containing single or multiple label) were put in scintillation vials, then 5 ml of Filter Count were added. The vials were tightly closed and then shaken for 5-10 h in order to dissolve the filter before counting. When [^{3}H] was present, the time of shaking had to be increased for 15 h in order to obtain reliable counts. With ^{32}P the minimum time was sufficient.

Glass filters with double ([^{14}C]/[^{3}H]) or triple labels ([^{14}C]/[^{3}H]]/[^{32}P]) were dried with diethyl ether/ethanol (1:1), then placed in the scintillation vials and counted with 5 ml of Filter Count after complete dissolution of the filter. Glass filters standards were prepared with defined amounts directly applied onto the filters in all possible combinations of isotopes used in the corresponding experiment. In that way the efficiency of isotope separation of the used programs could be controlled.

2.3.4 Cold TCA precipitation for the quantitative determination of aminoacylated tRNA

A 10 µl sample (normally two per assay) was delivered into a glass test-tube (1 x 10 cm) containing 20 µl of precipitation carrier solution ($tRNA^{bulk}$ 5 mg/ml). Two ml of ice cold TCA (10% w/v) was immediately added and mixed for 1-2 seconds. The aminoacylated tRNA was precipitated at 0 °C (ice bath) for 25 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 radioactivity adsorbed on the filter was then measured.

2.3.5 Polyuridin dependent Polyphenylalanin synthesis

In vitro protein biosynthesis is a standard experiment to examine the operability of the individual components of the system such as ribosomes, tRNA or S-100 enzymes for their operability. The reaction was accomplished under standard buffer finds in 100 µl with 24 pmol ribosomes or subunits.

The reaction mixture was incubated for 30 min with 37 °C. After addition of a drop of 1% w/v albumin solution the polyphenylalanin was precipitated by 2 ml 5% TCA. It was incubated for 15 min with 90 °C, so that the loaded [^{14}C]Phe-tRNA should be hydrolyzed and preceded as cold TCA precipitation. The radioactivity remaining on the filter amounted to in a successful experiment about 8000-10000 dpm.

2.3.6 Agarose gel electrophoresis of DNA and RNA

This technique was used for analysis of plasmid DNA after analytical and preparative isolation, as well as for restriction analysis and for the qualitative evaluation of rRNAs from 70S ribosomes, 50S and 30S ribosomal subunits.

The samples were prepared according to the type and the expected size of the nucleic acids to be separated. Plasmid DNA samples were prepared in a total volume of 6-10 µl in 5X agarose gel sample buffer including SDS (2% final concentration), in cases where the preparation was not highly purified (e.g. miniprep DNA). The amount of DNA was 0.2-1 µg per lane if intact plasmid preparations were analyzed, or 1.5-2 µg if fragments of 600 bp or less were expected. For rRNA analysis, 0.05 A_{260} units of the

corresponding ribosomal particles were incubated 2 min at 65 °C in 5X agarose gel sample buffer containing SDS (2% final concentration) and kept 10 min at room temperature before loading onto the gel.

The percentage of agarose used depended on the expected size of the nucleic acid to be separated: 0.8% for rRNA and 3 kb plasmids, and 1.5-2% for restriction fragments of 600 bp or less. The buffer systems used were TBE (for minipreps and restriction analysis) or TAE (for rRNA).

The agarose solution was heated in a microwave oven for not more than one min. After the solution was cooled to room temperature, 1.5 µl of ethidium bromide was added and the mixture was poured into the gel chambers:

a) Mini-gels: 9 x7 x 0.7 cm (total volume = 50 ml) with 14 or 28 wells (10 µl maximal sample capacity). Used mainly in routine checking of plasmid isolation, minipreps and restriction analysis. Run at 50-60 V.

b) Regular gels: 14 x 8 x 0.7 cm (total gel volume = 80 ml) with 14 or 28 sample wells (20 µl maximal sample capacity). Used in the analysis of small DNA fragments and rRNA. Run at 60-100 V.

The length of the nucleic acid molecules was estimated according to the migration of standards of known molecular weights.

2.3.7 Specific activity determination of labelled [^{32}P]-tRNA

Sometimes the dilution of the labeled tRNA with cold material can produce systematic errors that escape our attention. In order to determine the real specific activity of the labeled tRNA, the following strategy was applied. Since the tRNA is labeled at the 5' end, the 3' CCA end is free to be charged with its cognate amino acid. If the specific activity of the charging amino acid is known precisely, then the pmol of tRNA charged should correspond to the pmol of [^{32}P]-tRNA labeled. Thus, the ratio of the real pmol calculated for the charging tRNA versus the pmol estimated for the ^{32}P counts gives us the real specific activity of the labeled tRNA.

Under normal charging conditions described in the section 2.5.2.1, about 400 pmol of [^{32}P]-tRNA were charged with the appropriate radioactive amino acid. After 15 min of incubation at 37 °C, phenol and chloroform: isoamylalcohol extraction was performed

and the final aqueous phase recovered was subjected to a reversed phase HPLC run using a Nucleosil column 300-5 C8. A binary lineal gradient was applied at 0.5 ml/min and fraction size of 1 ml were collected. Aliquots of 200 µl of each fraction were withdrawn and counted in the respective isotope protocol (usually [^{32}P] and [^{14}C]). The A_{260} nm absorption profile elution coincided all time with counts for the isotopes used, demonstrating that tRNA ^{32}P-labeled was charged as well.

2.3.8 Western blot of LepA distribution in S30 fraction and membrane fraction

Immuno-analysis of the LepA protein location in *E. coli* K12 MG 1655 was performed in order to detect the distribution of LepA *in vivo*. For that purpose the following protocol was applied. Polyacrylamide gels were cast and run using the BioRad Mini-Protean II™ system. The polyacrylamide gel system used a 44.4% acrylamide solution consisting of acrylamide: bis-acrylamide at ratios of 150:1 and 49:1 in the separating and stacking layer, respectively. Generally, the final concentration of the acrylamide was 5% in the stacking layer and 8% in the separating layer. The overnight culture (in volume LB medium) was diluted 1:200 and grew until A_{600} ~0.6, harvested by centrifugation for 5 min at 4 °C/2000 *g*. The cells were resuspended in (volume) Qiagen cell resuspension buffer with 1mg/ml lysozyme and freeze-and-thaw 3 times. The S30 fraction and membrane fraction were separated by centrifugation for 30 min at 4 °C/18,000 *g*.

Samples of 10 µl of S30 fraction and 10 µl of membrane fraction were dissolved in protein loading buffer and heated at 95 °C for min before loading. Gels were run at 170 V for 2 h in 1xSDS-PAGE running buffer.

Control gels were soaked in Coomassie Blue R stain for >30 min, then left in a solution overnight with gently agitation. Gels were equilibrated in MQ-H_2O water for >2h before being dried between cellophane sheets in a perspex frame.

Proteins were transferred from polyacrylamide gels to nitrocellulose nitrate membranes using the BioRad Mini Tans-Blot™ system. A Tris-Glycine transfer buffer was kept cold using pre-frozen ice packs. Transfer proceeded at 100 V (300 mA) for 1h.

Transferred proteins were visualised by soaking the membrane in Ponceau S stain. BioRad broad range marker positions were marked with pencil. The stain was removed by rinsing in 1x TBS.

PVDF membranes were blocked overnight with 1xTBS, containing 1% (w/v) skim milk powder. Primary antibodies (anti-LepA) were hybridised for at least 1h in 1xTBS at RT with gently agitation. Primary antibodies were removed and kept for reuse. Filter were washed five times with 1xTBS for 5 min. Secondary antibodies (anti-sheep) were incubated with filters for 1h at RT. Excess secondary antibodies were removed by washing filter 3 times with 1xTBS.

HRP-conjugated secondary antibodies were detected by using ECL western blotting kit.

2.3.9 Western blot of EF-P distribution associated with ribosomes in S30 fraction

Over night EF-P over expression cell culture was diluted 1:100 and cultivated to A_{580} ~0.5. Repeat this step again and the second diluted culture just arrived A_{580} ~0.5. Immediately 20ml liquid N_2 was added and the flask was soaked into try ice/acetone solution and shaked violently for 30 seconds until the culture was almost frozen. The flask was soaked in H_2O/ice solution and the later steps must be performed at 4 °C. The culture was harvested and the S30 fraction was achieved as described above. $2A_{260}$ of S30 was diluted in 300µl standard buffer and loaded on SW40 15-45% sucrose gradient. The gradient was then collected and each fraction was checked by western blot also as described above.

2.3.10 Chemical probing and primer extension assay

Pi, PRE or POST (0.5µM) complexes were purified and LepA was added at a 5:1 ratio (protein to ribosome) with GTP or GDPNP 1:200 ratio (molar ratio nucleotide to factor). After the 37 °C incubation for 30min, 5 µL of the complexes were taken for nitrocellulose filter binding tests to determine the level of tRNA binding in the complex. The 90 µL of the 70S ribosome complexes were chemically modified by adding 1.9 µL of DMS (dimethyl sulfate) diluted (1:5) in ethanol (or only ethanol to the unmodified control)

and incubated for a further 10 minutes. The modification reaction was stopped by adding 25 µL of DMS stop buffer (1 M Tris-HCl [pH 7.5], 1 M ß-mercaptoethanol, 0.1 M EDTA) and 300 µL 95% ethanol. The rRNA was precipitated and resuspended in 200 µL TE/SDS (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% SDS, 5 mM EDTA) and phenol extracted three times (1 volume), followed by three chloroform extractions (1 volume). Finally, the rRNA was ethanol precipitated and resuspended in water to a final concentration of 0.1 µg/µL. Modification with 1-cyclohexyl-2-morpholino-carbodiimidemetho-p-toluensulfonat (CMCT) was initiated by the addition of 8.15 µl CMCT solution (84 mg/ml standard buffer) and was performed at 37 °C for 15 min. Pb(OAc)2 cleavage of 5 pmol ribosomal complexes was performed in 18 µl standard buffer for 5 min at 25 °C as described (Polacek et al., 2000). Primer extension products of modified rRNAs (Polacek and Barta, 1998) Primer extension analysis of the modified isolated rRNA was using 0.6 pmol of the appropriate primer, [^{32}P]-labelled at 5' end, was hybridized to approximately 1 pmol of rRNA, which served as the template for primer extension. The extension reaction was carried out using 0.4 units AMV reverse transcriptase (Roche) for 45 min at 42ºC in a buffer containing 122.5 mM Tris-HCl [pH 8.4], 11 mM $MgCl_2$, 15 mM KCl, 11 mM DTT, 250 µM dNTPs. For sequencing reactions, dideoxynucleotides were added to a concentration of 5 µM. were separated on 6% polyacrylamide gels and quantified using a Molecular Dynamics Storm PhosphorImager. I acknowledge the help of Dr. Norbert Polacek for these assays.

2.3.11 Toe-print assay

The mRNA MFKSIRYV was annealed to a ^{32}P-5'-end-labeled primer as described in (Hartz et al., 1988) and then used to program ribosomes for pre- (PRE) and post-translocation (POST) complexes. Briefly, 200 pmol re-associated 70S were incubated with 5 pmol mRNA:primer and 400 pmol of each tRNA $^{Met}_{f}$ and Ac-Phe-tRNA^{Phe} in standard buffer. Aliquots of the reaction mixture with 5 pmol 70S were withdrawn before and after EF-G dependent translocation reaction and used for toeprinting assays. The remaining post-translocational mixture of 275 µl was centrifuged through a 1ml 10% sucrose cushion in standard buffer (65,000xg for 18 h). The pellet was resuspended in 90 µl standard buffer and aliquotized in 15 µl portions.

7.5 pmol POST complexes in 15 μl were incubated for 30 min at 37 °C with 5 times excess of LepA and 200 times excess GDPNP or GTP and used for the toeprinting assay. The end-labeled primer on the mRNA was extended by 100 units of MuMLV reverse transcriptase (Fermentas) in the presence of dNTPs each 135 μM in standard buffer at 37 °C for 15 min. The reaction was stopped by 20 μl of loading buffer (9M Urea, 90mM TRIS, pH 8.3 at room temperature, 90 mM boric acid, 15 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and heated at 95 °C for 5 min. Toeprint reactions were analyzed on 8% urea-PAGE (8 M urea). The gels were quantified using a Molecular Dynamics Phosphor Imager.

2.3.12 GTPase assay

The reactions were set up as to maintain the standard buffer condition by making the reaction with 10% H-mix, 60% Tico and 30% H_2O. The ribosomes and protein were diluted in Tico buffer, which was considered in the final reaction. Generally the final ribosome concentration was 0.2μM and the final protein concentration was 0.02-0.2 μM. The premix contains the H-mix, 30% Tico, [^{32}P]γ-GTP (generally 50 μM with 50-1000 dpm/pmol), and H_2O. After the premix was prepared and distributed to the appropriate number of 1.5ml Eppendorf tubes (on ice) the reaction are made to the final volume by adding the remaining Tico, ribosomes and/or proteins. Generally the 70S or the protein or both were added immediately before starting the reaction. To start the reaction the reaction mixture is mixed well with the pipette and placed in a 37 °C water-bath. The reaction was incubated for the desired time and then the reaction stopped by pipetting 80% of the reaction into the stop solution and briefly vortexing. After all the reactions had been stopped they were vortexed 1min and then centrifuged (max) for 10 min. The hydrolyzed γP was extracted into the 2-butanol layer in a complex with the molybdate and separated from the aqueous layer during centrifugation. After centrifugation 150 μl of the 2-butanol layer (determine total volume of layer with pipette) was removed, and added to 5ml Ready value and counted via scintillation counting. The resulting counts have to be corrected for the volume of the organic layer counted and the extraction of 80% of the reaction volume. The pmols γP hydrolyzed can then be determined from the specific activity of the GTP solution.

2.4 Working with DNA

2.4.1 Preparation of E. coli competent cells for electroporation

The *Escherichia coli* strain CAN20-12E, deficient in several RNases (RNase BN^-, II^-, D^-, I^-), K12 derivative was used for preparation of competent cells.

One-liter culture of the *E. coli* strain of interest was grown to an A_{600} of 0.5-1.0. The cells were chilled on ice, and then pelleted by centrifuging, at 4000 rpm for 15 min at 4 °C in a GSA rotor, and resuspended in 1 volume of pre-chilled sterile MQ-H_2O. Bacterial cells were repeatedly centrifuged and washed in decreasing volumes of cold MQ-H_2O: two washes with 0.5 volume, one wash with 0.02 volume and final resuspension in 2-3 ml filter sterilised 10% (v/v) glycerol. Finally, the cell suspension was aliquotized in 40 µl portions, shock frozen in a dry ice-ethanol bath and stored at -80 °C.

The competence of the cells was assessed by transformation using the plasmid pSP65; using 10 ng of plasmid plus 40 µl of competent cells (processed according to the standard protocol using LB/ampicillin plates). The transformation efficiency was determined to be in the range of 0.5-1.5 x 10^6 transformants per µg of plasmid DNA. This level of competence was considered to be sufficient for our purpose.

2.4.2 Cloning strategies

All the DNA inserts were cloned between the 5'-3' ends of EcoRI and BamHI restriction sites, respectively. The T7 promoter was placed after the EcoRI restriction site.

The vector selected for the cloning purpose was the pSP65 or pSP64 (Promega). These vectors have the advantage of being high copy number plasmids, they do not contain a T7 promoter, and its restriction map allows the cloning between EcoRI and BamHI.

2.4.3 Restriction with EcoRI and BamHI

DNA samples were incubated with the desired restriction enzymes at a ratio of 1.5 units enzymes per µg of DNA for at least one hour. The temperature was the optimum

for each restriction enzyme. Double digestion was performed only if the two enzymes were active in the same buffer, otherwise a sequential digestion was performed.

The plasmid pSP65 or pSP64 (polyA tail) contain the β-lactamase gene (ampicillin resistance). The sequences for all mRNAs were cloned between the EcoRI and BamHI sites in front of the T7 promoter.

20 µg of pure pSP65 or pSP64 were incubated in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTE, with 30 units of EcoRI and 30 units of BamHI in a total volume of 400 µl. After three hours of incubation at 37 °C the reaction was stopped with the addition of 8 µl of 0.5 M EDTA, pH 8. The plasmid(s) cut was purified on 0.8% agarose gel using Qiagen gel extraction kit. The purity of the vector(s) was assayed on agarose gel 0.8%.

2.4.4 Digestion with alkaline phosphatase

In order to reduce the possibility that the vector closes again during the ligation step of the cloning procedure, plasmid prepared as mention before was dephosphorylated with calf intestine alkaline phosphatase (CIP).

In a total volume of 150 µl of CIP buffer (50 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 1 mM $ZnCl_2$), the cut plasmid was incubated at 37 °C for 15 minutes with 10 units of CIP. A second aliquot of CIP (10 units) was added and the incubation continued at 55 °C for 1 hour. At the end of the second incubation, the temperature was increased to 75 °C for 10 minutes in order to inactivate the phosphatase. The reaction mix was then extracted with phenol and the DNA was precipitated with ethanol. The recovered DNA was dissolved in 20 µl of buffer TE and stored at –20 °C.

2.4.5 Synthesis of dsDNA and ligation to a linearized plasmid

In order to obtain the dsDNA fragments containing the sequences to be cloned, the corresponding synthetic oligos were annealed and ‘’filled up’’ (Cobianchi and Wilson, 1987) using *E. coli* Klenow fragment of the DNA polymerase I (Klenow and Henningsen, 1970) in presence of dNTPs. The synthetic dsDNA was then ligated to a linear vector previously prepared.

2.4.6 Annealing and DNA filling reaction

250 pmol of a purified oligo were incubated 5 minutes at 75 °C with an equimolar amount of the corresponding complementary oligo in a total volume of 14.5 µl. The temperature was then slowly cooled down to 37 °C (in a period of 45 minutes). After the annealing incubation, the following components were added: 5.5 µl of dNTPs mix (dATP, dGTP, dCTP and dTTP, 10 mM each) and 2.5 µl of Klenow fragment enzyme (5 units/µl). The incubation continued for 45 minutes at 37 °C. After this incubation 75 µl of water was added and the mix was phenol extracted and the DNA was precipitated from the recovered aqueous phase with 3 volumes of ethanol.

2.4.7 Ligation to linearized plasmid.

In a final volume of 10 µl, about 100 ng of dephosphorylated plasmid, 60 ng of DNA insert and 3 U of T4 DNA ligase (6 U/µl) were incubated at 16 °C for 14 hours (final ionic conditions: $T_{50}M_{10}DTE_1ATP_1BSA_{0.025}$). In some opportunities the reaction mixture was supplemented with ATP 1 mM final concentration. When the incubation was finished, the ligation mix was immediately used for transformation of *E. coli* competent cells or stored at –20 °C.

2.4.8 Transformation

The electrocompetent cells (40 µl) were thawed on ice and mixed with no more than 2 µl of the ligation product (~100 ng of plasmid plus insert), then placed in an electrocuvette, and a PULSE of 25µF, 1.8 kV, 200 Ω was supplied by a Gene Pulser (Bio-Rad). The transformed cells were diluted with 1 ml of LB-medium and incubated for 1 h at 37 °C. Aliquots of 100-150 µl were plated on LB-agar plate with the appropriate antibiotic marker for selection. Additional LB plates were prepared with competent cells transformed with plasmid without DNA insert and without plasmid DNA in order to evaluate the ligation process and the competent state of the cells respectively. The selection of the clones containing the expected recombinant plasmids was done in two-step: first, inspection of the restriction pattern of plasmid DNA isolated from single bacterial colonies (miniprep procedure). Second, sequencing the plasmid DNA showing

a positive restriction pattern of the DNA insert using for that primers complementary to the plasmids pPS65 or pSP64.

2.4.9 Phenol/Chloroform extraction

The phenol/chloroform extraction was done in order to clean DNA or RNA from proteins. Nucleic acids solutions were mixed with 1 volume of re-distilled Phenol (70% in water). After 2 min vortex, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The aqueous phase (upper phase) was decanted carefully and extracted with 1 volume of chloroform:Iso-amylalcohol 24:1 (v/v). This suspension was shaken for 2 min and centrifuged at 13,000 rpm for 10 min at 4 °C. The upper phase was decanted and re-extracted once more with Chloroform: Isoamylalcohol. Both aqueous phases were combined and the DNA/RNA recovered (> 90%) was precipitated by ethanol.

2.4.10 Nucleic acid precipitation by ethanol or isopropanol

The DNA/RNA samples were precipitated by the addition of 2.5-3.0 volume of cold ethanol (100 %) or 0.7-1.0 volume of room temperature isopropanol. When the samples had a low salt content, 1/10 volume of 3M NaAc (pH 5.5) was added. This solution was mixed by inversion of the tubes and then incubated at -20 ° or -80 °C for a minimal time of two hours. In the case of isopropanol, the precipitation was done at room temperature for 10 min. After 30 min of centrifugation at 4 °C in an Eppendorf centrifuge, the nucleic acids were precipitated and then washed with 70 % cold ethanol. Finally, nucleic acids were dried at room temperature and dissolved in an appropriate volume of water or buffer (normally TE).

If the volume of nucleic acids to be precipitated was larger than 2 ml, Corex tubes of appropriate size were used, and the maximum RPM used in the centrifugation steps was never more than 7,000 for one hour.

2.4.11 Plasmid isolation (miniprep)

Small-scale preparations of plasmid DNA from 3 ml overnight cultures of bacteria were performed using QIAprep^{TM} spin miniprep.

Two ml of bacteria cultures (15-20 h) were centrifuged for 10 min at 5,000 rpm. The cells were sedimented and resuspended in 0.3 ml Buffer P1, then lysis was carried out for two min at room temperature by addition of 0.3 ml of Buffer P2. After neutralisation of the suspension plus the addition of 0.3 ml Buffer P3, the samples were mixed by inversion in order to precipitate the total proteins and the chromosomal DNA. This mixture was centrifuged for 20 min at 13,000 rpm (room temperature) and the DNA (supernatant) was decanted and precipitated with 0.7 ml of isopropanol (room temperature) and then centrifuged. The pellet washed with 70 % ethanol. Finally the plasmid recovered was diluted in 30-50 µl of buffer TE. The yield of plasmid obtained by this protocol was between 15-100 µg for overnight culture.

2.4.12 Plasmid preparation (maxi prep)

Large-scale isolations of plasmid DNA were prepared using Qiagen plasmid maxi kits. Flasks with 600 ml of LB medium were filled with 150 ml of starter culture. The bacterial cells were grown overnight and harvested in a Sorvall GSA rotor by centrifugation at 6,000 x g (6,000 rpm) for 15 min at 4 °C. The pellet was resuspended in 10 ml of Buffer P1. After the complete resuspension, 10 ml of Buffer P2 was added and the suspension mixed gently by inverting the tube 4-6 times and incubated at room temperature for 5 min. Genomic DNA, proteins and cell debris, were precipitated for 20 min on ice plus the addition of 10 ml of chilled Buffer P3. The white precipitate obtained was then centrifuged at 20,000 x g (12,000 rpm, rotor SS-34) for 30 min at 4 °C. The supernatant containing the plasmid DNA was removed and the sediment re-centrifuged one more time. Both supernatants were combined and applied to a Qiagen-tip 500 column previously equilibrated with 10 ml of Buffer QBT. The column was washed 2 x 30 ml of Buffer QC and the plasmid DNA eluted from the column with 15 ml of Buffer QF. The recovered DNA was precipitated with 10.5 ml of isopropanol (room temperature), and immediately centrifuged at 15,000 x g (11,000 rpm in a Sorvall SS-34 rotor) for 30 min at 4 °C. The DNA pellet was washed with 5 ml of room temperature 70 % ethanol and after centrifuging at 15,000 x g for 10 min at 4 °C, the DNA precipitated was air-dried and re-dissolved in a suitable volume of Buffer TE.

2.5 Working with RNA

2.5.1 Transcription

2.5.1.1 Run-off transcription with T7 polymerase

The synthesis of several mRNAs was performed using an *in vitro* transcription system with T7 RNA polymerase.

All the components indicated below except the T7 RNA polymerase were brought to room temperature prior to mixing in order to avoid precipitation of the DNA by spermidine and $MgCl_2$ (Milligan and Uhlenbeck, 1989). The order of component addition was also important because the enzymes and the RNase inhibitor could be inactivated if the ionic environment changes drastically. After the addition of polymerase, the reaction was incubated at 37 °C for 3-4 hours (preparative incubation) and stopped by adding 0.5 M EDTA, pH 8 (50 µl/ml).

Standard conditions are compiled in the following Table:

Order of addition	Component	Final concentration
1	Tris-HCl, pH 8 (37 °C)	40 mM
2	$MgCl_2$	22 mM
3	Spermidine	1 mM
4	NTP mix (ATP, GTP, UTP, CTP)*	3.75 mM
5	DTE	5 mM
6	BSA (RNase and DNase free)	100 µg/ml
7	RNase inhibitor (RNasin™)	1,000 units/ml
8	Inorganic pyrophosphatase (Ppase)	5 units/ml
9	Linearized plasmid template	20 pmol/ml
10	T7 RNA polymerase	40 µg/ml

*The components of the NTP mix were prepared as 100 mM stock solutions adjusted to pH 5.5-6.0 with 1 M KOH (in order to minimize the spontaneous hydrolysis) and stored at -80 °C before use.

2.5.1.2 PAGE purification of *in vitro* mRNA transcript

Gels of 14 cm x 16 cm glass plates were prepared for the mRNA purification. TBE buffer system containing 7.5 M urea (sequencing gel composition) was used. A special sample well 9 x 1 cm was made with an additional 2 mm thick spacer prepared for this purpose. The percentage of acrylamide used in every case was adjusted to the length of the RNA to be purified.

Length of RNA	% of acrylamide
15-36 mers	20%
36-50 mers	17%
50-80 mers	15%
100-150 mers	10%

Run-off i*n vitro* transcripts were dissolved in 200-300 µl of RNA denaturing sample buffer and incubated 2 minutes at 75 °C immediately before starting the run. The gel was pre-run at 400 Volts for 20 minutes. Prior to pour the samples into the gel, excess of urea diffusing from the gel to the wells was eliminated with electrophoresis buffer (1 X TBE).

The gel was run at 400 Volts until the xylene cyanol marker migrated 8-9 cm from the bottom of the well (the percentage of acrylamide used in the gel was chosen in order to ensure that the RNA with the expected length migrated above this marker). At this point the RNA band was localized by UV shadowing placing the gel on top of a fluorescent TLC plate covered by a clean and transparent plastic film and exposing it during a short time to UV light (240-280 nm). The portion of gel containing the RNA of the expected length was then excised with a sterile blade and crushed to small pieces by extruding it out with a sterile syringe barrel (5 ml) without needle. The disrupted gel was placed in a sterile 40 ml centrifuge tube (Sorvall) and RNA extraction buffer (6 ml) plus 6 ml of 70% phenol was added. The RNA was extracted for 12 hours with vigorous shaking at 4 °C. The RNA extracted was separated from the phenol phase by centrifugation at 10,000 rpm for 20 min at 4 °C in an HB-4 rotor (Sorvall). The aqueous phase was recovered and the phenol phase re-extracted two times more with 2 ml of RNA extraction buffer. The combined aqueous phases were treated once with chloroform: isoamylalcohol (24:1) and the RNA were precipitated with 2.5 volumes of

cold 100 % ethanol. After recovery of the precipitate by centrifugation (40 minutes at 10,000 rpm in a HB-4 rotor) and washing with 70% ethanol, the RNA was dissolved in water and stored at -80 °C is small aliquots.

2.5.1.3 Separation at the single nucleotide level (sequencing gel)

Maxam-Gilbert sequencing gels (30 x 40 x 0.04 cm) were used for separations at one nucleotide resolution. This type of gels is useful for DNA and RNA sequencing. It is used also for the analysis of radioactive species like *in vitro* transcripts, DNA or RNA oligonucleotides and the preparation of 5' [^{32}P]-phosphorylated-tRNA ((Gnirke and Nierhaus, 1989).

The percentage of acrylamide used for every analysis was selected according to the size of the species to be separated. The gel was pre-run for 60 min at 50 W before to load the sample. ^{32}P-tRNA labeled was diluted 1:1 with DNA denaturing sample buffer and incubated 2 min at 90 °C for denaturing (immediately before application). At the end of the pre-run, the sample wells were rinsed with electrophoresis buffer (TBE) in order to remove the urea diffused from the gel to facilitate the application of the sample. The running condition was set up to 50 W for 2-4 hours until the xylene-cyanol marker migrated 8-10 cm from the top of the gel. Once the run was finished, the gel was removed from the plates and placed onto an old film. The position of the labeled tRNA bands was detected by developing the gel (properly wrapped with saran-wrap) by autoradiography. The film developed was used to localise the tRNA band on the gel. Usually only two bands are obtained after developing the gel, the upper one (the longer molecule) corresponds to the expected size of the tRNA phosphorylated.

The region corresponding to the tRNA was carefully excised with a sterile blade and crushed into small pieces. The disrupted gel was placed in a 2 ml sterile Eppendorf and the tRNA was extracted for 12 hours with 2 volume of the RNA extraction buffer plus 1 volume of phenol saturated in H_2O under vigorous shaking at 4 °C. The RNA extracted was separated from the phenol phase by centrifugation at 13,500 rpm for 60 min at 4 °C and the phenolic phase was re-extracted once more with 2 volume of RNA extraction buffer. At the end, both aqueous phases were combined and chloroform: isoamylalcohol extraction was performed. The aqueous phase resulting of the latter extraction was immediately ethanol precipitated by shock-frozen in liquid nitrogen taking care the lid of

the Eppendorf tube was tightly closed. The pellet obtained, measured by the Geiger counter, was finally dissolved in the minimal volume of water and diluted until 3,000-10,000 dpm/pmol with the appropriate cold tRNA.

2.5.1.4 Gel filtration for the separation of RNA preparations from low molecular weight contaminants

The gel filtration technique was used for a quick separation of transcripts from low molecular weight contaminants (e.g. nucleotides, urea or SDS remaining from the gel electrophoresis purification). The NAP-25 column (from Pharmacia-LKB; packed with nucleic acid grade Sephadex G-25) was used for this purpose. Each column was used only once according to the recommended protocol from Pharmacia-LKB.

The column was equilibrated passing 15 ml of H_2O. The sample was then applied (20-30 A_{260} units of the desired RNA dissolved in 1-1.5 ml of H_2O or a suitable buffer) and adding 15 ml of H_2O developed the chromatography. Fractions of 1 ml were collected and the bulk of the RNA eluted (measured by the absorbance at 260 nm) was pooled and precipitated by ethanol.

2.5.1.5 List of messengers (mRNAs) used in this study

mRNA name	Sequence (5'-3') ***uuc(uuc)$_{11}$***: oligo-Phe **bold underlined:** Shine Dalgarno sequence ***bold italic*** : frameshifting window
MF	GGGAAAACAAAACAAAAC***AUGUUC***CAAAACAAAACAAAAC
MFStopC	GGGAAAACAAAACAAAAC***AUGUUCUGA***CAAAACAAAACAAAAC
MFKSIRYV	GGCAA**AGGAGGU**AUUAUUA***AUGUUCAAACGAUCAAUCUAC GUA*****UAA**UAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGGACA UCACAGAUUAACG.
MVF	GGGAAGAAAACACAT***ATGGTATTC***AAAGAAAAGAAAAGAAAA GAAAAGAAAATGGACTCAGAGCTACGGAAATATTCG
MVF+SD	GGGAAGAAAA**GGAGGT**CACAT***ATGGTATTC***AAAGAAAAGAA AAGAAAAGAAAAGAAAATGGACTCAGAGCTACGGAAATATTC G

2.5.2 tRNAs

2.5.2.1 Analytical tRNA aminoacylation

This type of assay was used for the estimation of the amino acid acceptor activity of tRNA samples, the discrimination of the charging activity from different S-100 preparations, as well as for the optimization of the aminoacylation conditions for different tRNAs.

The standard assay (10 µl) contained 50-100 pmol of tRNA; 3 to 10 molar excess of radioactive amino acid ([^{3}H] or [^{14}C]; 100-2,000 dpm/pmol), variable amounts of tRNA-free S-100 (typically between 1 to 3 µl) and ATP (3 mM final concentration), under standard binding conditions ($H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$). After 15 min incubation at 37 °C the amount of aa-tRNA synthesised was determined via cold TCA precipitation.

2.5.2.2 Analytical enzymatic de-aminoacylation of aminoacyl-tRNA

The optimization of an enzymatic de-aminoacylation assay was done under ionic conditions similar to those of the aminoacylation assays, but replacing the deacylated tRNA for aa-tRNA and the ATP for AMP and PP_i.

A 10 µl assay contained 10-50 pmol of aa-tRNA (5-10 µl per determination were normally used), 2-4 µl of tRNA free S-100 (fractions SII or SIII depending on which had the highest corresponding tRNA synthetase activity), 3 mM AMP and 3 mM PP_i (added from a 100 mM Na_4PP_i stock solution). The final ionic conditions were $H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$ (standard conditions). The PP_i was always added shortly before the enzyme preparation in order to reduce the risk of depletion of this component by precipitation (an insoluble complex with magnesium is readily formed), or by action of pyrophosphatases before the de-aminoacylation process takes place. At the end of the incubation time the amount of remaining aa-tRNA was determined via cold TCA precipitation.

The optimal time of incubation was determined in every case by kinetic analysis of the de-aminoacylation reaction.

2.5.2.3. Preparative tRNA aminoacylation and subsequent acetylation

The preparative aminoacylation of specific tRNAs with the cognate amino acid was achieved by the synthetase activity present in the S-100 preparation. 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 aminoacyl-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 labeled ([^{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). The final ionic conditions fixed for the tRNA charging was $H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}ATP_3$. Before the addition of the S-100 enzyme fraction, the pH was adjusted between 7.5 -8 units with 1 N KOH.

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-chloroform, 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 lyophilised (~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. The yield of the charged fraction varied according to the specific tRNA between 40% and 95% due to the different activities of the cognate synthetase and the stability of the resulting aminoacyl-tRNA (Hentzen et al., 1972). In general the less stable tRNAs give lower yields due to *a posteriori* spontaneous de-aminoacylation.

The preparation of aminoacyl-tRNA was normally submitted to a purification step using reversed-phase HPLC (section 2.5.2.5) before used in functional analysis or further processed in order to obtain N-acetyl-aminoacyl-tRNA.

Upon treatment with acetic anhydride, an aminoacyl-tRNA is converted to its N-acetyl-aminoacyl-tRNA derivative with high yield. The extent of reaction has been reported to be usually above 90%, which constitutes the major advantage of the method, but some additional acetylations in the bases of the tRNA body has been detected after alkaline hydrolysis of the acetylated product and paper electrophoresis analysis (Haenni and Chapeville, 1966). However, these additional modifications do not seem to affect significantly the biological properties of the product, since it has been shown that acetyl-aminoacyl-tRNAs obtained through treatment with an α-NH_2 group specific acetylating agent, the N-hydroxysuccinimide ester of acetic acid (Rappoport and Lapidot, 1974), were functionally equivalent to those obtained using acetic anhydride. In this work, the acetyl-aminoacyl-tRNAs were synthesized using the acetic anhydride method.

Since the acetylation reaction proceeds at low pH, the solution of aminoacyl-tRNA (25 A_{260} unit/ml) was acidified by addition of 1/10 of the volume of 3M NaAc, pH 5.0, and kept on ice during all the subsequent manipulations. Per one ml of this solution 50 µl of acetic anhydride were added, carefully mixed and incubated on ice for 15 min. The acetic anhydride addition was repeated three times more and after the last 15 min period the tRNA was precipitated by addition of 2.5 volumes of cold ethanol.

After recovery of the acetyl-aminoacyl-tRNA by ethanol precipitation and before the final purification by reversed-phase HPLC, the preparation was tested for the presence of remaining aminoacyl-tRNA by means of analytical enzymatic de-aminoacylation reaction (section 2.5.2.2). When the de-aminoacylation test revealed the presence of more than 2% of aminoacyl-tRNA, a preparative de-aminoacylation was performed before the acetylaminoacyl-tRNA was submitted to the last purification step.

2.5.2.4 Preparative de-aminoacylation of aminoacyl-tRNA remaining in the N-acetylaminoacyl-tRNA fraction

The aminoacyl-tRNA synthesis is a reversible reaction. In the presence of the corresponding synthetase and the products of an aminoacylation, pyrophosphatase and AMP, the aminoacyl-tRNA is fast and quantitatively split producing deacylated tRNA and free amino acid with concomitant formation of ATP (Berg et al., 1961). When the aminoacyl moiety attached to the tRNA is modified, as in the case of N-acetylaminoacyl-tRNA, the cognate synthetase does not recognise it as substrate and consequently does

not catalyses the reverse aminoacylation (Haenni and Chapeville, 1966). This discrimination power of the synthetases was used in the definition of a de-aminoacylation assay, which removes any significant amount of aminoacyl-tRNA present in the preparation of N-blocked aminoacyl-tRNA.

Between 10 and 30 A_{260} units of N-acetyl-aminoacyl-tRNA at a final concentration of 4-8 pmol/µl were incubated with the same relative amount of tRNA-free S-100 fraction used for the aminoacylation assay (preceding section). Instead of ATP and the cognate amino acid, AMP and pyrophosphate (Na_4PPi) were added to the reaction both at a final concentration of 6 mM. The time of incubation was the optimum determined by analytical assays using aminoacyl-tRNA as substrate. After the incubation, small aliquots were withdrawn and precipitated with 10% cold TCA. The ratio of the counts (or pmol) recovered after precipitation with respect to the initial total counts (or pmol) put to precipitate gives us the efficiency of the de-aminoacylation assay. The rest of the material is extracted with 75% phenol and precipitated with ethanol as indicated in the previous sections. The recovered material was stored at -80 °C before purification *via* HPLC chromatography.

2.5.2.5 Reversed-Phase HPLC purification of aminoacyl-tRNA and acetylaminoacyl-tRNA

The development of techniques like the Benzoyl-DEAE-cellulose chromatography (Gillam et al., 1967) and the reversed-phase chromatography (Kelmers et al., 1971) for the separation of tRNA mixtures was an important pre-requisite for the study of the structure and function of the tRNA molecule. These techniques were soon adapted for the separation of deacylated and aminoacylated forms of certain tRNAs (Walters and Novelli, 1971; Wimmer et al., 1968). On the other hand, 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 (Hartwick et al., 1979). Following this idea, Odom et. al., (Odom 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 Phe-tRNA as well as AcPhe-tRNA from *E. coli* and is described here for AcPhe-tRNA.

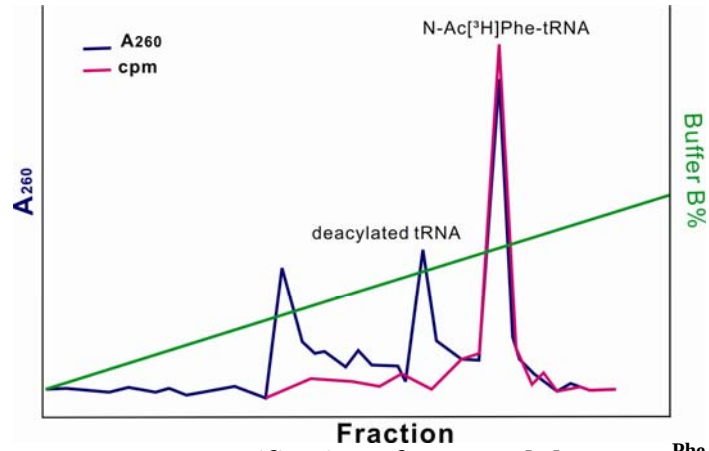

Figure 2.5.2.5 **Purification of N-AcetylPhe-tRNA^{Phe} from *E. coli* by reversed phase HPLC after de-aminoacylation**

An aliquot of 1-2 ml comprising 20-50 A_{260} units of Acetyl-Phe-tRNA^{Phe} (labeled with [^{3}H] or [^{14}C]), prepared as described in the previous section, was spun down for 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 (400 mM NaCl, 10 mM $MgAc_2$, 20 mM NH_4Ac, pH 5.0) 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 (60% Uvasol-methanol, 400 mM NaCl, 10 mM $MgAc_2$, 20 mM NH_4Ac, pH 5.0) 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 eluted first and the charged form eluted later (AcPhe-tRNA^{Phe}; Figure 2.5.2.5).

2.5.2.6 Preparation of N-formyl-methionyl-tRNA $^{Met}_{f}$ (E. coli)

The specific aminoacylation of tRNA $^{Met}_{f}$ from *E. coli* and the formylation of the resulting fMet-tRNA $^{Met}_{f}$ were performed enzymatically in a single incubation step in the presence of methionine and formyl donor. The formylase and the Met-tRNA synthetase enzymes both are present in the tRNA-free S 100.

2.5.2.6.1 Preparation of the formyl donor

The formyl donor, N^{10}-formyltetrahydrofolic acid, was prepared from commercial folinic acid (N^5-formyltetrahydrofolic acid) calcium salt: 12.5 mg of folinic acid were dissolved in 1 ml of 50 mM ß-mercaptoethanol, then 110 µl of 1 M HCl were added and the solution was incubated at room temperature (precipitated material could be re-dissolved by slight warming).

The incubation was stopped when the absorbance at 355 nm reached a maximum (this takes approximately three hours), indicating the conversion of N^5N^{10}-methenytetrahydrofolic acid. In this intermediate form the reagent could be stored at -80 °C before use.

The final form of the formyl donor, N^{10}-formyltetrahydrofolic acid, was obtained by neutralisation of the intermediate solution with the addition of 1/10 volume of 1 M Tris-HCl, pH 8.0, and 1/10 volume of 1 M KOH. The quantitative conversion is achieved after 15-30 min at room temperature. In this period the solution loses its slight cloudy and yellow colour.

2.5.2.6.2 Synthesis and purification of fMet-tRNA $^{Met}_{f}$

The following components were added to an aqueous solution containing 40 A_{260} units of deacylated tRNA $^{Met}_{f}$ (1550-1670 pmol/A_{260}): a 5-fold excess of radioactive methionine (labeled with [^{14}C], [^{3}H] or [^{35}S]), about 700-fold of formyl donor, and an optimised amount of tRNA-free S100 fraction (20-50 µl/A_{260} of tRNA $^{Met}_{f}$). The conditions of reaction were adjusted to $H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$, pH 7.5, 3 mM ATP. After 20 min incubation at 37 °C the reaction was stopped by addition of 1/10 of the volume in 3M

sodium acetate, pH 5.0, and a phenol extraction was performed followed by an ethanol precipitation.

The co-precipitated formyl donor and the residual free methionine were separated from the tRNA by means of a gel filtration step (section: 2.5.1.4) before the product was purified by reversed-phase-chromatography.

The formylation was in general quantitative and the efficiency of aminoacylation ranged between 80-90 %. The figure 2.5.2.6.2 shows the profile of the reversed phase HPLC purification of the fMet- $tRNA_f^{Met}$ preparation. The elution gradient was optimized in analytical scale. The column used for this preparation was Nucleosil 300-5 C_4 equilibrated with 400 mM NaCl, 10 mM $MgAc_2$, 20 mM NH_4Ac pH 5.0 (buffer A), at a flow rate of 0.5 ml/min. The elution was done with a gradient of buffer B (60% methanol in buffer A). The recovered fMet- $tRNA_f^{Met}$ (80%) was of high purity (1760 pmol/A_{260}).

2.5.2.7 Labelling of deacylated tRNA with γ-[^{32}P]-ATP

Deacylated tRNA was labeled by exchanging the 5' terminal phosphate group with [^{32}P]-phosphate (Chaconas and Sande, 1980), according to the method described by Gnirke et. al. (Gnirke et al., 1989) with some modifications. The exchange was achieved in a two-step procedure: 1) digestion with alkaline phosphatase in order to hydrolyze the 5' phosphate group, and 2) phosphorylation with γ-[^{32}P]-ATP in the presence of T4 polynucleotide kinase (Richardson, 1981). The tRNA was labeled at high specific activity and after the separation in a sequencing gel (section 2.5.1.3) the pure radioactive tRNA was diluted with cold tRNA to the desired specific activity.

2.5.2.7.1 Dephosphorylation of tRNA with alkaline phosphates

The standard dephosphorylation reaction contained 1 A_{260} unit of deacylated tRNA with 5 units of alkaline phosphatase (from calf intestine) in 100 µl total volume. The final ionic concentrations were 50 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 0.5 mM EDTA. The reaction mix was incubated for 45 min at 55 °C. The reaction was stopped by addition of 1/10 volume of 3M NaAc, pH 5.0. The mix was extracted with an equal volume of 75% phenol for 5 min followed by a 5 min re-extraction with chloroform-

isoamylalcohol (25:24:1). The tRNA was finally precipitated from the aqueous phase by addition of 2.5 volumes of ethanol. After 30 min at - 80 °C or 2 hours at -20 °C, the tRNA was recovered by centrifugation, washed with 75% ethanol and re-dissolved in 30 µl H_2O.

2.5.2.7.2 [5'] Phosphorylation with γ-[^{32}P]-ATP

An aliquot of 200 pmol of dephosphorylated tRNA was incubated with 20-50 µCi of γ-[^{32}P]-ATP in 40 µl total volume in the presence of 20 units of a T4 polynucleotide kinase. The final ionic concentration was fixed to 50 mM Tris-HCl, pH7.5, 10 mM $MgCl_2$, 1 mM EDTA, 6 mM ß-mercaptoethanol. After 60 min incubation at 37 °C the complete reaction mix was lyophilized and re-dissolved in 5 µl of RNA denaturing sample buffer. The sample was then denatured at 80°C for one min and applied to a 15% polyacrylamide-urea gel sequencing gel (Maxam and Gilbert, 1977). The electrophoresis was performed at 50 W until the xylene cyanol dye migrated 8-10 cm from the top (one nucleotide resolution). The gel was then transferred to a used film and a short time (1 min) autoradiography was done in order to localize the labeled product. The radioactive tRNA band was excised and extracted with a procedure analogous to that described in section 2.5.1.2.

The [^{32}P]-tRNA recovered was immediately diluted with pure cold tRNA of the same class to yield specific activities ranging from 3,000 to 10,000 dpm/pmol. At concentrations below 70 pmol/µl and specific activities below 10,000 dpm/pmol a radiolysis was not detectable even after 6 weeks storage at -80°C.

2.6 Preparative Methods

2.6.1 Large-scale cultures of Escherichia coli

Large scale cultures from *Escherichia coli* K12, CAN/20-12E (RNase I$^-$, RNase II$^-$, RNase D$^-$, RNase BN$^-$, RNase T$^-$, (Zaniewski et al., 1984) were performed in a Bioengineering Fermenter. Hundred liters of sterile L-medium, supplemented with sterile glucose solution (20%) up to 0.5%, were inoculated with 2.5 L of an overnight culture of the appropriate strain, and allowed to ferment at 37 °C. The bacterial growth was

followed by determination of the optical density at 560 nm. When the culture reached the early logarithmic phase (0.5 A_{560}/ml), the cells were harvested using a continuous flow centrifuge (Padberg, model 41) operating at 2,000 rpm for 20 minutes. The cells were shock frozen in liquid nitrogen and stored at -80 °C. The yield of the fermentation was of 1-1.3 gram of wet cells per liter of medium.

2.6.2 Isolation of 70S ribosomes from Escherichia coli

The isolation of ribosomes and the soluble enzyme fraction (S-100) was performed according to the procedure described in Rheinberger *et al.* with slight modifications (Rheinberger et al., 1988), using a near *in vivo* ionic condition ($H_{20}M_6N_{30}SH_4$: Tico buffer) that ensures to obtain ''tight-couple'' 70S ribosomes (Hapke and Noll, 1976). In a typical preparation ~300 grams of frozen *E. coli* cells were thawed while re-suspending in ~600 ml (double amount) of Tico buffer, and recovered by centrifugation at 8,000 rpm for 15 minutes in a Sorvall GSA rotor (4 °C). This mixture was transferred to a Retsch-Mill, and the cells were ground for about 40 minutes. After addition of Tico buffer (1.5 ml per gram of cell) the cells were further mixed for 10 minutes. The pellets of the cells were then weighted and dissolved in Tico buffer in proportion as 1 ml per gram. Such a suspension should easily go through the tip of 10 ml glass pipette. When this condition was reached, everything was applied to the Microfluidizer apparatus, which was washed and equilibrated with Tico buffer prior. Within this apparatus cells are pushed under high pressure (18 atm), through a tiny channel; which leads to their breakage. Then, collected suspension was centrifuged and resulting supernatant was transferred to the clean tubes and centrifuged again. The supernatant was centrifuged at 16,000 rpm (30,000 x g) for 45 minutes in a SA-600 rotor. The pellet (cell debris) was discarded and the supernatant (S-30) containing ribosomes and soluble enzymes was further centrifuged at 22,000 rpm (30,000 x g) for 17-20 hours in a 45 Ti rotor in order to sediment the 70S ribosomes. The pellet was resuspended in Tico buffer and again centrifuged in a SA-600 rotor at 8,000 rpm for 10 minutes in order to eliminate the non-dissolved aggregates. The ribosomes in suspension (crude 70S) were then shock-frozen in liquid nitrogen in aliquots containing 6,000-9,000 A_{260} units and stored at -80 °C. The supernatant was processed as the S-100 fraction.

The yield of crude 70S ranged between 300 and 400 A_{260} units per g of wet cells. A complete separation of tightly coupled 70S ribosomes from ribosomal subunits was achieved upon two consecutive zonal centrifugation steps of samples containing 5,000-9,000 A_{260} units of crude 70S preparation through a sucrose gradient (0-40% sucrose in Tico buffer). The gradient was pumped out the rotor using a solution containing 50% of sucrose in MQ. After the first zonal centrifugation (16 hours at 21,000 rpm in a Beckman Ti XV rotor) the fractions containing 70S were pooled and the ribosomes sedimented *via* a centrifugation step (24,000 rpm for 24 hours in a 45 Ti rotor). The sediment was resuspended in a small volume of Tico buffer and applied to a second zonal centrifugation under the same conditions. The resulting tightly coupled 70S ribosomes were essentially free from 50S subunits (main contaminant after the first zonal centrifugation). The 70S pellet was resuspended in Tico buffer, aliquotized in 50 µl portions, shock-frozen in liquid nitrogen and stored at -80 °C. The yield of tight couple 70S ribosomes ranged between 10 and 20% of the total A_{260} units initially applied to the zonal (Figure 2.6.2 A).

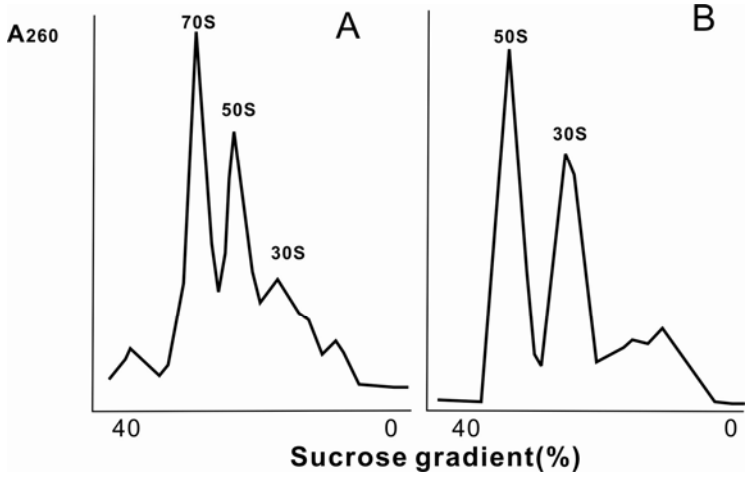

Figure 2.6.2 **Absorption profile of zonal gradient centrifugation of ribosomes.** (A) 70S tight coupled ribosomes ($H_{20}M_6N_{30}SH_4$) (B) 30S and 50S ribosomal subunits in dissociating conditions ($H_{20}M_1N_{200}SH_4$)

2.6.3 Preparative isolation of 30S and 50S subunits

The 30S and 50S ribosomal subunits were isolated by zonal centrifugation using a linear sucrose gradient from 0 to 40% in 20 mM Hepes, 1 mM $MgCl_2$, 200 mM NH_4Ac, 4 mM ß-mercaptoethanol (dissociating conditions). For each zonal centrifugation, 4,000-6,000 A_{260} of crude 70S ribosomes were used. The centrifugation was performed using a Beckman zonal rotor Ti15 (0-40% sucrose) at 22,000 rpm for 17 h at 4 °C. The gradient was pumped out the rotor using a solution containing 50% of sucrose in MQ.

After the zonal centrifugation two pools were made with fractions containing the 30S and 50S subunits, respectively. The 30S and 50S subunits were pelleted in 45 Ti rotors (Beckman) at 35,000 rpm, for 22 h at 4 °C. The pellets were resuspended in 3 ml of Tico buffer or re-associated buffer. In order to eliminate large aggregates, the resuspended material was once more centrifuged in a SS-34 rotor (Sorvall) at 7,000 rpm for 15 min at 4 °C. The concentrations of the 30S and 50S subunits were determined by absorbance at 260 nm and the suspension was divided in small aliquots, frozen in liquid nitrogen and stored at -80 °C. The typical yield starting from 5,000 A_{260} of 70S was 1,000 A_{260} and 1,200 A_{260} of pure 30S and 50S subunits, respectively (Figure 2.6.2 B).

2.6.4 Preparation of re-associated 70S

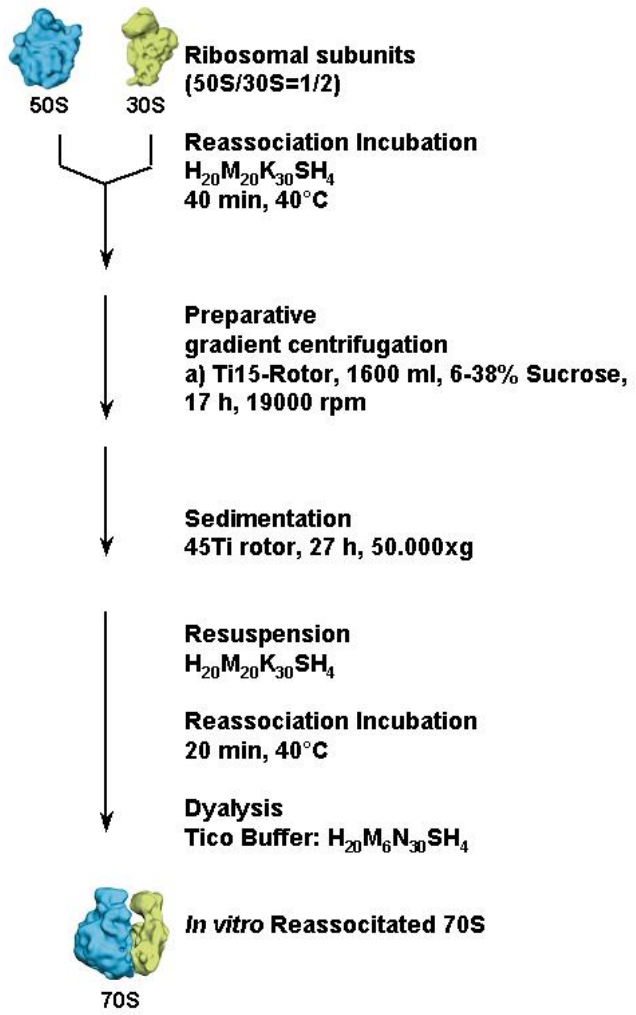

Figure 2.6.4 **Protocol for the re-associated 70S preparation**

The tight couple ribosomes are derived partially from polysomes and thus contain still some tRNAs and small fragments of mRNA. In order to get a very pure preparation of ribosomes free of these ligands, the ribosomal subunits purified by sucrose gradient were incubated in the presence of high Mg^{2+} concentrations to form re-associated 70S ribosomes (Figure 2.6.4; (Blaha et al., 2002)). Re-associated ribosomes are more efficient in both tRNA binding and in poly(U) dependent poly(Phe) synthesis, as compared to tightly coupled ribosomes.

The latter ones still contain mRNA fragments and tRNA (on average about 0.6 tRNA per 70S; (Remme et al., 1989)). If the subunits contain intact rRNA, 6000 A_{260} of purified 30S and 50S ribosomal subunits at molar ratio of 1:1 of A_{260} were diluted in re-association buffer until 40-140 A_{260}/ml and incubated for 60 min at 40 °C in a water bath with gently agitation. Adaptation buffer was prepared when ribosomal subunits were dissolved in Tico buffer. The final ionic concentration was adjusted to that of the re-association conditions ($H_{20}M_{20}K_{30}SH_4$ pH 7.5). It is important to note that excess of 30S subunits minimise the amount of free 50S subunit, thus it improves the separation of the re-associated 70S ribosomes from the 50S subunits in the following gradient centrifugation (Blaha et al., 2000).

After the first incubation before the samples were applied to the gradient centrifugation, a second incubation was performed for 10 min at 4 °C. Then the particles were subjected to a gradient centrifugation (0%-40% sucrose) in re-association buffer and centrifuged for 17 h at 18,000 rpm, 4 °C in a Beckman zonal rotor. The gradient was fractionated and the fractions corresponding to the 70S peak were pooled and centrifuged at 24,000 rpm for 24 h, 4 °C in a 45 Ti rotor in order to pellet the re-associated 70S ribosomes. The use of higher centrifugation rates is not recommended, because it may lead to pressure-induced dissociation of the ribosomes. The ribosomes were resuspended in re-association buffer ($H_{20}M_{20}K_{30}SH_4$, pH 7.5) and incubated once more for 20 min at 40 °C. This additional incubation improves the re-association process. The 70S re-associated ribosomes were clarified by low speed centrifugation and then dyalized against Tico buffer ($H_{20}M_6N_{30}SH_4$, pH 7.5): 100X volume/ 3X 45 min. The concentration of ribosomes was determined at 260 nm. Small aliquots were prepared, shock frozen in liquid nitrogen and stored at -80 °C (Figure 2.6.4). Due to the high concentration of the re-associated 70S was usually 400-700 A_{260}/ml so that a dialysis against Tico was not necessary.

2.6.5 Quality and functionality determination of the ribosomes preparation

The quality of the ribosome preparation can be tested using three assays: (1) A SW 40 run is performed (gradient 15-45% sucrose in standard buffer conditionor re-association buffer, 18 h, 18,000 rpm, 4 °C) in order to check the homogeneity of the

ribosomal particles (30S and 50S subunits) and the 70S ribosomes (tight couples or re-associated). (2) RNA gels are done in order to check the integrity of the ribosomal RNA. By means of this analysis degradation of the 16S and 23S rRNA can be detected. Both, the analytical sucrose gradient and the RNA gel analysis provide information about the structural integrity of the ribosomal particles. (3) The activity in the poly(U)-dependent poly(Phe) synthesis is the third criterion for estimating the activity of the ribosomes. The latter assay is described in section 2.7.1.1.

2.6.5.1 Analytical sucrose gradient centrifugation

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 (15-45% (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 (Beckman). In the SW 40 up to 10 A_{260} 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 (Figure 2.6.5.1).

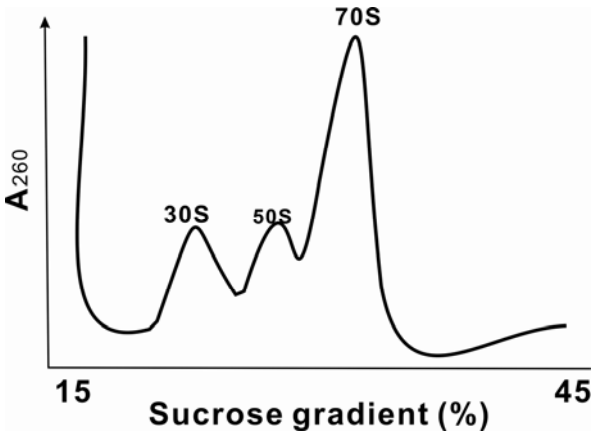

Figure 2.6.5.1 **Profile absorption of SW40 gradient centrifugation of re-associated 70S**

2.6.5.2 Integrity of rRNA: 1D tube gel analysis

The integrity of the rRNAs of both ribosomal subunits and 70S ribosomes (tight couple and re-associated) was determined by SDS polyacrylamide electrophoresis for rRNA. Each gel, a plastic tube (0.5 cm x 12 cm) was filled (10 cm) with 2 ml of SDS-3.1% AA/BAA gel solution, and the polymerization was started by the addition of 1 µl of TEMED and 40 µl of 10% APS. The total polymerization took 1-2 h at room temperature. On the top of the gel-solution and before completion of polymerization, 200 µl of water was added to ensure the formation of a flat surface. Per gel, 30 µl of 50S, 30S or 70S ribosomes diluted in Tico buffer (0.5-1 A_{260} units) were mixed with SDS (final concentration 1%) and incubated at 70 °C for 2 min. After cooling, 10 µl of marker solution (60% sucrose, 0.1% BPB) was added and the mixture was applied to the gel.

The tube gels were placed in the electrophoresis chamber and at the bottom of each tube a dialysis membrane was fixed in order to prevent gel sliding from the tube. TBE 1X plus SDS (up to a final concentration of 0.1 g/l) was used as running buffer. Before loading the samples, the gels were pre-run for 15 min at 1.5 mA/gel. After loading the samples, gels were run (anode at the bottom, 4 °C) for 2 h at 0.5 mA/gel and then for further 2 h but at 1.5 mA/gel (until the marker reached the bottom). Gels were scanned at 260 nm in a Gilford 250 photometer. By means of this method, not only the damage of the rRNA could be estimated, but also the degree of cross-contamination of the subunits (Figure 2.6.5.2).

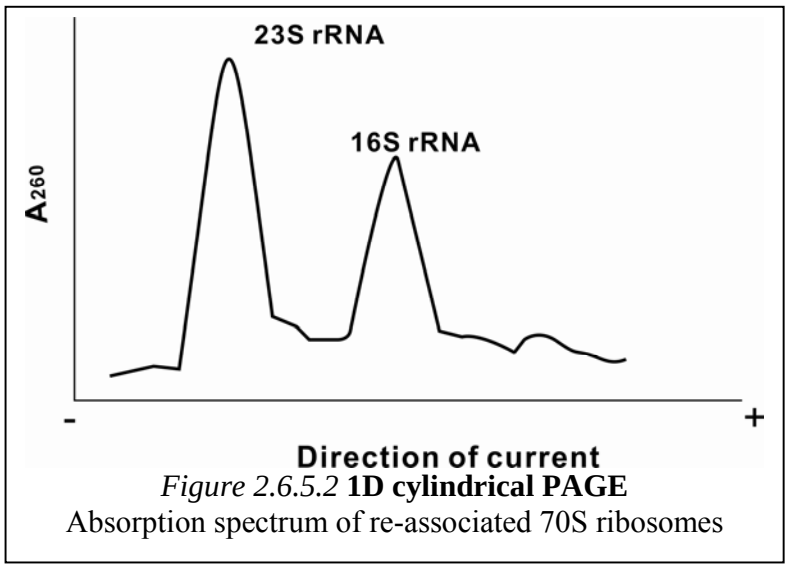

Figure 2.6.5.2 **1D cylindrical PAGE**
Absorption spectrum of re-associated 70S ribosomes

2.7 In vitro *systems*

2.7.1 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).

2.7.1.1 Poly(U)-dependent poly(Phe) synthesis

Under standard conditions (15 µl of reaction mix), the assay contained, $H_{20}M_{4.5}N_{150}SH_4Spd_2Sp_{0.05,}$ the binding reaction was done by incubating 25 µg of poly(U), 0.2 A_{260} of re-associated 70S ribosomes sometimes primed with a two molar excess of acetyl ([^{3}H] or [^{14}C])Phe-tRNA^{Phe}; specific activity: 1,000-3,000 cpm/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 25 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 twice with 10% TCA and once with 5 ml of di*ethyl*ether/ethanol (1:1) to remove the TCA and to dry the filters. The radioactivity adsorbed on the filters was measured as described before.

2.7.1.2 Determination of the AcPhe-tRNA^{Phe} binding

The binding of N-acetyl([^{3}H] or [^{14}C])Phe-tRNA^{Phe} to ribosomes was determined by nitro-cellulose 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 standard buffer

condition ($H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$) 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 estimated as function of the amount of radioactivity retained on the filters and, this value was normalised to pmol bound per pmol of ribosomes (ν).

2.7.2. 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). 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 N-acetyl-aminoacyl-tRNA and forms an acyl-puromycin derivative (Allen and Zamecnik, 1962) when the ribosomal A site is free. 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 activity (Figure 2.7.2).

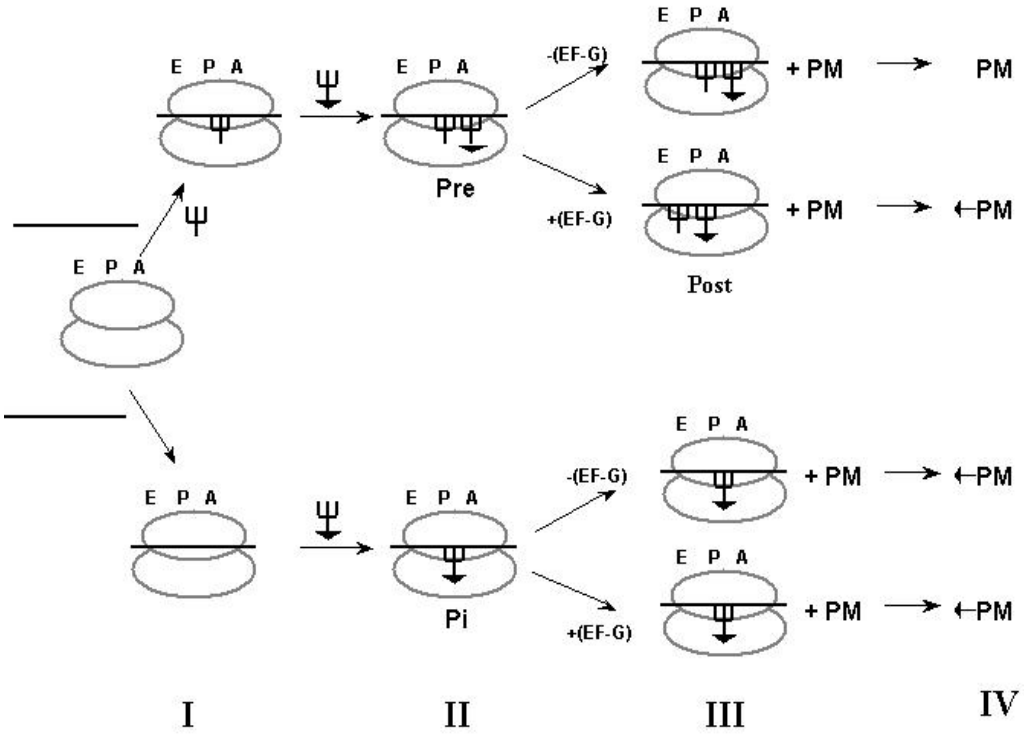

Figure 2.7.2 **Watanabe assay**.
General picture of the modified non-enzymatic A site tRNA binding. (I) 70S programmed with mRNA and P site occupation of deacylated tRNA. (II) Pi or Pre-translocational complex formation after addition of Acetylated tRNA or fMet-tRNA$_f^{Met}$. (III) EF-G dependent translocation. (IV) 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 (section 2.7.3).

The final ionic condition used in this experimental scheme was standard conditions. A typical experiment was conducted as follows:

2.7.2.1 First step: P site binding or P_i complex formation

P_i 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 molar 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.

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.

For a complete set of samples analyzed in all four steps with duplicated binding determinations and puromycin reaction the indicated amounts and the volume was increased up to 10-fold.

2.7.2.2 Second step: A site binding and PRE complex formation

Keeping constant the ionic conditions, 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.

2.7.2.3 Third step: Translocation reaction

At this step, a GTP-mix (5 µl per aliquot) was added to P_i or PRE complexes maintaining constant the standard 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 (0.1-0.4-fold EF-G per ribosome). Control aliquots contained standard buffer condition instead of EF-G. After the addition of EF-G the aliquots were incubated for 10 min at 37 °C.

2.7.2.4 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 standard buffer condition (final concentration = 0.7 mM) were added to two aliquots from every group (± EF-G), while the other two received 2.5 µl of standard 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 on 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 hydrocloride, the pH of the solution has 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 - can be kept at –80 °C 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. to test a new heteropolymeric mRNAs).

2.7.3 Di-peptide formation

The di-peptide system was an extension of the Watanabe assay described before. Pi and PRE complexes were formed as described above for the Watanabe protocol except that aliquots of 10-50 pmol of Pi or POST complexes were added by LepA•GTP (0.3x 70S) or LepA buffer and incubated at 37 °C for 30 min, followed by an enzymatic A site binding of aminoacyl-tRNA ([^{3}H]Lys-tRNA^{Lys} in complex with EF-Tu·GTP (ternary complex).

LepA•GTP was formed by mixture of LepA:GTP (1:250) in LepA buffer. Ternary complex formation was achieved by incubating a mixture containing 1.5-fold excess)

over ribosomes and 1.5-fold EF-Tu per aminoacyl-tRNA for 5 min at 37 °C. GTP was also present at a final concentration of 2.5 mM. The final volume per aliquot was 12.5 µl, and the final ionic conditions were the standard conditions. Aliquots were withdrawn and the binding was evaluated. The rest of the samples were subjected to total phenol extraction in order to analyse di-peptide synthesis. After phenol extraction and ethanol precipitation, the pellet was dissolved in 60 µl of 0.3 M NaOH and incubated for 30 min at 50 °C in order to hydrolyse all RNA. Then 15 µl of 1M HCl was added and the final solution was subjected to a reverse phase chromatography on a C18 column (HPLC). A binary linear gradient was applied at 0.5 ml/min. The buffer system consisted of 0.1% TFA (buffer A) and 60% acetonitrile with 0.1% TFA (buffer B). Fractions were collected and the elution of single amino acid and the di-peptide formed was monitored by radioactivity. Di-peptide was identified in that fraction where the elution of both radioactive amino acids (Ac[^{14}C]Phe and [^{3}H] Lys) coincided and the ratio of pmols corresponding to these amino acids was one. Di-peptides were normalised to the active fraction of ribosomes determined by the amount of AcPhe incorporated (Figure 2.7.3). A mixture of Ac [^{14}C]Phe, [^{3}H]Lys and Ac[^{14}C]-Phe-[^{3}H]Lys could be satisfactorily separated.

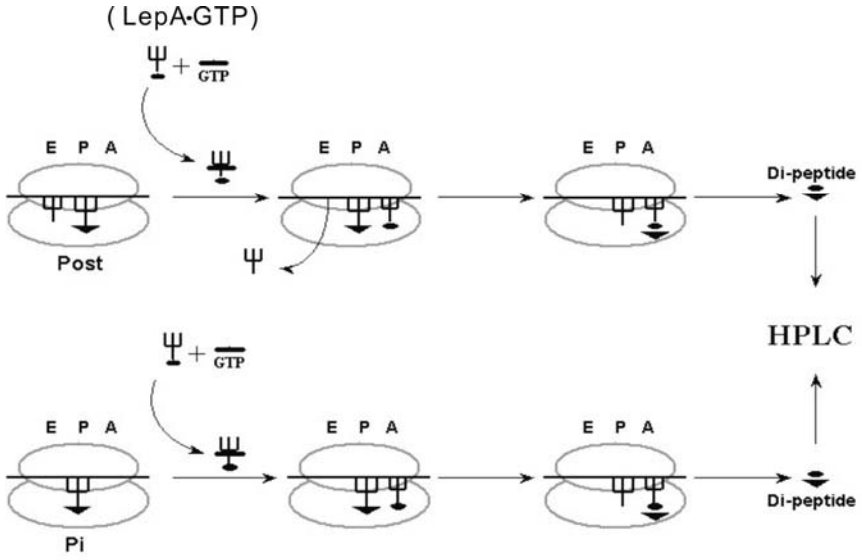

Figure 2.7.3 **Di-peptide analysis**
Pi or Post-translocational complex are formed like in Watanabe assa.y These complex then are subjected to an enzymatic A site binding in presence of [^{3}H]Lys-tRNA^{Lys}:EF-Tu:GTP (ternary complex). HPLC analysis of the total di-peptide formed is done after phenol extraction, ethanol precipitation and total RNA degradation of ribosomes.

In the case of MVF±SD mRNA, the f[^{3}H]Met-tRNA$_{f}^{Met}$ was first bound to P-site. The cognate A-site tRNA was [^{14}C]Val-tRNA and non-cognate tRNA is [^{3}H]Asp-tRNA. Enzymatically binding was achieved by mixing of these two tRNAs containing 1.5-fold excess over ribosomes and 1.5-fold EF-Tu per aminoacyl-tRNA for 5 min at 37 °C. The binding assay and dipeptide analysis are the same as described above.

2.7.4 RNase assay

Detection of RNase contamination was performed by a novel method developed by V. Marquez in the Nierhaus group (Márquez, 2002). MF-mRNA* (MF for methionine and phenylalanine, respectively) was labeled with γ-[^{32}P] as was described at section 2.5.2.7.2. Around 10,000 dpm of [^{32}P]-MF mRNA per reaction was incubated in a total volume of 15 µl with the components to be tested for RNase contamination (usually 5 µl). The final concentrations were adjusted with 10x standard buffer. After incubation at 37 °C for 3 min, RNA loading buffer was added and samples were heated at 95 °C for 2 min. Polyacrylamide gel 14 cm x 16 cm (15%) was pre-run at 400 volts for 20 min. Prior to sample-application excess of urea was removed. The gel was run at 400 volts until the xylene cyanol marker migrated to a position located 8-9 cm from the bottom of the gel. The electrophoresis was stopped; the electrophoresis chamber disassembled and the gel edges were carefully dried with absorbent paper. Then the gel was wrapped with Envoplast plastic paper and stored in a PhosphoImager cassette over night. The next day, the detector was scanned and the gel discarded.

2.7.5 RTS system

2.7.5.1. RTS 100 High Yield *E. coli* Kit

The Roche batch system was adjusted to 10 µl volume instead of 50µl suggested by Roche.

According to the protocol, the contents of the kit were reconstituted with a supplied Reconstitution buffer (Table 2.7.5.1_1). The reaction mixes were prepared according to the Table 2.7.5.1_2 and 8 µl were distributed to separate vials, DNA template was added after this. When checking antibiotics' effect on RTS system,

antibiotics solved in H_2O is added which compensate the H_2O volume from DNA templat. For the LepA influence assay, 0.3 x LepA per 70S (15.6pmol 70S/ 10µl reaction) was added into the solution and the control was the same volume LepA buffer (Hepes 20mM, KCl 500mM and ß-SH 4mM). Standard incubation temperature was 30°C. Samples were introduced into ProteoMaster instrument and incubated for 6 h at 900rpm. Then the machine will automatically stop and deduce the temperature to 8°C for the maturation of the reporter protein GFP for 20 h. To quantify the translation fidelity (active fraction) from each 10 µl reaction, 1.5 µl was analyzed on a 15% PAGE, under denaturing conditions with 2% SDS according to (Laemmli and Favre, 1973) or after a maturation period of about 16 h at 4°C under native conditions (Maniatis et al., 1982). On each gel, reference lanes were included with a known amount of pure and active GFP protein (Roche). The denaturing gels were run for 3 h at 150 V, which enabled good separation of the GFP protein band from neighbor bands of the S30. The SDS-PAGE gels were scanned and the GFP bands quantified and the total amount of GFP was determined by comparison with the reference GFP protein. The active GFP present in each sample was calculated by measuring the fluorescence 430–580 nm of the GFP proteins in the native PAGE. The reference GFP were arbitrarily assigned as 100% and the relative activity of the newly translated GFP protein was calculated. On average, the activity of the GFP from the coupled *in vitro* system performed in the absence of antibiotic was 50% of that of the reference GFP. To calculate the translational fidelity, the amount of active GFP protein was compared with the total amount of GFP protein with/without antibiotic. A decrease in translational fidelity will result in a corresponding decrease in active protein, since misreading events will result in the incorporation of incorrect amino acids, eventually leading to loss of activity (Dinos et al., 2004).

Table 2.7.5.1_1 **Reconstitution of reaction components (RTS 100 HY *E. coli* Kit)**

Solution	Reconstitution procedure
1. *E. coli* Lysate	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer, mix carefully by rolling or gentle shaking. DO NOT VORTEX!
2. Reaction Mix	Reconstitute the with 0.30 ml of Reconstitution Buffer, mix by rolling or shaking

3. Amino Acids	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer, mix by rolling or shaking.
4. Methionine	Reconstitute the lyophilizate with 0.33 ml of Reconstitution Buffer, mix by rolling or shaking.
5.Reconstitution Buffer	1.6 ml; Ready-to-use solution; stable at 2–8°C, can also be stored at – 15°C to – 25°C.
6. Control vector GFP	Briefly centrifuge down the content of the bottle and reconstitute the lyophilizate with 50 µl of sterile DNase- and RNase- free water The solution is stable at –15°C to –25°C

Table 2.7.5.1_2 **Preparation of working solutions (RTS 100 HY *E. coli* Kit)**

Contents	**Preparation of working solution for one 50 µl reaction**
Reaction Solution	Into one of the supplied reaction tubes pipette the following components: 1. 12 µl *E. coli* Lysate 2. 10 µl Reaction Mix 3. 12 µl Amino Acids 4. 1 µl Methionine 5. 5 µl Reconstitution Buffer 6. 0.5 µg of circular DNA template or 0.1–0.5 µg of linear template in 10 µl of water or TE-buffer. Mix carefully by rolling or gentle shaking. DO NOT VORTEX!

2.7.5.2 RTS 500 High Yield *E. coli* Kit

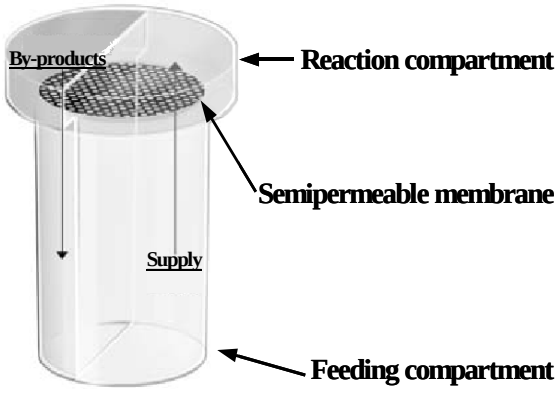

Figure 2.7.5.2 **Reaction device for RTS 500**

The principle of RTS 500 reaction is that transcription and translation take place simultaneously in the 1 ml reaction compartment, and substrates and energy components essential for the reaction are continuously supplied via a semipermeable membrane from the 10 ml feeding compartment (Figure 2.7.5.2) through

the same principles of continuous exchange cell-free protein synthesis as suggested by Spirin (1988). Essential compartments and membrane are indicated. Arrows within the device indicate direction of the flow membrane potentially inhibitory reaction by-products are diluted via diffusion from reaction compartment into feeding compartment (RTS 500 HY E. coli Kit). The reaction device is supplied together with the kit.

Lyophilised reagents provided with RTS 500 Kit were reconstituted according to the Kit directions (Table 2.7.5.2_1).

Table 2.7.5.2_1 **Reconstitution of RTS 500 reaction components (RTS 500 HY *E. coli* Kit)**

Content	Reconstitution/Preparation of working solution
E. coli Lysate	Reconstitute the lyophilizate with 0.525 ml of Reconstitution Buffer, mix carefully by rolling or gentle shaking. DO NOT VORTEX!
Reaction Mix	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer mix by rolling or shaking.
Feeding Mix	Reconstitute the lyophilizate with 8.1 ml of Reconstitution Buffer, mix by rolling or shaking.
Amino Acid Mix	Reconstitute the lyophilizate with without Methionine 3 ml of Reconstitution Buffer, mix by rolling or shaking.
Methionine	Reconstitute the lyophilizate with 1.8 ml of Reconstitution Buffer, mix by rolling or shaking.
Reconstitution Buffer	Ready-to-use solution stable at 2-8°C, but can also be stored at –15 to – 25°C.

The working solutions were prepared from reconstituted reagent according to the RTS 500 Kit protocol (Table 2.7.5.2_2). The following Reaction solution was loaded into 1 ml reaction compartment of the supplied reaction device. The Feeding solution was loaded into feeding compartment with care avoiding air bubbles. The loaded reaction device was introduced into ProteoMaster instrument and incubated at 30°C.

After the reactions, 5 to 10 µl were taken for the SDS-PAAG electrophoretic analysis of a total fraction and the rest of the reactions was stored at + 4°C overnight to gain the active form and then checked on the native PAGE. The GFP is tagged with streptividine and purified with *Strep*-tag® Starter Kit.

Table 2.7.5.2._2 **Preparation of working solution for RTS 500 (RTS 500 HY *E. coli* Kit)**

Content	Reconstitution/Preparation of working solution
Feeding Solution	Add 2.65 ml of the reconstituted Amino Acid Mix without Methionine and 0.3 ml of reconstituted Methionine to Feeding Solution 3. Mix by rolling or shaking. Total volume of Feeding Solution is 11 ml.
Reaction Solution	To the content of *E. coli* lysate, add 0.225 ml of the reconstituted Reaction Mix, 0.27 ml of the reconstituted Amino Acid Mix without Methionine and 30 µl of reconstituted Methionine. Add 10–15 µg of the DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. Total volume of reaction solution is 1.1 ml. DO NOT VORTEX.

2.8 Computational analysis

2.8.1 Secondary structure prediction of synthetic RNA and estimation of its ΔG° of formation

The secondary structure and the corresponding minimum free energy for synthetic RNA molecules designed and produced *via in vitro* transcription was determined with the program MFOLD available on internet: http://mfold.burnet.edu.au/

The program MFOLD prediction was used to determine families of minimal secondary structures for an RNA molecule. The sequence of the synthetic mRNAs designed in this work showed a modest degree of secondary structure upon FOLD analysis.

2.8.2 Protein sequence analysis

Database searches for orthologs of *E. coli* and yeast LepA and EF-G were carried out using BLASTP with standard parameters and protein databases of organisms with

completely sequenced genomes that were downloaded from the Integr8 web site. Orthology was assigned based on best reciprocal hits. Additionally, the INPARANOID database and the NCBI non-redundant protein database were screened for additional homologs that were tested for orthology by phylogenetic analysis. Subcellular localization of proteins was predicted using CHLOROP and MITOP. Multiple alignments were constructed using the MAFFT software for each common structural domain of EF-G and LepA separately because domain order is only partially conserved between these proteins. Only regions with sufficient sequence similarity for unambiguous alignment were considered. Alignments for individual domains were concatenated and used for phylogenetic analysis. Phylogenetic analysis was carried out with the MEGA software (version 3.1). Pair-wise sequence distances were obtained by Maximum Likelihood estimation on the basis of the JTT substitution rate matrix with the assumption of a uniform distribution of rates across sites. Phylogenetic trees were reconstructed using the Neighbor Joining algorithm. Statistical support values for internal branches of the tree were obtained from 1,000 bootstrap samples and their analyses. Trees were calculated on a reduced set of organisms and on a large set of organisms. The essentials of the phylogenetic tree already emerged during the analysis of the smaller data set. Due to the higher number of taxa the second data set offers an enhanced resolution of internal branching patterns in the bacterial subtree and confirms results from the first data set. I thank Dr. Eike Staub for his help with this informatic evaluation.

2.8.3 Protein Modeling

E. coli LepA structure was generated in analogy to the EF-G structure according to (Andersen et al., 2003) and compared with that of ternary complex aa-tRNA•EF-Tu•GTP.