Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Suppliers

Following reagents were supplied by the corresponding manufacturers.

Amersham-Bucher, Braunschweig

γ^{32}P-Adenosine-5'-triphosphate
L-^{14}C-Phenylalanine
L-^{3}H-Valine

Beckman, München

Ready Value (scintillation cocktail)
Ultracenrifuge tubes

Biolabs, New England (U.S.A)

Restriction endonucleases
Reaction buffers
T4 DNA ligase, 6 U/µl

Biorad, Richmond (U.S.A)

Ammoniumperoxidisulphate
SDS

Boehringer, Mannheim (now Roche Pharmaceuticals)

Adenosine-5'-triphosphate
Alkaline phosphatase (CIP)
Ampicilline
dNTPs
Guanosine-5'-triphosphate
T4 Polynucleotide kinase
Polyuridine (poly(U))
Pyruvate kinase (10 mg/ml)

Calbiochem, Frankfurt

HEPES

Difco, Detroit (U.S.A)

Bacto-agar
Bacto-pepton
Bacto-tryptone
Yeast-extract

Fluka, Neu-Ulm

Spermidine
Spermine

Fuji, Tokio (Japan)

Medical X-ray films

Gibco-BRL, Eggenstein

Agarose (Ultra Pure)
Sucrose (Ultra Pure)
Urea (Ultra Pure)
TEMED

Kodak Eastman, Rochester (U.S.A)
X-ray films XAR-5

Merck, Darmstadt
All chemicals used in the laboratory and all essential amino acids
Bromophenol blue
Glycerol (87%)
Ethanol (Ultra Pure)
Ethidium bromide (1%)
Methanol (Ultra Pure)
Urea
Tris
Xylencyanol

Molecular Dynamics, Amersham Biosciences (UK)
Image Quant Cassettes

Packard, Frankfurt
Filter Count (Scintillation Cocktail)

Pharmacia, Uppsala (Sweden)
BSA (DNase and RNase free)
Nap-25 and Nap-5 Columns
Polyuridine (poly(U))
Sephadex
Sephacryl S-300 Spun Column
Sephacryl S-400 HR

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

Qiagen, Düsseldorf
Qiagen Maxi Prep Tip 500
Qiagen Midi Prep Tip 100
Qiagen Mini Prep
Quiquick Nucleotide Removal kit

Roth, Karlsruhe
40% Acrylamide
Rotiphenol
Rotiphenol/chloroform/isoamylalcohole

Sartorius, Göttingen
Nitrocellulose filter (Nr. 11306)

Schleicher and Schüll, Dassel
Selecta Glass Filter (Nr. 6)

Serva, Heidelberg
Acrylamide
Bis-acrylamide
Puromycin
SDS
Spermidine
Spermine

Sigma, Deisenhofen
L-Amino acids
NTPs-tris salt

Whatman, Springfield Mill (GB)
Paper filters

2.1.2 Bacterial Strains and Plasmids

E. coli Can/20-12E: this strain is an *E. coli* K12 derivative (Zaniewski et al., 1984). *E. coli* K12, a rod-shaped gram-negative bacterium, lives as a harmless inhabitant of the human lower intestine and is widely used in medical and genetic research. Can/20-12E is exoribonuclease and endonuclease deficient (Rnase I^-, Rnase II^-, Rnase D^-, Rnase BN^-, Rnase T^-).

Plasmid pSP65: pSP65 is a pUC13 derivative with a SP6-promoter. This plasmid is resistant against ampicillin (Melton et al., 1984).

Plasmid pSTtPheF: PSTtPhe is a plasmid carrying $tRNA^{Phe}$ insert with a Fok1 restriction side right after the CCA end (Schäfer, 1997).

2.1.3 Media, Gel Solutions, Buffers

2.1.3.1 Media

Luria-Bertani (LB) medium

Bacto-Tryptone/Peptone	10.0	g
Yeast Extract	5.0	g
NaCl	5.0	g
1M NaOH	1.0	ml
ddH_2O	ad 1.0	L
		pH 7.4

For optimal conditions add 20% (w/v) glucose; autoclave.

L-Agar Plate

Agar added to LB	15.0	g/L

Sterilize and distribute 16-20 ml aliquots in sterile plates while it is still liquid (when required, antibiotics were added just before the pouring to the plates).

Antibiotic Solution

Ampicillin	100.0	mg/ml

Ampicillin dissolved in H_2O were filter sterilized through a 0.45-μm filter (Schleicher-Schüll, Dassel Germany). Solutions were stored at –20 ℃ in light-impenetrable Eppendorfs.

2.1.3.2 Gel Solutions and Electrophoresis Buffers

Agarose Gel Solution (1%)

10X TBE	5.0	ml
Agarose	0.5	g
ddH_2O	ad 50.0	ml

Acryladime-Bisacrylamide (38/2) Gel Solution (40%)

Acrylamide	38	% (w/v)
N,N’methylenebisacrylamide	2	% (w/v)

Acrylamide solutions were filtered through 3MM paper and stored at 4 ℃ in dark bottles.

Acrylamide Urea Gel Solution (13%)

10X TBE	50	ml
Acrylamide-Bisacrylamide (40%)	162.5	ml
Urea (7M)	210	g
ddH_2O	ad 500	ml

DNA Gel Loading Buffer (6X)

(for the agarose gel)

Ficoll 400	15	%
Bromophenolblue	0.25	% (w/v)
Xylencyanol	0.25	% (w/v)

RNA Gel Loading Buffer

(for acrylamid-urea gels)

Tris.HCl, pH 8 (20 °C)	10	mM
EDTA	1	mM
Urea	7	mM
Bromophenolblue	0.05	% (w/v)
Xylenecyanol	0.05	% (w/v)

TBE (10X)

Tris	108.0	g
Boric Acid	55.0	g
EDTA	9.6	g
ddH_2O	ad1,000.	ml

2.1.3.2.1 Gel Staining and De-staining Solutions

Ethidium Bromide (10 mg/ml) One gram of ethidium bromide was added to 100 ml of H_2O. Solution should be stirred for several hours to ensure that the dye has dissolved. After dissolving it should be kept in dark bottle.

Toluidine Blue

Toluidine blue	1	g
Acetic acid (glacial)	100	ml
ddH_2O	900	ml

After mixing the solution must be filtrated through paper Whatman N° 1 in order to eliminate the non-dissolved dye.

Coomasie Brillant Blue

Coomasie Brillant Blue R250	2,5	g
Methanol	450	ml
ddH_2O	450	ml
Acetic acid (glacial)	100	ml

The dye is dissolved in the methanol, then water is added, and finally acetic acid. After mixing thoroughly, the solution must be filtrated through paper Whatman N°1 in order to eliminate the non-dissolved dye.

Destaining Solution

Ethanol (technical grade)	100	ml
Acetic acid (technical grade)	70	ml
ddH_2O	830	ml

2.1.3.3 Plasmid DNA Isolation Buffers

P1

(Resuspension buffer for the Qiagen-plasmid preparation)

Tris-HCl, pH 8.0 (0 ℃)	50	mM
EDTA	10	mM

P2

(Cell lysis buffer for the Qiagen-plasmid preparation)

NaOH	200	mM
SDS	1	% (w/v)

P3

(Neutralization buffer for the Qiagen-plasmid preparation)

KAc, pH 5.5	3,000	mM

Buffer QBT

(Equilibration buffer for the Qiagen column)

MOPS, pH 7.0	50	mM
NaCl	750	mM
Triton X-100	0.15	%
Isopropanol	15	% (v/v)

Buffer QC

(Washing buffer for the Qiagen column)

MOPS, pH 7.0	50	mM
NaCl	1,000	mM
Isopropanol	15	% (v/v)

Buffer QF

(Elution Buffer)

MOPS, pH 8.2	50	mM
NaCl	1,000	mM
Isopropanol	15	% (v/v)

2.1.3.4 Buffers for the RNA Transcription

10X Transcription Buffer

Tris-HCl (pH 8.0 at 37 °C)	400	mM
$MgCl_2$	220	mM
DTE	50	mM
Spermidin	10	mM

RNA Extraction Buffer

(Extraction from acrylamide gel)

Tris-HCl**,** pH 7.8	10	mM
NaCl	100	mM
DTE	1	mM
EDTA	1	mM
SDS	1	% (w/v)

QA Buffer

MOPS, pH 7.0	50	mM
NaCl	400	mM
Isopropanol	15	% (v/v)

QAT-Buffer

MOPS, pH 7.0	50	mM
NaCl	400	mM
Triton	0.15	% (v/v)
Isopropanol	15	% (v/v)

QR Buffer

MOPS, pH 6.6	67	mM
NaCl	900	mM
Isopropanol	20	% (v/v)

QRU Buffer

MOPS, pH 7.0	50	mM
NaCl	900	mM
Isopropanol	15	% (v/v)
Urea	6,000	mM

2.1.3.5 Buffers for the in Vitro Translation System (Watanabe System)

Since β-mercaptoethanol is interfering with iodine cleavage, this reagent has been taken out from buffers on purpose.

Binding Buffer ($H_{20}M_6N_{150}Sp_{0.05}Spd_2$)

Hepes-KOH, pH 7.6 (0 ℃)	20	mM
$Mg(Ac)_2$	6	mM
NH_4Ac	150	mM
Spermine	0.05	mM
Spermidine	2	mM

Tico Buffer ($H_{20}M_6N_{30}$)

Hepes-KOH, pH 7.6 (0 ℃)	20	mM
$Mg(Ac)_2$	6	mM
NH_4Cl	30	mM

HM_6K ($H_{20}M_6K_{150}DTE_1Gly_{10\%}$)

Hepes-KOH, pH 7.6 (0 ℃)	20	mM
$Mg(Ac)_2$	6	mM
KCl	150	mM
Dithioerythriol	1	mM
Glycerin	10	% (v/v)

Watanabe-MixI (6mM non-enzymatic system) ($H_{60}M_{18}N_{690}Sp_{0.25}Spd_{10}$)

Hepes-KOH, pH 7.6 (0 ℃)	60	mM
$Mg(Ac)_2$	18	mM
NH_4Ac	690	mM
Spermine	0.05	mM
Spermidine	10	mM

Watanabe-MixII (6mM non-enzymatic system) ($H_{100}M_{30}N_{750}Sp_{0.25}Spd_{10}$)

Hepes-KOH, pH 7.6 (0 ℃)	100	mM
$Mg(Ac)_2$	30	mM
NH_4Ac	750	mM
Spermine	0.25	mM
Spermidine	10	mM

Watanabe-MixIIe (6mM enzymatic system) ($H_{40}M_{12}N_{300}Sp_{0.25}Spd_{10}$)

Hepes-KOH, pH 7.6 (0 ℃)	40	mM
$Mg(Ac)_2$	12	mM
NH_4Ac	300	mM
Spermine	0.25	mM
Spermidine	10	mM

Watanabe-MixIII (6mM enzymatic-system) ($H_{66.7}M_{20}N_{500}Sp_{0.25}Spd_{10}$)

Hepes-KOH, pH 7.6 (0 ℃)	66.7	mM
$Mg(Ac)_2$	20	mM
NH_4Ac	500	mM
Spermine	0.25	mM
Spermidine	10	mM

2.1.3.6 Buffers for the HPLC Run

(Buffers used for the column Eurosil Bioselect 300 C-8)

Buffer A

NaCl	400	mM
$Mg(Ac)_2$	10	mM
NH_4Ac (pH 5.0)	20	mM

Buffer B

NaCl	400	mM
$Mg(Ac)_2$	10	mM
NH_4Ac (pH 5.0)	20	mM
Uvasol Methanol	60	% (v/v)

2.2 Analytic Methods

2.2.1 Photometric Measurements

2.2.1.1 Spectrophotometric Determination of the Amount of DNA or RNA

The concentration of oligonucleotides, DNA and RNA was determined by absorption measurement in a Hitachi U-3000 spectrophotometer. The measurements were performed at 260 or 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in a sample, whereas the ratio between the reading at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the nucleic acid.

A_{260}/A_{280} = 1.8 for pure DNA*****

A_{260}/A_{280} = 2.0 for pure RNA*

***** This coefficients are valid for high molecular weight species (Berger, 1987). In the case of oligonucleotides (less than 100 bases) the base composition can have a significant effect on the extinction coefficient. This means that the A_{260}/A_{280} ratio will change with the sequence. Extreme cases are A rich sequences (A_{260}/A_{280} ratio bigger than 2.2) and C rich sequences (A_{260}/A_{280} ratio smaller than 1.5).

Conversion factors used for the quantification of DNA and RNA are:

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 then 25 bases)	20	µg
1 A_{260} unit of single stranded DNA (30-80 bases)	30	µg

2.2.1.2 Determination of ribosome and nucleic acid concentration

The concentration of 70S ribosomes and 30S and 50S subunits was determined photometrically at 260 nm, using the following molar extinction coefficients:

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
30S	1.4×10^7 $M^{-1} \times cm^{-1}$	1 A_{260} unit = 72 pmol

In order to make an estimation of the molar extinction coefficient of the synthetic RNA obtained via *in vitro* transcription, the base composition of those products was used together with the following parameters:

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 calculated as the sum of the coefficients corresponding to each base (Wallace and Miyada, 1987) minus a 10 % of this total in order to to account for the hypochromicity (Gnirke, 1988).

5S rRNA	1 A_{260} unit = 986 pmol
23S/5S rRNA	1 A_{260} unit = 36 pmol
16S rRNA	1 A_{260} unit = 72 pmol
tRNA	1 A_{260} unit = 1500 pmol
MF-mRNA (46 nt)	1 A_{260} unit = 1750 pmol
MFV-mRNA (49 nt)	1 A_{260} unit = 1650 pmol

2.2.2 Radioactivity Measurements

The radioactivity measurements were performed using a liquid scintillation counter Wallac Rack Beta model 1209 or a Rack Beta model 1409. The treatment of the samples before measurement was done according to their physical nature and isotope content:

2.2.2.1 Liquid samples

Radioactive liquid samples with a volume of 1 ml or less were put to the scintillation vials (plastic, 20 ml maximal volume), then 5 ml of Ready Value (Beckman) or Filter Count (Packard Bioscience) were added. After quick and vigorous shaking (10 sec), the samples were immediately counted.

2.2.2.2 Nitrocellulose filters (containing single or multiple labels)

Samples filtrated through the nitrocellulose filters were put in scintillation vials, then 5 ml of Filter Count was added. In order to dissolve the filter before counting, vials were shaken in cold room for 5 to10 h. When ^{3}H was present, the time of mixing had to be increased to 15 h in order to obtain reliable counts. For ^{32}P the minimum time was enough.

2.2.2.3 Glass fiber filters with $^{14}C/^{3}H$, $^{14}C/^{32}P$ or $^{14}C/^{3}H/^{32}P$ triple labels

After passing through the samples from glass filters, they were dried with diethyl ether/ethanol (1:1) and then placed in scintillation vials. After adding 5 ml of Filter Count and shaking in the cold room for 5 to10 h, measurement was done.

2.2.2.4 Controls for (multiple) labels

Control vials can be prepared in two different ways. First way is to put 10,000 cpm from each radioactivity directly to the vials. After addition of 5 ml of Filter Count, measurement can be done. For the multiple labels, a distinct amount of each isotope alone and in all possible combinations of the radioactivities

were prepared in the same way. The second method follows the same logic but in this case radioactivities were put on the nitrocellulose filter first and dissolved in the presence of 5 ml Filter Count with overnight shaking. For controls glass vials are preferential and they can be kept in the cold room roughly one-two months.

2.2.3 Cold trichloroacetic acid (TCA) precipitation assays

Radioactively labeled RNAs such as aminoacyl-, acetylaminoacyl-tRNA or 5' labeled tRNA can be tested for reaction efficiency after charging, purification, or deacylation assays by cold TCA precipitation.

To 2 ml of ice cold 10% (w/v) TCA, 20 µl of a precipitation carrier solution (10 mg/ml $tRNA^{bulk}$ from yeast total tRNA) and 5-10 pmol sample are immediately added and mixed by 1-2 seconds vortexing. The RNA precipitated at 0 ℃ for 20-30 min before filtration. The precipitation mix was then collected on glass fiber filters washing them with 2 ml of cold 10% TCA two times, once with 2 ml diethylether/ethanol (1:1) in order to dry the filters. The radioactivity adsorbed to the filter was then measured in scintillation counter as indicated in the section: 2.2.2.

2.2.4 Electrophoresis Techniques

2.2.4.1 Agarose Gel Electrophoresis

This technique was used for analysis of plasmid DNA preparation (small or large-scale isolation) and restriction digestions. The samples were prepared according to the type and the expected size of the nucleic acids to be separated. The percentage of agarose used in every case depends on the expected size of the nucleic acid to be separated. For example, 0.8% is good for 3 kb plasmids and for rRNA and 1.5-2% is for restriction fragments of 600 bp or less. To be able to load the plasmid DNA and digested ones on the same gel, 1% was preferred in all our experiments (Figure 2.2.4.1.1). The amount of DNA was 0.2-1 µg per lane with a volume of 5-10 µl in 1X agarose

sample buffer. TBE was used as a buffer since it has significantly higher buffering capacity than TAE. Since the electrophoretic mobility of the linear double-stranded DNA is reduced by ~15% in the presence of the ethidium bromide, staining was done by immersing the gel in H_2O containing ethidium bromide (0.5 μg/ml) for 20-30 minutes at room temperature. Destaining is not usually required but possible background of unbound dye can be removed by soaking the stained gel in H_2O or 1mM $MgSO_4$ for 10 minutes at room temperature.

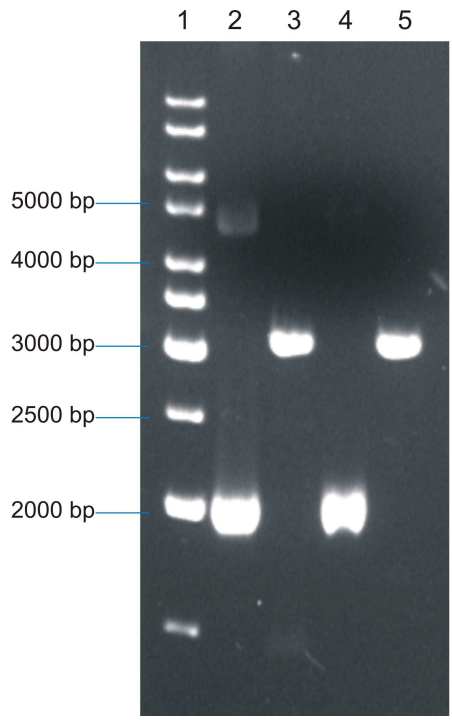

Figure 2.2.4.1.1: 1% agarose gel for the plasmid digestion. Lane 1 is the DNA marker. Lane 2 and 4 are the uncut plasmid DNA, lane 3 and 5 are the cut ones.

2.2.4.2 Denaturing Urea-Polyacrylamide Gel Electrophoresis

Denaturing urea-polyacrylamide gels were used in a quality check of *in vitro* transcribed RNAs, and in the purification of 5'-labelling of transcribed RNAs as well as in footprinting experiments. For routine checking of *in vitro* transcript, an electrophoresis was performed in a ''midi-sequencing gel'' with the dimensions14 x 16 x 0.1 cm. Maxam-Gilbert sequencing gels (30 x 40 x 0.04 cm) were used for separations at one nucleotide resolution mainly in the footprinting experiments and in the purification of labeled RNAs. The percentage of acrylamide used for every analysis depends on the size of samples to be separated.

PAA (%)*	*BPB* (bp)*	*Xc* (bp)*
5	35	130
6	26	106
7.5	22	90
8	19	75
10	12	55
15	10	40
20	8	24

* PAA: polyacrylamide, BPB: bromophenolblue, Xc: xylenecyanol

The running conditions were:

Type of gel	*Pre-run*	*Run*
Midi-sequencing gel	30 min at 8 W	30 min-1 h at 8 W
Maxam-Gilbert	45 min at 60 W	45 min-75 min at 60 W

2.3 Preparative Methods

Here the preparation of 70S ribosome, 30S and 50S subunits and S100 will be explained. 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 ionic conditions ($H_{20}M_6N_{30}SH_4$: Tico buffer) that ensure to obtain ‘’tight-couple’’ 70S ribosomes (Hapke and Noll, 1976). The protocol to isolate the 70S particles is identical with subunit preparation except that the crude 70S pellet is resuspended in Tico buffer ($H_{20}M_6N_{30}SH_4$) instead of dissociation buffer ($H_{20}M_1N_{200}SH_4$). Briefly, the cells are mechanically lysed and the large cellular debris pellet via two low-speed spins (max. 30,000 g). An overnight spin (about 50,000 g) pellets the ribosomes. The pellet is resuspended in dissociation buffer or Tico buffer for 70S preparations and loaded on a sucrose gradient to separate the subunits. The subunits are pellet overnight via a high-speed spin and resuspended in Tico buffer.

Everything should be done in the 4 ℃ room, except for thawing the cells. Extreme caution should be used not to introduce RNases into the samples.

2.3.1 Ribosome Preparation from E. coli

2. 3.1.1 Large Scale Cultures of E. coli

Large biomasses from *Escherichia coli* K12, CAN/20-12E (Rnase I^-, Rnase II^-, Rnase D^-, Rnase BN^-, Rnase T^-, (Zaniewski et al., 1984)) were usually purchased from CDN Ltd., Tallinn in Estonia, that were bred under steady-state conditions and thus contained exclusively log-phase cells. In rare cases we ferment *E. coli* strains in our 100 L Bioengineering fermenter in Berlin. In the latter case hundred liters of sterile L-medium, supplemented with sterile glucose solution (20%) up to 0.5%, were inoculated with 2.5 liter of an overnight culture of the appropriate strain, and allowed to ferment at 37 ℃. The bacterial growth was followed by determination of the optical density at 650 nm. When the culture reached the early logarithmic phase (0.5 A_{650}/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 ℃. The yield of the process was 1-1.3 gram of wet cells per liter of medium.

2.3.1.2 Preparation of 70S Ribosome

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 ℃). The cell pellet was weighted, and a double amount of aluminium oxide (Alcoa-305) was added. 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 homogenate was then centrifuged at 8,000 rpm for 10 minutes in a GSA rotor (4 ℃) in order to remove the Alcoa and the unbroken cells. 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) during 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 during 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 ℃. The supernatant was processed as indicate in section 2.3.1 in order to obtain the S-100 fraction.

The yield of crude 70S ranged between 300 and 400 A_{260} units per g of wet cells processed. A complete separation of tight couple 70S ribosomes from the ribosomal subunits was achieved upon two consecutive zonal centrifugation steps of samples containing 6,000-9,000 A_{260} units of crude 70S preparation through a sucrose gradient (6-38% sucrose in Tico buffer). 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 by centrifugation at 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 tight couple 70S ribosomes were essentially freed 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 ℃. The yield of tight cou ple 70S ribosomes ranged between 10 and 20% of the total A_{260} units initially applied to the zonal.

2.3.1.3 Preparation of Ribosomal Subunits

The 30S and 50S ribosomal subunits were isolated by zonal centrifugation using a linear sucrose gradient from 0 to 40% in dissociation buffer. For every zonal run 3,000-6,000 A_{260} of 70S ribosomes were used. The centrifugation was performed using a Beckman zonal rotor Ti15 at 22,000 rpm for 17 h at 4 ℃. The gradient was pumped out the rotor using a solu tion containing 50% of

table sugar in water. After the zonal centrifugation two pools were made with the fractions containing the 30S and 50S subunits respectively. The 30S and 50S subunits were pelleted in 45Ti rotors (Beckman) at 35,000 rpm, for 22 h at 4 ℃. The pellets were resuspended in 3 ml of in Ti co buffer and centrifuged in a SS-34 rotor (Sorvall) at 7,000 rpm for 15 min at 4 ℃, in order to eliminate the aggregate material. The concentrations of the 30S and 50S subunits were determined by their optical absorbance at 260 nm. The suspension was divided in small aliquots, frozen in liquid nitrogen and stored at -80 ℃. The typical yield starting from 5,000 A_{260} of 70S was 1,000 and 1,200 A_{260} of pure 30S and 50S subunits, respectively.

2.3.1.4 Preparation of Reassociated 70S

Reassociated ribosomes are the ones that 70S ribosome is formed in the presence of high Mg^{2+} concentrations from ribosomal subunits purified by sucrose gradient. Reassociated ribosomes are pure when compared to the tight-coupled ribosomes since tight-coupled ribosomes still carry some tRNAs and mRNA fragments after purification.

The 50S and 30S ribosomal subunits are mixed in a molar ratio of 1:1 of A_{260} units, diluted to a final concentration of 40-140 A_{260}/ml in either adaptation buffer (if ribosomal subunits are in Tico buffer) or reassociated buffer to get final concentrations of $H_{20}M_{20}K_{30}SH_4$, pH 7.5. By using an excess of 30S the amount of amount of free 50S subunit is minimized, 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 incubation (10 min at 4 ℃), the samples are subjected to the gradient cen trifugation (10%-30% sucrose) in re-association buffer, $H_{20}M_{20}K_{30}SH_4$, and centrifuged for 17 h at 18,000 rpm, 4 ℃ in a Beckman zonal rotor. The gradie nt was fractionated and the 70S peak were pooled and pelleted by centrifugation at 50,000xg for 27 h, 4 ℃ in a 45 Ti-rotor. The use of higher centrifugat ion rates is not recommended, because it may lead to pressure-induced dissociation of the ribosomes (Gross et al., 1993). The ribosomes were resuspended in reassociation buffer and incubated 20 min at 40 ℃. Aft er the solution has

been clear by low speed centrifugation, 70 ribosomes were dyalized against Tico buffer ($H_{20}M_6N_{30}SH_4$, pH 7.5) three times each 45 min. The concentration of ribosomes was determined from A_{260}. Small aliquots (50 µl) were shock frozen in liquid nitrogen and stored at -80 ℃.

The quality of the preparation can be checked using three assays (Blaha et al., 2000): (1) A SW 40 run is performed (a gradient of 10-30% sucrose in reassociation buffer, 16 hours at 22,000 rpm, about 34,000xg and 4 ℃) in order to test the homogeneity of the reassociated 70S. (2) RNA gels run in order to test the intactness of the ribosomal RNA. 16S and 23S RNA have to be essentially free from breaks. (1) and (2) establish the structural integrity of the particle. (3) The activity in the poly(U)-dependent poly(Phe) synthesis system is an important criterion for establishing the activity of the reassociated 70S preparation (Bommer et al., 1996).

2.3.2 Preparation of the S-100 Fraction from E. coli

The S-100 fraction containing all the soluble proteins, which are necessary for the protein biosynthesis process (i.e. translation factors and aminoacyl-tRNA synthetases), was prepared from the supernatant obtained after the sedimentation of the crude 70S preparation (see section 2.3.1.2). In order to eliminate residual ribosomal particles (mainly 30S ribosomal subunit in this case), this fraction was centrifuged during 4 hours at 100,000 g (38,000 rpm, 45 Ti rotor). The upper two thirds of the supernatant were recovered by careful decantation and dialized (using Sartorius dyalisis membranes; M.W. cut off = 3,000) during 15 hours against ~20 volumes of Tico buffer (4 times changed) in order to eliminate the low molecular weight components. After dialysis the S-100 fraction was shock-frozen in small aliquots and stored at -80 ℃.

2.3.2.1. Preparation of S-100 tRNA-free

The preparation of a tRNA-free S-100 fraction is important for the partial isolation and concentration of the aminoacyl-tRNA synthetases as well as for the elimination of endogenous RNA (mainly tRNA), free amino acids and other soluble factors that could interfere with the tRNA aminoacylation and other functional experiments. The separation is achieved with the use of DEAE-cellulose (Durnoff and Maitra, 1971; Woodwar et al., 1974; Yang and Novelli, 1971) since this material will bind strongly the negatively charge RNA, allowing the selective elution of the protein fraction containing the translational factors and synthetases. This approach has the additional advantage of eliminating the bulk of RNase activity contained in the original S-100, since these proteins are normally not bound to the matrix, when they are in free form (most of them have strong basic character), but are retained in a complex with tRNA.

A portion of 15 grams of pre-swollen microgranullar DEAE-cellulose was suspended in 300 ml of buffer TMK-500 and allowed to sediment for 30 minutes at 90 ℃. The supernatant was then discarded and a new portion of TMK-500 was added, allowing resuspention and sedimentation of the celullose again. After decant the supernatant newly, the same procedure was repeated three times but using buffer TMK-150. In a final equlibration step, the DEAE cellulose was again resuspended in TMK-150 and incubated overnight at 4 ℃. At this level the matrix has been equlibrated (pH of the supernatant should be 7.5; when this was not the case, the buffer was changed again) in the buffer TMK-150 and is ready to use.

After decanting excess of buffer, 150 ml of S-100 were mixed with the matrix. This mix was maintained at 0 ℃ during 2 hours with occa sional agitation and then centrifuged at 10,000 x g for 30 minutes in a Sorvall HB-4 rotor (8,000 rpm; 4 ℃). The supernatant (S1) was collected, and the matrix was sequentially treated with the buffers TMK-150, TMK-200, and TMK-500 (all in a 150 ml portions) yielding the supernatant SII, SIII, and SIV, respectively. All the supernatant were dialysed against Tico buffer and centrifuged at 10,000 x

g for 30 minutes in order to eliminate the residual matrix. The absorbance at 230, 260, and 280 nm of every fraction were determined as well as the synthetase activity by means of analytical aminoacylation of tRNA (section 2.6.1). The fractions SII and SIII contained the highest synthetase activity and were essentially free of endogenous tRNA. These fractions were stored in small aliquots at -80 ℃.

2.4 Genetic Methods: Working with DNA

In this part of methods section, cloning strategies will be discussed. The whole procedure can be divided into five main steps.

(1) Preparation of competent cells
(2) Preparation of vector
(3) Preparation of the insert
(4) Ligation and Transformation
(5) Plasmid DNA isolation and sequencing for the right colony.

In this work, MFV-mRNA was cloned.

2.4.1 Preparation of E. coli Competent Cells

One liter culture of the *E. coli* strain of interest was grown to an OD_{600} of 0.5-1.0. The cells were chilled on ice, and then pelleted by centrifuging, at 4,000 rpm for 15 min at 4℃, and resuspended in 1 volume o f pre-chilled sterile H_2O. Bacterial cells were repeatedly centrifuged and washed in decreasing volumes of cold H_2O: two washes of 0.5 volume, one wash of 0.02 volume and final resuspension in 2-3 ml filter sterilized 10% (v/v) glycerol. The cells were either used immediately or aliquotized in 40 µl, shock frozen in liquid nitrogen and stored at -80 ℃.

2.4.2 Preparation of the Vector

The plasmid that was used as a vector was pSP65. This high copy plasmid does not have a T7 promoter. That is why inserts were prepared with the T7 promoter.

2.4.2.1 Restriction digestion with BamHI and EcoRI

DNA samples were incubated with the desired restriction enzymes (in this case BamHI and EcoRI) at a ratio of 2-4 units enzymes per one μg of DNA for at least 3 hours. The temperature was optimum for each restriction enzyme. Double digestion was done in the buffer where both enzymes are active. 20 μg of plasmid were incubated with 40 units of BamHI and 40 units of EcoRI in a total volume of 500 μl.

2.4.2.2 Alkaline phosphatase digestion

In order to prevent the vector circulization during the ligation step (2.4.3.3) the plasmid was dephosphorylated with calf alkaline phosphotase (CIP) after restriction digestion and phenol-chloroform purification. In a total volume of 150 μl of CIP buffer the cut plasmid was incubated at 37 ℃ for 15 min with 10 units of CIP. A second CIP (10 units) was added and the incubation continued at 55 ℃ for 1 hour. At the end of the second incubation, the temperature was increased to 75 ℃ for 10 min in order to inactivate the phosphatase. The reaction was then extracted with phenol and the DNA was precipitated with EtOH. The recovered DNA was dissolved in 20 μl H_2O and stored at –20 ℃.

2.4.3 Preparation of the insert

The insert that will be explained here is the dsDNA, which is the substrate for the transcription of MFV mRNA.

2.4.3.1 Designing of the Oligomers

The sequence of the construct is as following:

5‘- CG GGATCC TAATACGACTCACTATA GGGAAAAG AAAA/G AAAAG
***Bam*H1 T7 Promoter**

AAA ATG TTG GTT AAAAG/ AAAAG AAAAG AAATATT GAATTC CG -3’
Met Phe Val *Ssp*I *Eco*RI

For this construct, two oligomers were designed which are complementary to each other in the 23 bases. **/** shows the borders of the overlapping area.

Oligomer 1

5‘-CG GGATCC TAATACGACTCACTATA GGGAAAAG AAAAG AAAAG AAA ATG TTG GTT AAAAG-3’

Oligomer 2

Oligomer 2 is **complementary** to the sequence below.

5‘- G AAAAG AAA ATG TTG GTT AAAAG AAAAG AAAAG AAATATT GAATTC CG- 3’

2.4.3.2 Annealing and DNA Filling and Digestion

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 the presence of dNTPs.

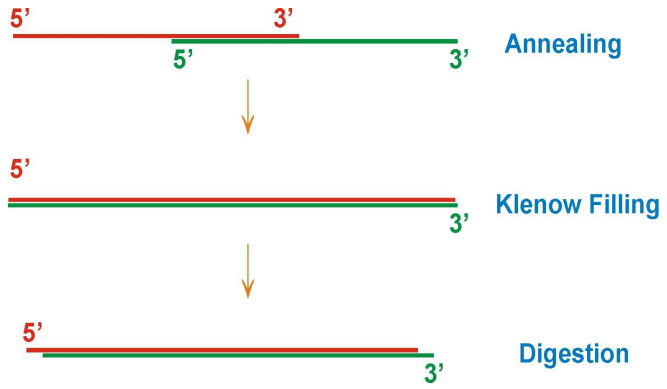

Figure 2.4.3.2.1: Main steps to prepare an insert for cloning

Annealing reaction was as following: 250 pmol of a purified oligo were incubated 5 minutes at 75 ℃ with an equimolar amount of the corresponding comple-mentary oligomer in a total volume of 15 µl. The temperature was then slowly downs to 37 ℃ in a period of 45 minutes to 1 hour. For the filling reaction, 2.5 µl H_2O, 2.5 µl of One-Phor-All buffer (Pharmacia) , 2.5 µl of dNTPs mix (dATP, dGTP, dCTP and dTTP, 20 mM each) and 2.5 µl of Klenow fragment enzyme (5 units/µl). The reaction was incubated for 45 minutes at 37 ℃. After incubation 75 µl of water was added and the mix was phenol extracted and the DNA was precipitated from the recovered aqueous phase with 2.5 volumes of ethanol. It is dissolved in 82 µl H_2O and restriction digestion was done as explained in the section 2.4.2.1 with the final volume 100 µl. Restriction digestion was done as explained in the section 2.4.2.1.

2.4.3.3 Ligation

Ligation reaction was carried on the 16 ℃ for maxi mum 16 hours with 80-100 ng of linearized dephosphorylated plasmid, 30-60 ng of DNA insert and 2 U of T4 DNA ligase. In some cases ligation buffer contains ATP. In the absence ATP to the 1 mM final concentration should be added. After incubation, ligation mix can be stored at -20 °C or immediately used for transformation of *E. coli* competent cells.

2.4.4 Transformation

For electroporation, 1-2µl plasmid DNA (typically 1-5ng) or 2µL resuspended ligation (~100 ng of plasmid plus insert) was pipetted into a cold BioRad Gene Pulser® cuvette (0.2cm electrode gap). A 40µL aliquot of electrocompetent cells was added carefully (to prevent any air bubbles in the cell suspension) to a pre-chilled cuvette. After careful mixing the cell suspension was tapped to the bottom of the cuvette, which was placed in a BTX Electro Cell Manipulator® 600 and charged with a current (2.45kV, 5-6ms, 129 ohm resistance, 12.25kV/cm). The cells were allowed to recover in 1ml LB medium without antibiotic for 1h at 37 ℃, then plated on selective medium.

2.4.5 Plasmid Isolation

2.4.5.1 Small Scale Preparation of Plasmid DNA (Miniprep)

Small-scale preparations of plasmid DNA from 2 ml overnight cultures of bacteria were performed using the solutions of Qiagen plasmid kit without any column.

Single colonies were selected to inoculate 2 ml of LB medium, which contains 100 μg/ml ampicillin as a final concentration. The cultures were grown over 12 h at 37 °C (early stationary phase) and harvested by centrifugation (Eppendorf Centrifuge 5415C) 5 min at 6,000 rpm in 1,5 ml Eppendorf tubes if the rest were kept in glycerol stock, otherwise 2,0 ml Eppendorf tubes. After removal of the supernatant the cell pellets were resuspended in 300 μl P1 buffer which contains RNase with a final concentration of 100 μg/ml to digest the RNA. When pellet is resuspended, 300 μl P2 (alkaline lysis) buffer should be added and mix gently by inverting the tube couple of times. It should be kept in room temperature less then 5 min, until you see clearing or gooey white stuff that indicate lysis. Violent mixing at this stage can shear DNA especially if plasmid DNA is large. Addition of 300 μl P3 causes a white precipitation. This solution has a high-salt concentration ad causes genomic DNA and cell debris to clump up and precipitate. Plasmid DNA is small enough to remain soluble. Next step is the centrifugation at 12,000 rpm for 15 min. Supernatant was transferred to a new tube and EtOH precipitation was performed with the addition of 2.5 volume EtOH. The precipitate was recovered by centrifugation (45 min, 4 ℃, 12,000 rpm in Eppendorf centrifuge 54 17R). The pellet was washed with 70% ethanol, briefly dried in room temperature and resuspended in 20-30 μl H_2O, stored at –20 ℃. This DNA can be used for transform ation, restriction digestion, and sequencing.

Glycerol Stock: The positive clones were stored as frozen stocks. This was done with cultures grown in LB medium in the presence of an appropriate antibiotic until to the early stationary phase. Two aliquots of 250 μl were mixed

with 250 µl of glycerol storage solution in screw capped vials, and stored at -80 ℃.

2.4.5.2 Large Scale Preparation of Plasmid DNA

Large-scale isolations of plasmid DNA were prepared using Qiagen plasmid maxi kits.

A single colony from a freshly streaked selective plate was picked and inoculated a starter culture of 2 ml LB medium containing the appropriate selective antibiotic. After approximately 8 h incubation at 37 ℃, 300 rpm, the starter culture was diluted 1/500 to 1/1,000 into selective LB medium. For high-copy plasmids 100 ml LB medium was inoculated. For high copy number plasmids 100 ml cultures grown for 16h was sufficient to yield 1-2 mg DNA. Cells were pelleted by centrifuging at 5,000 rpm, for 15 min, in the Sorvall using a GS3 rotor. After harvesting, the protocol from Qiagen plasmid purification handbook was followed. DNAs eluted from column with 15 ml elution buffer and collected in 30 ml Corex tubes. Nucleic acids were precipitated by adding 0.6 volumes of isopropanol and leaving at –20 ℃ for 2-4 h. The nucleic acid was pelleted by centrifuging at 6,000 rpm for 1h at 4 ℃ (Sorvall rotor HB-4). Pellets were washed with 70% EtOH and air-dried and resuspended in 500 µl of H_2O.

2.5 Working with RNA: In Vitro Transcription and Site-Specific Binding of Thioated tRNAs to Probe Ribosome - tRNA Interactions

In this part of the methods, the normal and thioated RNA transcription, purification, 5’-labeling and footprinting experiments will be explained. During this experiments extreme caution should be used not to contaminate the samples with RNases.

2.5.1 Run-off Transcription with T7 Polymerase

In vitro transcription systems allow us to synthesize large amounts of RNA molecules functionally equivalent to the native ones. A general scheme for the synthesis of RNA using purified T7 polymerase is as following: As a first step, the sequence to be transcribed is inserted into a plasmid vector downstream from the promoter (see section 2.4.3.1). A template for the run off transcription is prepared by cutting the plasmid DNA with a restriction enzyme downstream of the inserted DNA sequence. When a proper salt buffer, ribonucleoside triphosphates, and T7 RNA polymerase are added to the DNA template, transcription begins specifically at the promoter and proceeds through the cloned DNA sequence and terminates when it reaches the end of the linear template. RNA polymerase reinitiates transcription at the promoter many times so that a number of RNA copies are made from every molecule of DNA template.

2.5.1.1 Transcribed RNAs Used in this Thesis

MF-mRNA is a 46 nucleotide length mRNA which contains a methionine, **M**, (AUG) and a phenylalanine, **F**, (UUC) codon for tRNA binding in the middle of $(A_4G)_3$ motive.

5’-GGGAAAAGAAAAGAAAAGAAA**AUGUUC**AAAAGAAAAGAAAAGAAAU-3’

MFV-mRNA is a 49 nucleotide length mRNA which contains a methionine, **M**, (AUG), a phenylalanine, **F**, (UUC), and a valine, **V**, (GUU) codon for tRNA binding in the middle of $(A_4G)_3$ repeating motive:

5’-GGGAAAAGAAAAGAAAAGAAA**AUGUUCGUU**AAAAGAAAAGAAAAGAA AU-3’.

tRNA^{Phe} is a 76 nucleotide long tRNA cloned in plasmid pxxFok1 (Schäfer, 1997).

2.5.1.2 In vitro Transcription

The in vitro transcription system with T7 RNA polymerase that we are using in our laboratory was optimized as a starting point according to the papers (Milligan and Uhlenbeck, 1989; Weitzmann and Cooperman, 1990). In the table below, the components and the order of component additions is given. Pipetting order is important since the enzyme and the RNase inhibitor could be inactivated if the ionic environment changes drastically. After the addition of polymerase, the reaction was incubated at 37 ℃ for 8-10 hours and stopped by adding EDTA to a final concentration of 25 mM.

After stopping the reaction, products were analyzed in the acrylamide gel in case of optimization (analytic transcription) experiments or purified by one of the proper methods explained in section 2.3.1.4 in the case of preparative assay.

Standard conditions for in vitro transcription assays

order of addition	Component	Final concentration
1	Tris-HCl, pH 8 (37 ℃)	40 mM
2	MgCl2	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^{TM})	1,000 units/ml
8	Inorganic pyrphosphatase (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 ℃ before use.

2.5.1.3 In vitro Transcription with Thioated RNA

Main steps and the idea of transcribing the phosphorothioated tRNAs are same as explained above. The thioation of the tRNA at A, C, G, or U positions, respectively, is obtained by replacing a certain amount of the nucleotide of interest in the reaction with the corresponding [αS]-NTP. It was optimized to 20% (Dabrowski et al., 1995), but the experiments here were done with transcribing tRNA with 1.3-1.5 thioated nucleotide per tRNA ratio to eliminate the possibility of incorporation of more then one thio-nucleotide. The reaction volume per tube is maximal 400 µl. Higher volumes somehow decrease the efficiency of the reaction. After incubating the reaction at 37 ℃ for 8-10 hours, the reaction was stopped by adding EDTA to a final concentration of 25 mM.

2.5.2 Purification of Transcriptions

Purification of the transcribed RNAs can be done either via gel or column purification, depending on the product. If there are other bands besides the main one in the product, the best way for the purification is the gel purification by cutting the main band.

2.5.2.1 Purification via Polyacrylamide Gel Electrophoresis

Purification of a transcribed RNA via gel is the best way, especially when there is more than one band. For this reason, the MFV mRNA produced by in vitro transcription was purified by gel electrophoresis.

The sample produced by in vitro transcription using 50 µg (25 pmol) of plasmid template was first purified with phenol-chloroform before going to the gel purification. After EtOH precipitation samples dissolved in 150 µl of H_2O and 150 µl of RNA denaturing sample buffer and incubated 2 minutes at 80 ℃ just before loading to the 13 % acrylamide-urea gel. Glass plates (14 cm x 16 cm) were sterilized in 180 ℃ for 3 h, before pouring the gel. A special

sample well 9 x 1 cm was made with an additional 2 mm thick spacer prepared for this purpose. The gel was pre-run at 8 Watts for 30 minutes and the well was rinsed with electrophoresis buffer (1 X TBE) in order to eliminate the excess of urea diffusing from the gel prior the application of the sample. After loading the sample, the gel was run at 8 Watts 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 UV shadowing (240-280 nm) localized the RNA band. The portion of gel containing the RNA of the expected length was then cut with a sterile blade and crushed into the small pieces inside of a sterile 30 ml corex tube. Disrupted gel pieces covered with 3 ml of RNA extraction buffer, 3 ml of 70% phenol, and the RNA extracted during 12 hours in vigorous vortex. At the end of this extraction, the phases were separated by 45 minutes centrifugation at 6,000 rpm in a HB-4 rotor (Sorvall), the aqueous phase was recovered and the phenol phase re-extracted one more time with 2 ml of RNA extraction buffer. The combined aqueous phases were purified once with chloroform: isoamylalcohol (24:1) and the RNA precipitated with 2.5 volumes of cold EtOH. After recovery of the precipitate by centrifugation (1h at 6,000 rpm in a HB-4 rotor) and washing with 70% ethanol, the RNA was dissolved in water, optical measurement was done for the concentration calculations and stored at -80 ℃ is small aliquots.

2.5.2.2 Purification via gel filtration

Since there is only one main band in the transcribed tRNAs, they were purified by QIAGEN RNA/DNA purification kit directly after transcription reaction. Purification was performed with QIAGEN-tip 100 for 300-400 μl, 8-10 hours reaction. Column was equilibrated with 3 ml QRE Buffer. Meanwhile, reaction sample was mixed with 1 ml QRL1 Buffer and then 9 ml Buffer QRV2 which creates optimal conditions for binding RNA to resin. After application of the sample to the column, the column was washed with 12 ml Buffer QRW. Elution was done with 6 ml preheated (45 ℃) Buffer QRW. Sample was collected to the 30 ml sterilized (3h at 180 ℃) Corex tubes. Precipitation was done with 2.5 volume EtOH, keeping it overnight in –20 ℃. The mixture was

centrifuged for 1h at 4 ℃ (rotor HB-4), and the RNA pellet washed with 70% EtOH, dried in room temperature for 10 min and dissolved in 200-400 µl H_2O according to yield. Aliquots were taken immediately and stored at –80 ℃ right after shock freeze.

2.5.3 5'-Labeling of RNAs with [γ- ^{32}P] ATP

In this study mRNA and tRNAs were labeled from the 5'- ends by exchanging the 5' terminal phosphate group with the γ-phosphate of [γ-^{32}P]ATP (Chaconas and Sande, 1980), according to the method described by Gnirke et al. (Gnirke and Nierhaus, 1989) with some modifications. The labeled mRNAs are used for the RNase (see section 2.7.1). tRNA labeling was executed for footprinting experiments. 5' labeling is a 2 step procedure. The first step is the dephosphorylation (hydrolysis of the 5' phosphate group) with alkaline phosphatase. Second step is the re-phosphorylation with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase.

2.5.3.1 Dephosphorylation of RNA with alkaline phosphatase

Generally, the removal of 5' phosphates from nucleic acids is used to enhance subsequent labeling with [γ-^{32}P]ATP, to reduce the circulization of plasmid vectors in ligation reaction (see section 2.4.2.2) and to render DNA susceptible or resistant to other enzymes that act on nucleic acids.

In our experiments here, dephosphorylation reaction contained 500 pmol RNA with 2.5 units of alkaline phosphatase (from calf intestine) in 50µl of 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 50 ℃. This temperature is important for partial denaturation of the 5' of the $tRNA^{Phe}$, which increase the yield of dephosphorylation, since 5' end of the tRNA has a base pair. The reaction was stopped by addition of 1/10 volume of 3 M sodium acetate, pH 5.0, and phenol chloroform extraction followed. This step is extremely important to remove the enzyme for a safe next step, namely phosphorylation. The sample

was precipitated from the aqueous phase by addition of 2.5 volumes of EtOH. After freezing the samples in liquid nitrogen, the RNA was recovered by a 1 h low-speed centrifugation for 45 min, washed with 70% EtOH and dissolved in 10 µl H_2O.

2.5.3.2 Phosphorylation of RNA

The removal of 5' phosphates from nucleic acids with phosphatases and their readdition in radiolabeled form by bacteriophage T4 polynucleotide kinase is a widely used technique for generating ^{32}P-labeled probes.

In the table below, the components were given. The end volume of the reaction was 40 µl. Incubation time and temperature is optional with either 4 ℃ for 15 hours or 37 ℃ for 1 hour. After incubation , the reaction volume was decreased by speed vacuum to roughly 5 µl to being able to load to one slot of gel and before loading to the gel 5 µl of RNA denaturing sample buffer was added. The sample was then denatured at 80 ℃ for one minute and applied to a 13% polyacrylamide-urea gel sequencing gel (Maxam and Gilbert, 1977), run at 50 W until Xylene Cyanol dye migrated 8-10 cm from the top (one nucleotide resolution). The important feature of this gel is that it contains 30% acrylamide on the bottom part (3-4 cm height) to collect the non-incorporated radioactive nucleotides and decrease the possibility of radioactive contamination while working. This part was cut and discarded right after stopping the gel. The rest part of the gel, which contains the labeled RNAs, was transferred to a used film and covered with transparent plastic wrap and a short time (1 min) autoradiography was done in order to localize the labeled product. The radioactive tRNA band was cut and extracted with a procedure similar to the one described in the section 2.5.2.1.

Right after the extraction of labeled RNAs, dilution was done with cold RNA of the same class to give specific activities ranging between 5,000 to 10,000 dpm/pmol depending on the experimental purpose. Immediate dilution and making aliquots are very much important to prevent the radiolysis effect.

Component	Final concentration
Tris-HCl, pH 7.5 (0 ℃)	50 mM
EDTA	1 mM
$MgCl_2$	10 mM
β-Mercaptoethanol	6 mM
Dephosphorylated RNA	2.5 pmol / µl
[γ-^{32}P]ATP (~3.5 µCi / pmol)	0.4 pmol / µl
T4 Polynucleotide kinase	0.5 U / µl

2.5.4 Aminoacylation of the 5'-[^{32}P] Labeled tRNA

Since the main procedure of aminoacylation of 5'-labeled tRNA is similar with the nonlabeled ones, please see the section 2.6.1 for more detailed explanation about the methodology.

2.5.5 Footprinting Experiments with Phosphorothioated tRNAs

Phosphorothioate method is one of the footprinting techniques and plays an important role as tool to assess the contact patterns of RNAs within ribonucleoprotein complexes. It was introduced by Eckstein and coworkers in the mid of 80s to analyze the contacts of $tRNA^{Ser}$ with its cognate synthetase (Schatz et al., 1991). It is applicable to both RNA and DNA. The method is based on the fact that small and chemically inert iodine (I_2) molecule can cleave the phosphate-sugar backbone of phosphorothioated RNA. In this RNA, a sulphur atom is replacing a nonbridging O_2 at the phosphate group. Usually replacement of the oxygen atom with a sulfur atom does not affect the functional spectrum of a molecule because the negative charge of the phosphate group is retained and also the size of the sulfur atom is only slightly larger than the O_2 atom. Thioated tRNA transcripts have been shown to be active in aminoacylation, ternary complex formation, and poly(Phe) synthesis (Dabrowski et al., 1995). But at certain positions such a replacement can interfere with the binding of a tRNA to a ribosomal site, for

example, by changing the tRNA structure (Smith and Nikonowicz, 2000) or by removing an oxygen atom of the phosphate group that was involved in the coordination of a Mg^{2+} atom. This in itself can provide interesting insights into the functional importance of the certain phosphate groups. Furthermore, it is essential to determine these positions before undertaking further analyses. This experimental strategy to identify the residues essential for binding is called interference strategy, because modifications that interfere with the complex formation are identified. tRNAs that exhibit interference, *i.e.* have a reduced ribosome binding ability due to thioation at distinct sites, can be identified by iodination after extraction of the tRNAs from the ribosome complexes. Bands corresponding to interference sites will be weaker than the corresponding bands of a control-tRNA solution pattern. The remaining majority of nucleotides, which do not interfere with complex formation, can be analyzed in a second strategy of this method, namely, the protection approach.

Very general scheme of the phosphorothioate experiments is shown in Figure 2.5.5.1. This scheme is changing according to specific experiments and will be explained occasionally. In the footprinting experiments, complex formation, iodine cleavage, removal of unbound tRNAs, extraction of ribosomal proteins, gel electrophoresis and the analysis of the bands are the main steps. In the following parts of this section, these steps will be introduced and discussed.

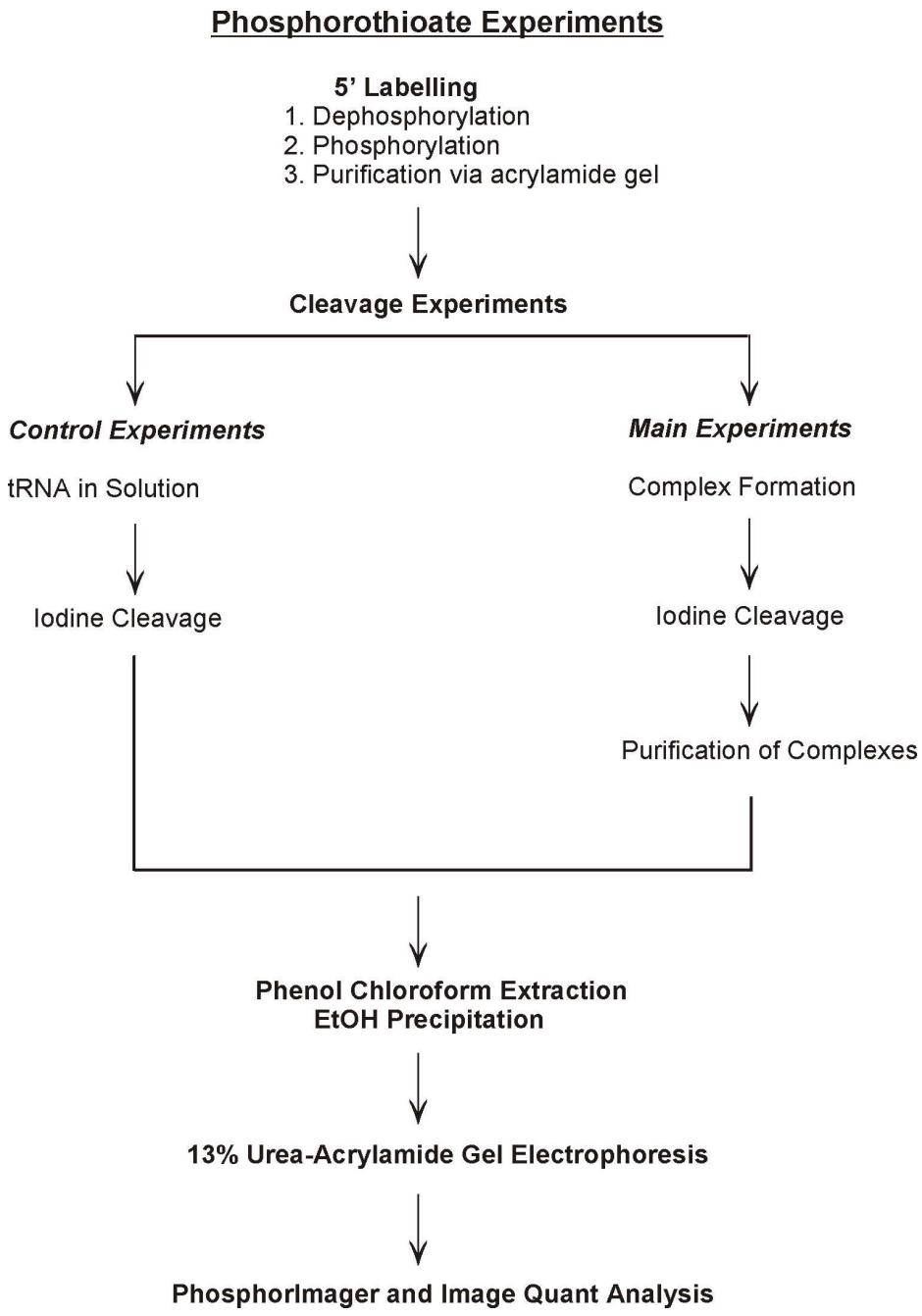

Figure 2.5.5.1: General schema of the phosphorothioate experiments.

2.5.5.1 Functional Complex Formation

In order to carry out footprinting of the ribosome on thioated tRNA species occupying different ribosomal sites, the following complexes can be defined as representing the main states of the elongating ribosome in vitro.

1. Deacly-tRNA in the P-site.
2. Ac-aminoacyl-tRNA in the P-site.
3. Deacyl-tRNA in the P-site and Ac-aminoacyl-tRNA in the A-site (pre-translocational state).
4. Deacyl-tRNA in the E-site and Ac-aminoacyl-tRNA in the P-site (post-translocational state).
5. Ac-aminoacyl-tRNA or deacyl-tRNA in the P-site and Aminoacyl-tRNA in the A-site (pretranslocational state).

The investigation of the protection patterns of tRNAs bound to the ribosome can be performed for all ribosomal-binding sites with these defined states. The [^{32}P]5'-thioate-tRNA is bound to the site chosen for investigation. It is very important to put control reactions where the tRNA is free in solution. The detailed pipetting scheme of the complex formation will be explained in the section 2.6.2.

2.5.5.2 Iodine Cleavage

Iodine (I_2) cleaves the RNA at the thioated position with an efficiency of approximately 5%. Cleavage reaction initiates with the nucleophilic attack of sulfur atom towards the I_2. The nucleophilic interaction of the 2'-OH group of sugar leads either directly to cleavage or to a phosphotriester intermediate, which may be hydrolyzed inducing cleavage or restoring the phosphodiester bond. If the RNA is labeled at one of its ends, e.g. with ^{32}P at its 5' end, the cleavage pattern can be monitored on a sequencing gel. The reaction scheme is shown in Figure 2.5.5.2.1.

2.5.5.3 Purification of Ribosomal Complexes

Ribosomal complexes can be separated from free ligands by several methods such as spun columns, centrifugation of the complexes through a sucrose cushion or gel filtration by gravity flow.

i. Isolation of ribosome complexes via spun column

100-125 µl sample containing 0.5-5 A_{260} of ribosomal complex is loaded onto a cDNA spun column S300 (Pharmacia) that has been pre-equilibrated with binding buffer $H_{20}M_6N_{150}Sp_{0.05}Sp_2$. The column was centrifuged for 1 min at 1500 rpm at 4 ℃ in a Sorvall HB4 rotor. The flow-t rough (fraction 1) is collected in a microcentrifuge tube. The next fractions were collected by loading 100-125 µl of binding buffer and repeating the centrifugation procedure (see section 3.1.3).

ii. Sucrose cushion centrifugation

A 100-200 µl sample containing 1-5 A_{260} of ribosome in binding buffer $H_{20}M_6N_{150}Sp_{0.05}Sp_2$ is loaded on a 1-2 ml 10% sucrose cushion in binding buffer. A polycarbonate centrifuge tube (Beckman) was used. After centrifugation for 18 h at 4 ℃ and 40,000 rpm in a Beckman TLA 100.3 rotor, the pellet is resuspended in 100 µl of binding buffer. The yield is about 50-60% of input.

RNA Sequencing via Phosphorothioate Method

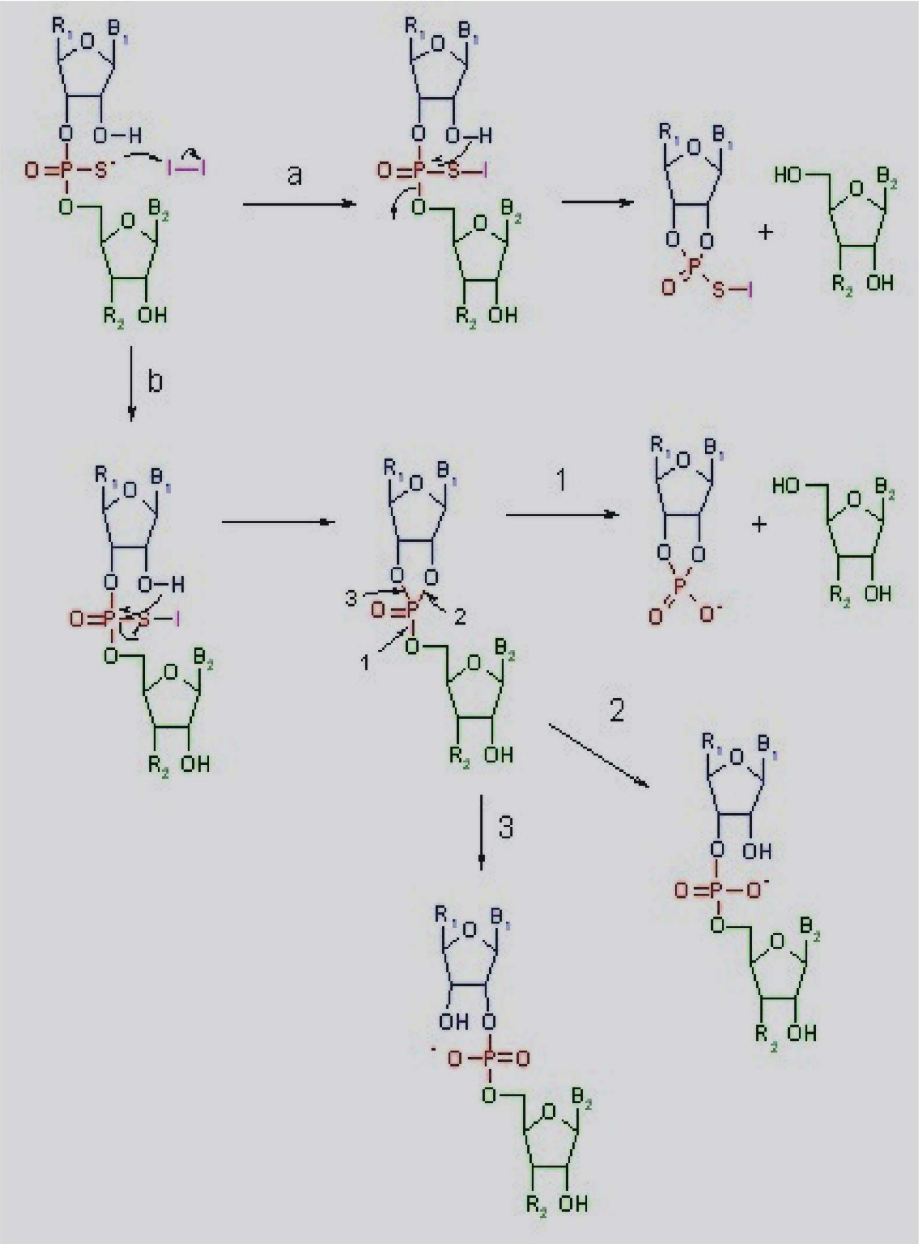

Figure 2.5.5.2.1: Reaction schema of the iodine cleavage.

2.5.5.4 Phenol-Chloroform Extraction

Phenol extraction phase was done overnight in the cold room with strong vortexing. It is very important to remove all ribosomal proteins from the sample in order to get more clear and sharp bands. Phenol extraction was repeated one more time (30 min). After chloroform extraction, samples were precipitated with EtOH precipitation and washed with 70% EtOH. Pellet was dissolved in 6 µl H_2O and 6 µl loading buffer and 2 times 1 µl sample was measured in scintillation counter to adjust the amount to load to the gel.

2.5.5.5 Gel Electrophoresis

13% denaturing polyacrylamide sequencing gels (acrylamide/bis-acrylamide: 19/1, 7M urea; 3,000-10,000 dpm/lane) were run as it was explained in section 2.2.4.2.

2.5.5.6 Image Quant Analysis of Gels

13% denaturing polyacrylamide sequencing gels (acrylamide/bis-acrylamide: 19/1, 7M urea; 3,000-10,000 dpm/lane) were exposed for 12 h to 16 h on a PhosphorImager™ (Molecular Dynamics™). Evaluation of the scanned gel was performed with the package program ImageQuant Vers.3.3™ (Molecular Dynamics™).

The experiments were repeated up to four times and the data were normalized as follows. Assuming that the results of two experiments performed under identical conditions are considered, the first normalization concerned variation in loading between respective thio-A-lanes (input normalization). Here the total counts of the two lanes were normalized, and the intensity of each band was multiplied by the normalization factor. Input normalization was performed for both complex and solution cleavage experiments individually. The second normalization is between the two sets of data (bound tRNA *versus* tRNA in solution) and essentially followed an identical procedure, where the protection value regarding the amount of $tRNA_{bound}/tRNA_{solution}$ for a distinct band was calculated using the normalized

intensities of corresponding bands derived from a tRNA in a complex and a tRNA in solution, respectively.

2.6 In Vitro Systems

2.6.1 Aminoacylation of tRNAs

Aminoacylation of a tRNA is prepared by incubation of 10-50 A_{260} units of tRNA with three- to five-fold molar excess of radioactively labeled (^{3}H or ^{14}C) amino acid and an optimal amount of a tRNA free S100 enzyme which is usually about 40 µg of protein per 10 A_{260} units of tRNA (see section 2.3.1.1). Before the addition of the S-100 enzymes, the pH of the reaction mix was adjusted to 7.5 with 1 N KOH. Buffer system is changing for the transcribed tRNA and the native ones. Charging efficiency of transcribed tRNAs is relatively higher in the conventional buffer system ($H_{20}M_{10}N_{100}SH_5ATP_3$), however native tRNAs are giving more or less same yield with the conventional or polyamine buffer system ($H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}ATP_3$, see section 3.1.4). After 10-15 min incubation at 37 ℃, the reaction mixture is extracted with one volume of 70% phenol and radiolabeled aminoacyl-tRNA recovered by ethanol precipitation before the final purification step, which is reversed-phase HPLC. With the addition of acetic anhydride, a non-purified aminoacyl-tRNA is converted to its N-acetyl-aminoacyl-tRNA derivative with a yield of above 90%. Acetic anhydride is added (1/30th of the sample volume) and the mixture incubated at 0 ℃ for 15 min. This procedure is repeated three times more. The N-acetyl-aminoacyl-tRNA is precipitated with two volume of ethanol and resuspended in H_2O.

N-acetyl-aminoacyl-tRNA and aminoacyl-tRNA were made free of nucleotides and lower molecular weight materials by a gel-filtration step performed on NAP-25 columns before purification by reversed-phase HPLC on a Nucleosil 300-5 C4 column equilibrated in buffer A (400 mM NaCl, 10 mM MgAc, 20 mM NH_4Ac, pH 5.0). For elution a buffer B (60% Uvasol-methanol, 400 mM

NaCl, 10 mM MgAc, 20 mM NH_4Ac, pH 5.0) was applied with an increasing gradient. 1 ml fractions were collected and the absorbance at 260 nm was monitored while elution procedure was taken. The order of elution is first deacyl-tRNA, then aminoacyl-tRNA and finally N-acetyl-aminoacyl-tRNA.

Analytic aminoacylation assays are performed with 0.01-0.03 A_{260} units of tRNA in the 30 µl of reaction mix under the same conditions stated earlier. After a 15 min incubation at 37 ℃ the amount of ami no acid incorporated was determined by precipitation with ice-cold trichloroacetic acid (10%), filtration through glass-fiber filters, and counting in a scintillation counter.

2.6.2 Preparation of Defined Functional Complexes

The partial reactions of the ribosomal elongation cycle as well as the functional competence of the ribosomes and translational factors can be studied using an experimental approached based on the procedure described by Watanabe in 1972 (Watanabe, 1972). The two main states of the ribosome in the ribosomal elongation cycle are called the state before translocation or PRE and the state after translocation or POST state. The ability to produce the homogenous PRE and POST states is due to the high-energy barrier between the two states. During elongation EF-Tu and EF-G catalyze the transition from POST to PRE and from PRE to POST, respectively (Nierhaus, 1996).

This method allows a controlled stepwise execution of the partial reactions of the ribosomal elongation cycle, thus providing a tool for the precise determination of the occupation of the three tRNA binding sites on the various complexes (Figure 2.6.2.1).

In the first step a 70S-mRNA-tRNA complex is formed, in which the tRNA is located in the ribosomal P-site. Either deacyl-tRNA or Ac-aminoacly-tRNA is used. Since elongation ribosome always carries two tRNAs, a programmed ribosome that carries only one tRNA occupying the P site mimics an initiation

complex and is called P_i complex (**i** for **i**nitiation). The α-amino group of the Ac-aminoacyl-tRNA is blocked by an acetyl residue, and therefore the tRNA behaves like a peptidyl-tRNA. A P_i complex is assayed with and without EF-G in the puromycin reaction. No difference in the reactivity with puromycin indicates 100% P-site occupation.

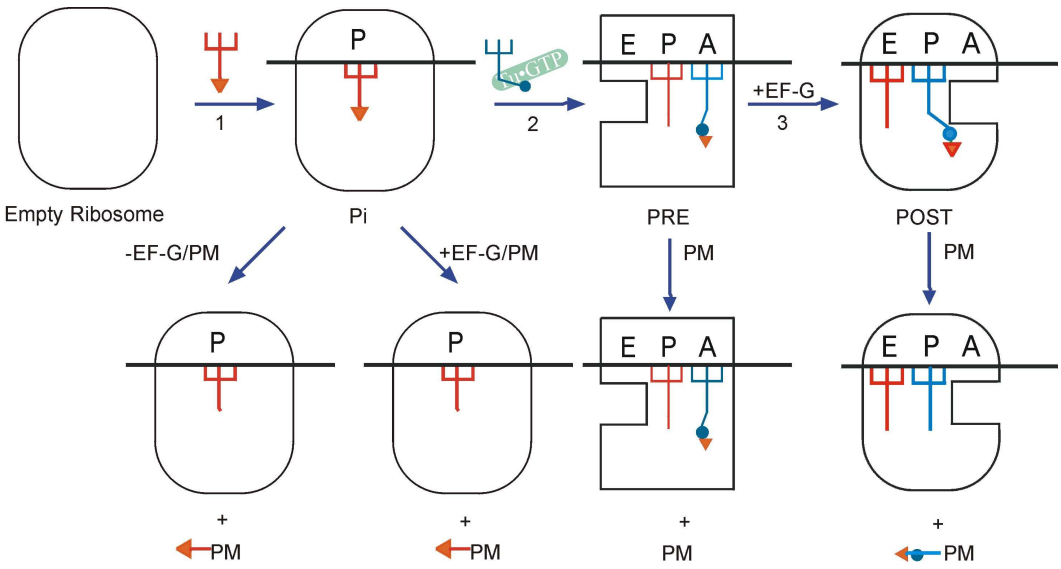

Figure 2.6.2.1: Reaction scheme for the preparation of ribosomal functional complexes carrying tRNAs in defined positions. The Pi complex carries only one tRNA, namely, peptidyl-tRNA in the P site, the PRE complex has a deacyl-tRNA in the P site and a peptidyl-tRNA in the A site, which move to the E and P sites on translocation, respectively.

In the second step the A- or E- site can be filled with the corresponding cognate tRNA. When the A site is filled, peptidyl transfer occurs immediately since it is a fast step.

In the third step, upon the addition of EF-G the PRE complex is translocated to the POST complex, where the tRNAs are now located in the P and E sites.

The efficiency of the translocation reaction and /or the binding state of the tRNAs can be determined in a fourth step with puromycin (analogue of the 3' aminoacylated end of a tRNA) reaction. This antibiotic reacts specifically with the P-site bound acyl-tRNA, if the ribosomal A site is free, forming an acyl-puromycin derivative which is used in order define the location of a charged tRNA on the ribosome (Allen and Zamecnik, 1962). While the peptidyl P-site

bound tRNA reacts with it, A-site aminoacyl or peptidyl bound tRNA will not react (Traut and Monro, 1964). Thus, the addition of EF-G has no effect in the binding state and therefore in the puromycin reactivity of a P-site bound aminoacyl- or peptidyl-tRNA, while the A site bound species should show a translocation factor-dependent puromycin reaction.

Reaction Steps:

The complete protocol contains 4 different steps. Below the volumes and amounts are given for a single determination assay. These values can be increased up to 10 fold according to the purpose of the experiment. Also, in each step different mixes and buffers were used to maintain the final ionic condition of the binding buffer $H_{20}M_6N_{150}SH_4Sd_2Sp_{0.05}$ throughout the assay.

P site binding: Construction of the Pi Complex

For construction of a Pi complex, 5-10 pmol of re-associated 70S ribosomes were incubated in a volume of 12.5 µl with 6-10 molar excess of either poly(U) or heteropolymeric mRNA over ribosomes and deacylated tRNA in a 1.5-2 fold molar ratio to ribosomes for 15 min at 37 ℃.

A site binding: Construction of PRE Complex

A site binding is divided into two types, namely enzymatic or non-enzymatic binding.

While constructing a PRE complex, the first site to be occupied with a tRNA is the P site. After this has been accomplished an aminoacyl-tRNA is added in a 0.8-2 fold molar ratio to ribosomes in case of non-enzymatic A-site binding. For enzymatic A-site binding a ternary complex (aminoacyl-tRNA•EF-Tu•GTP) is formed immediately before its addition to the binding assay: aminoacyl-tRNA (1-2 pmol per pmol of 70S ribosomes), 0.5 mM GTP, and EF-Tu (1.2 pmol per pmol of aminoacyl-tRNA) is pre-incubated for 2 min at 37 ℃ under the ionic conditions of the binding buffer and is added to the reaction mixture. The mix was, then incubated for 30 min at 37 ℃ for b inding.

Translocation: Construction of POST Complex

POST complexes were constructed via an EF-G (0.3 pmol / pmol 70S) dependent translocation of the PRE complex. 2.5 µl of HMK buffer containing EF-G was added and incubated for 10 min at 37 ℃.

Puromycin Reaction

Generally, for puromycin reaction 6 samples were used. 2.5 µl of binding buffer to two control-samples (without puromycin and ±EF-G) as background for the puromycin reaction was added. Besides, 2.5 µl of puromycin stock solution (10 mM in binding buffer, final concentration 0.7 mM) was added to four samples (two with and two without EF-G) in order to determine the amount of A-site occupation. Incubation was done either at 37 ℃ for 10-15 min or at 0 ℃ for about 12 h and the reaction was sto pped by adding 32.5 µl of 0.3 M sodium acetate, pH 5.5, saturated with $MgSO_4$. The amount of aminoacyl-puromycin formed was determined by extraction with 1 ml of ethyl acetate after 1 min vortexing. After 10 min incubation at 0 ℃, 1 min centrifugation at 13,000 rpm in the microfuge, in order to get the phase separation, measurement of the radioactivity contained in an aliquot of the organic phase was done by taking 800 µl and counting in scintillation counter.

Filter Binding Assay

Nitrocellulose filter assay is a method to measure the tRNAs on the complex. Complexes were mixed with 2 ml ice-cold binding buffer and immediately filtrated through the nitrocellulose filters. Filters were washed two times with ice cold binding buffer and radioactivity retained on the filter is determined by liquid scintillation counting. Binding assays included reactions without ribosomes as controls in order to determine the filter background. This background is normally low (below 10% of the binding signal) and directly proportional to the concentration of the radioactive component in the assay.

2.7 Some Additional Methods

2.7.1 RNase Test

5'-^{32}P labeled MF-mRNA as described at section 2.5.3 was used to follow the possible contamination. For this purpose, a minimum 2,000 - 3,000 dpm or higher amount of labeled mRNA was incubated with the samples that should be controlled for the RNase contamination in a total volume of 5-10 µl. To enhance the reaction for the possible RNase degradation in the case of contamination, samples were incubated at 37 ℃ for 5 min. After addition of RNA loading buffer and denaturing at 85-90 ℃ for 1 min, samples were loaded onto a 13% polyacrylamide gel. Gel was run in 8 Watt for 45 min up to 1 hour. The gel was transferred to a used x-ray film as a physical support, wrapped with plastic wrap and stored in a PhosphorImage cassette overnight (12-14 hours). Since the cassette is sensitive to the radioactivity and ^{32}P has a high energy radiation, there is no need to dry the gels to get good resolution.

2.7.2 Gel filtration method (Sepharcryl S-400 HR) for poly(U) fractination

Poly(U) from Boehringer Mannheim is not of uniform length, but rather a mixed population. Since for a phosphorothioated experiments short length (30-40 mer) poly(U) was needed, the poly(U) was fractionated by gel filtration.

2.7.2.1 Preparing the Gel Suspension:

Equilibration was done in the cold room. A bottle with Sephacryl HR was shaked gently to homogenize the solution (150 ml), then the content was poured into a one-liter-graduated cylinder, which contained 850 ml buffer (300 mM NaAc, pH 5.5, with 2% MeOH), stirred with glass rod to make homogenous suspension free from aggregates and then one has to wait until all Sephacryl has been settled to the bottom of the cylinder.

2.7.2.2 Assembling and Packing the Column

A glass column (65 cm length, 3 cm diameter) was attached to a holder vertically and a tube on the bottom of the column was closed with a stopper. The same buffer that was used to homogenize the solution was degassed beforehand and poured into the column. There should be no air bubble while pouring. A two liter funnel was connected to the upper side of column. Gel was re-suspended and poured into the funnel in one continuous motion. Pouring down a glass rod held against the wall of the funnel helps to prevent formation of air bubbles. The cap of the funnel was covered, and left overnight until the column is well packed.

2.7.2.3 Equilibration of the Column

The funnel was removed and the upper part of the column was connected to a reservoir by tubing. The stopper was removed from the bottom and connected to a UV detector and fraction collector. The flow rate was adjusted by the height distance between reservoir and collector (10 ml elution in 75 min). A safety loop was made between column and UV detector. The column was equilibrated with 400ml of running buffer.

2.7.2.4 Analytical Run

Poly(U) from Boehringer Mannheim was dissolved in three ml H_2O (820.6 A_{260}/ml, 2462 in 3ml). 1/10 volume of it was loaded onto the column (300 µl poly(U) + 300 µl 60% Glucose + 2.4 ml H_2O) with a Pasteur pipette. The sample should make a layer. 10 ml fractions were collected. Fraction 11-26 were selected and precipitated with isopropanol.

2.7.2.5 Preparative Run

The procedure for the preparative run is the same as in the analytic one. The rest of the poly(U) (~ 2.5 ml) was loaded to the column. Fractions were collected and after EtOH precipitation and spectrophotometric measurement, samples were subjected to a gel (see section 3.1.2).

2.7.2.6 Calculations

In order to calculate the concentration of the each fraction, firstly the distance between the markers and their loading slots on the gel was measured. The nucleotide length of each marker was converted into logarithm. A graph (nucleotide (ln) versus distance on gel) was drawn. The distance of each fraction from the slot was measured. Since it is a kind of smear rather than one sharp band, the-mid point was chosen for the real value and the both ends of smear were measured for the deviations. These values were plotted to the same graph (x-axis) and y values found and converted to nucleotide number with inverse (ln). In order to calculate as pmols, we need to know the molar extinction coefficient of U which is $1x\ 10^{4}\ M^{-1}\ x\ cm^{-1}$. Example of the calculations and the table of the calculation results were given in Results (see section 3.1.2).